Paul-Ehrlich-Institut 

Dissertation

To obtain the academic degree
Doctor in Science, Doctor philosophiae naturalis (Dr. phil. nat.)

Genetic modification in hematopoietic stem cells, using lentiviral vectors, to target protein expression in megakaryocyte and platelets

Submitted to the Department of Biochemistry, Chemistry and Pharmacy

Johann Wolfgang Goethe University

Frankfurt am Main – Germany

Presented by

Lisette Johana Latorre Rey

Born in Caracas, Venezuela

Frankfurt am Main, October (2018)

(D30)

vom Fachbereich 14 der

Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan:	Prof. Dr. Clemens Glaubitz
Gutachter:	Prof. Dr. Rolf Marschalek
	Prof. Dr. Ute Modlich
Datum der Disputation:	05. März. 2019 10:00 am

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt und andere als die in der Arbeit angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus anderen Schriften entnommen sind, habe ich als solche kenntlich gemacht. Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegen. Des Weiteren bin ich mit der späteren Ausleihe meiner Doktorarbeit an die Fachbereichsbibliothek einverstanden.

Frankfurt, den 30.10.2018

_____

Lisette Johana Latorre Rey

“I was taught that the way of progress is neither swift nor easy”

Marie Curie

Illustration by John Tenniel

"Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that! "

The Red Queen said to Alice…….

In*: "**Through the looking-glass, and what Alice found there** "*(1871), by Lewis Carroll

Dedicado a mis padres

Alvaro y Rosmira

En profundo y eterno agradecimiento a su amor infinito, dedicación y ejemplo

Dedicated to my parents

Alvaro and Rosmira

In deep and eternal gratefulness to his infinite love, dedication and example

Acknowledgments

Nunca el hombre en su carrera se encuentra solo, jamás; siempre, adelante y atrás encontrará quién le guíe y aliente. Vive, sonríe, ten valor y llegarás.

We are never alone along the way, we always find someone who care for us and guide us. Live, smile, have courage and you will be able to make it possible.

Amalia Domingo Soler

Sevilla 1835 - Barcelona 1909

After all these challenging, amazing and stirring years, I would like to thank all who jointed me, inspired me, guide me and helped me to accomplish my deepest and coolest dream.

I would like first to thank to God, for allowing me, despite all the difficulties, to bring this so important goal for me to fruition; for all the blessings, and the life full of wonderful opportunities that gave me, to continue developing myself not just as a scientist, but as human being as well.

The illustrious Goethe University Frankfurt and the Paul Ehrlich Institute, for open me the doors as student and as a scientist; There, I had the opportunity to find within their corridors and lecture halls people who inspired me with their example and helped me in the development of this project.

I would also like to thank the two people who guided me during all these years and helped me on my development as scientist and as a professional:

To Prof. Dr. Ute Modlich, for her trust on giving me the opportunity to work on this project, and for open me the doors of her lab. I would like to thank you for your continuous support and your painstaking and careful guidance since I began my PhD, not just in the academic issues but in those outside of them as well. For let it me be part of all changes that we went through as a group since I began. Your decision of taking me as your student changed my life for good and gave me the opportunity to accomplish this so important and meaningful goal for me. For everything and forever my deepest gratitude.

To Prof. Dr. Rolf Marschalek, I would like to express my eternal and deepest gratitude for all his support and his excellent guidance during all these years. I would like to thank you for all your advices, recommendations and orientations during our long discussions that helped me on the development not just of this work, but also for me as scientist.

I would also like to thank to all my colleagues in the laboratory, for the good times and for everything I could learn from them. Specially I would like to thank Niels Heinz for all the help and orientations I received from him at the beginning of my PhD, as well as Saskia Kohlscheen and Franziska Schenk for their contribution and their help during the development of this work.

The good friends become family, and during my stay here in Germany I had the opportunity to meet and get to know wonderful people. I would like to deeply thank you all for the amazing time and beautiful moments that I have expend with all of you; thank you for helping with your company to raise my mood, to cheer me up, as well as to drain and forget my continuous stress. Thank you for teaching me to appreciate and to know more about this beautiful country to which I have so much to be thankful. Ein großer Teil meines Erfolges gehört zu euch alle.....Vielen lieben Dank!

Quisiera agradecer infinitamente también a mi querida Amparito y Asia por su apoyo y su cariño incondicional desde siempre.

And I cannot stop mentioning the most important people in my life, to whom I owe and dedicate all my achievements, no matter how small they may be; who helped me and gave me their support and their love when I needed it the most: My parents, Alvaro and Rosmira; I definitely do not know what would I have done without you, thanks for always being with me and for giving me the strength through your love to always continue. You, my biggest gift and my greatest Blessing. I love you infinitely and time will not be enough to thank you for all you have done for me since always.

No puedo dejar de mencionar a las 2 personas más importantes en mi vida, a quienes debo y dedico todos mis logros sin importar cuan pequeños sean: Mis padres: Álvaro y Rosmira, quienes siempre me dieron su apoyo incondicional y a través de su amor infinito, me dieron la fortaleza para continuar cuando más lo necesitaba. Ustedes son y serán mi más grande regalo y mi gran bendición; el tiempo no será suficiente para agradecerles todo lo que con tanto esmero y sacrificio han hecho por mí.

To all of you who were beside me and help me along the way, my deepest and infinite thanks.

Lisette

Table of contents

Zusammenfassung…… 1

Chapter 1……..... 8

Introduction

1.1. Hematopoietic Stem Cell (HSCs) and Hematopoiesis…….... 9

1.2. Origin of platelets: Megakaryopoiesis……..... 12

1.3. Transcriptional control of megakaryopoiesis…..……..……..... 13

1.4. Cytokines that regulate platelet formation…….. 15

1.5. Megakaryocyte maturation…… 17

1.6. Biogenesis of secretory granules……. 19

1.7. Platelets: more than a budded particle. Platelets as protein producers…… 25

1.8. DHL in the blood: Platelets as natural carrier and delivery cells…… 26

1.9. Platelets are multifunctional cells…… 30

1.10. Physiology of platelets. Activation and secretion…….. 32

1.11. Lentiviral vectors and their used in Gene Therapy……..... 37

1.12. Lineage specific vectors…… 45

2. Concept of the study…… 46

3. Specific aims of the project……. 51

Chapter 2…….. 53

Materials and Methods

2.1 Materials……... 54

2.1.1 Technical Equipment……. 54

2.1.2 Expendable items……... 55

2.1.3 Fine Chemicals…….. 56

2.1.4 Cytokines……... 58

2.1.5 Buffers and Solutions…… 58
2.1.6. Kits……... 59
2.1.7. Enzymes……... 60
2.1.8. Plasmids……... 61
2.1.9. Primers……. 65
2.1.10. Antibodies…….. 66
2.1.11. Bacterial strains, Cells and Cell lines and culture media…….. 67
2.1.12. Mice……... 68
2.2. Molecular biology methods…… 69
2.2.1. Generation of lentiviral vectors…… 69
2.2.2. Bacteria transformation and cultivation…… 70
2.2.3. Blue white screen…….. 71
2.2.4. Isolation of plasmid DNA from bacteria……... 71
2.2.5. Polymerase chain reaction (PCR)…….. 72
2.2.6. Separation of DNA by electrophoresis…….. 73
2.2.7. Extraction of DNA from agarose……... 73
2.2.8. DNA restriction digests……. 74
2.2.9. Ligation of DNA……..... 74
2.2.10. DNA concentration and purity…….. 74
2.2.11. Sequencing…… 75
2.2.12. Real-time PCRs……. 75
2.3. Cell culture methods…….. 77
2.3.1. Cultivation of cells…….. 77
2.3.2. Viral particle production……. 77
2.3.3. Transduction of cell lines…… 78
2.3.4. Isolation and transduction of murine lineage marker-negative (lin-) BM cells…….. 78
2.3.5. Transduction of primary BM cells…….. 79

2.3.6. Hematopoietic differentiation in-vitro…… 80
2.3.7. Platelets isolation…… 80
2.3.8. Flow cytometry analysis from in vitro experiments……... 81
2.3.9. Fluorescence microscopy of cultivated cells…….. 83
2.3.10. Immunofluorescence staining on whole femora cryosections…… 83
2.3.11. Immunostaining for confocal laser scanning microscopy…….. 84
2.3.12. Mouse experiments……. 86
2.3.13. Histopathology…… 87
2.3.14. Blood counts and flow cytometry……... 87
2.3.15. Cytospins……. 87
2.3.16. May Grünwald-Giemsa staining……. 88
2.3.17. Statistical analysis……... 88
Chapter 3 ……. 89

Megakaryocyte specific vectors

3.1. Generation of in vitro megakaryocytes…… 90
3.2. Design of lentiviral vectors for megakaryocyte-specific expression……... 92
3.3. Activity of lineage-specific promoters in in-vitro megakaryocytes…… 94
3.4. Activity of lineage specific promoters in in-vitro non-megakaryocytic cells……. 97
3.5. MK-specific lentiviral vectors confer high GFP expression in platelets……. 99
3.6. MK-specific lentiviral vectors show lower activity in hematopoietic non-target cells 103
3.7. Expression from MK-specific lentiviral vectors in HSPC…… 108
3.8. Expression in MKs in the BM in situ 117
3.9. Mpl expression from MK-specific lentiviral vectors improves Mpl-/- megakaryopoiesis…… 120

Chapter 4…… 127
4.1. Targeting proteins to the alpha granule of platelets by sorting signals 128

4.1.1 Primary megakaryocytes contain alpha granules expressing P-selectin and von Willebrand factor…… 128
4.2.1. Intra-alpha granule targeting……. 132
4.2.2 Intragranular targeting. Lineage specific GFP targeting vector……... 135
4.3 Transmembrane alpha granule targeting…….. 143
4.3.1 Targeting d2eGFP to the membrane of alpha granules by using the P selectin sorting signal…….. 144
4.3.2 Targeting of human coagulation Factor VIII to the membrane of alpha granules... 148
4.4. Genetically modified platelets……. 161

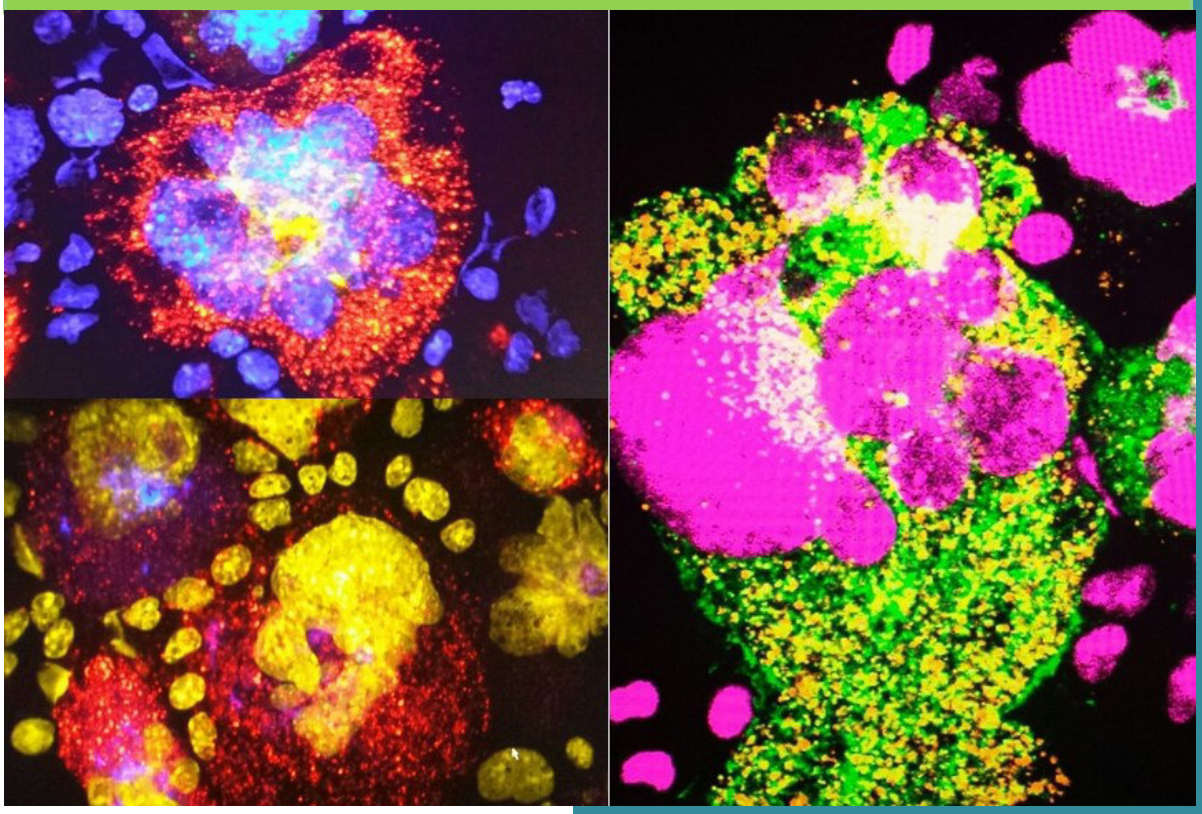
Chapter 5…….. 170

Discussion

5.1. Generating genetically modified platelets, safety considerations for gene therapy. 171
5.2. Targeting transgene expression to megakaryocytes and platelets by lineage-specific lentiviral vectors…… 173
5.3 Difference of expression regardless the similarities about transcription factor binding sites…… 176
5.4. Other studies restricting protein expression…….. 177
5.5 Use of murine promoter fragments in human cells. Is it relevant for therapeutic purposes?..... 179
5.6. Effect of the vector copy number and vector integration site on the differential percentage and intensity of expression……... 181
5.7. Sorting Signal motifs……. 183
5.8. FVIII gene therapy approaches…….. 193
5.9. What still needs to be done…… 199
5.10. Safety issues: Possible limitations that still need to be addressed……. 200
Outlook……... 203
Summary……. 206

2018

Zusammenfassung



Genetische Modifikation in hämatopoetischen Stammzellen mit lentiviralen Vektoren zur gezielten Protein-Expression in Megakaryozyten und Blutplättchen

Lisette Johana Latorre Rey
Paul Ehrlich Institute

Einleitung

Blut ist ein flüssiges Gewebe, das aus zellulären und löslichen Komponenten besteht. Die visköse Flüssigkeit kann frei innerhalb der Blutgefäße im ganzen Körper zirkulieren. Der zelluläre Teil des Bluts besteht aus Zellen, die sich zwar in ihrer Funktion unterscheiden, aber aus einer gemeinsamen Stammzellpopulation entstehen. Diese hämatopoetischen Stammzellen (HSC) befinden sich im Knochenmark und differenzieren sich in verschiedene hämatopoetische Vorläuferzellen. Durch diesen Prozess der Differenzierung und Spezialisierung wird eine konstante Menge an wichtigen Blutzellen täglich neu produziert. Der Hauptbestandteil des Blutes besteht aus den roten Blutkörperchen (oder Erythrozyten), die für den Transport von Sauerstoff zuständig sind; dem gegenüber stehen die weißen Blutkörperchen (oder Leukozyten), deren Hauptfunktion die Durchführung gerichteter Immunreaktionen ist. Diese spezialisierten, weißen Blutkörperchen sind im Wesentlichen die sog. Basophile, Eosinophile, Neutrophile, Lymphozyten und Monozyten. Eine dritte Gruppe sind die Blutplättchen (oder Thrombozyten), die von Megakaryozyten (MKs) gebildet werden und deren wesentliche Funktion die Durchführung einer koordinierten Blutgerinnung ist. Aufgrund ihrer relativ leichten Gewinnung sind Blutzellen diejenigen Zellen, an denen man rasch genetische Untersuchungen durchführen kann. Zudem eignen sich Blutzellen als Ziele für eine gezielte, genetische Modifikation. Sie erlauben zum Beispiel die permanente oder kurzfristige Expression therapeutischer Proteine. Dies ist davon abhängig, welche der Blutzellen hierbei verwendet werden, da sie eine unterschiedliche Lebensdauer aufweisen. Auch Thrombozyten eignen sich dazu prinzipiell, und haben den zusätzlichen Vorteil, dass sie ihre Proteinladungen in einem kontrollierten Prozess (nach der Aktivierung der Thrombozyten) in den extrazellulären Raum abgeben zu können. Aus diesen Grund sollten in dem vorliegenden Forschungsprojekt genetisch modifizierte Megakaryozyten, "rekombinante Thrombozyten", erzeugt werden. Dazu sollte das rekombinante Protein in sekretorischen Alpha-Granula angereichert werden, um dann nach einem externen Stimulus (die Aktivierung der Thrombozyten) sezerniert zu werden und den therapeutischen Effekt zu erzielen. Während der Aktivierung von Blutplättchen würden dann potentiell therapeutische Proteine entweder als lösliches Protein, zusammen mit den natürlichen intragranularen Proteinen, in den extrazellulären Raum freigesetzt werden, oder aber als Transmembranprotein auf der Zelloberfläche von Plättchen exponiert werden. Für eine nachhaltige Therapie muss aber in Zukunft auch darüber nachgedacht werden, ob man solche Veränderungen direkt in den hämatopoetischen Stammzellen durchführen sollte. Ein Nachteil dieses Verfahrens wäre dann eine Expression in allen davon ableitbaren Zellen. Dies könnte durch Linien-spezifische Promotoren umgangen werden.

Ziel des Projekts

In einem ersten Ansatz sollte deshalb die Spezifität und Aktivität von sechs verschiedenen Promotorfragmenten in lentiviralen Vektoren vergleichend bewertet werden. Dazu wurden folgende Promotoren getestet: der murine Platelet factor 4 (mPf4) 1222 bp (-1074 to +148), das humane Glycoprotein Ib alpha (hGP1BA) 595 bp (-265 to +330), je ein kurzes und ein längeres Fragment des humanen Glycoprotein 6 (hGP6 /

hGP6s) 351 bp (-322 to +29) / 726 bp (-697 to +29), sowie der humane Glycoprotein 9 (GP9) Promoter 794 bp (-782 to -12). Diese Promotorfragmente wurden als Promotoren in selbst inaktivierte lentivirale Vektoren (SIN) einkloniert, um ein verstärkten grün fluoreszierendes Protein (eGFP) als Gen-Reporter zu exprimieren, um so die Promoter Aktivität *in vitro* und *in vivo* zu testen und zu bewerten. Als ubiquitär und nicht spezifischer Kontrollvektor sollte der humane PGK-Promotor in dem lentiviralen Vektor verwendet werden; als zweite Kontrolle wurde der Milz-Fokus-bildende Enhancer/Promotor (SFFV), im Kombination mit dem LTR-getriebenen gammaretroviralen Vektor verwendet. Um Proteine in sekretorische alpha-Granula der Megakaryozyten und Thrombozyten zu exprimieren, wurden das Signalpeptid des Zytokins RANTES verwendet. RANTES wird normalerweise von T-Zellen exprimiert und sezerniert. Das Signalpeptid wurde als N-terminale Signalsequenz an das destabilisierte GFP, d2eGFP (RANTES.d2eGFP) fusioniert. Das Sortiersignal ist eine kurze Peptidsequenz (VIATLKDGRK), die das lösliche Protein über das ER-System und Golgi-Apparat in den sekretorischen Vesikeln abliefert. Um Proteine auf der Oberfläche von Vesikeln zu exprimieren, wurde entweder die transmembranäre-granula targeting-Sequenz von P-selektin (die Transmembrandomäne und der zytoplasmatische Schwanz (als TDCT bezeichnet) an das C-terminale Ende des humanen Gerinnungsfaktor-VIII fusioniert (bezeichnet als BDcohFVIII_TDCT oder FVIII_TDCT). Die TDCT Proteinsequenz sorgt dann dafür, dass das entsprechende Protein in der Membran der Alpha-Granula angereichert wird. Für die Expression der Proteinfusionen wurden der SFFV-Promotor oder der Plättchenfaktor-4-Promotor als Promotor in den SIN-lentiviralen Vektoren verwendet. Beide Strategien wurden *in vitro* getestet, indem transduzierte Vorläuferzellen *in vitro* in Megakaryozyten differenziert wurden. Zudem wurden auch die genetisch modifizierten Zellen mittels Laser Scanning Confocal Microscopy (LSM) auf die Kolokalisation von unterschiedlichen Proteinen hin untersucht. Fluoreszenzintensität und Kolokalisierungskoeffizient (WCC) wurde dabei bestimmt, indem man den von Willebrand-Faktor und/oder P-Selektin als Alpha-granula-marker entweder mit dem rekombinant-exprimierten GFP, FVIII oder FVIII_TDCT verglichen hat. Für die Analysen wurden die Zellen, die mit den gezielten Vektoren transduziert wurden, mit den Zellen, die mit den Nicht-gezielten Vektoren transduziert wurden, verglichen. Die Kolokalisationsanalyse wurde auf Einzelzell-niveau durchgeführt.

Ergebnisse

Alle zu testenden Vektoren wurden auf Titer zwischen $1x10^6$ - $1x10^7$ transduzierenden Partikeln/ml eingestellt. Um die Spezifität der Expression zu untersuchen, wurden Linien-Marker-negative Zellen aus dem Knochenmark von C57BL/6-Wildtyp-Mäusen isoliert, und 24 Stunden mit Zytokinen-kultur stimuliert, bevor sie anschließend zweimal mit dem lentiviralen Vektoren transduziert wurden. Die Zellen wurden anschliessend entweder in Flüssigkulturen mit 50 ng / ml hTPO zu MKs differenziert, oder in halbfestes Differenzierungsmedium eingebracht, um sie in Kolonietests zu analysieren. Transduzierte Zellen wurden auch in konditionierte C57BL/6-Empfängermäuse transplantiert, um Analysen an Zellen aus der Milz, des peripheren Blutes

oder dem Knochenmark durchzuführen. Die GFP-Expression wurde mittels Durchflusszytometrie in den jeweiligen Zellpopulationen gemessen. Die Analyse von *in vitro* differenzierten MKs zeigte, dass der Prozentsatz der GFP-Expression nach der MK-Differenzierung, zwischen 2,4 und 5-fach höher war in Vergleich zu den nicht-differenzierten transduzierten Linien-marker negativen Kontrolle Zellen. Die Stärke der GFP-Expression konnte auch durch die Fluoreszenzintensität abgeschätzt werden. Mit Ausnahme des hPGK-Kontrollvektors, bei dem keine Unterschiede beobachtet wurden, stieg die GFP-Intensität von MK-Vektoren mit dem Grad der Polyploidisierung (eine MK-Reifung Zeichen in späteren Stadien der MK-Differenzierung) deutlich an. Eine parallele Analyse der GFP-Expression in nicht-megakaryozytären Zellen aus den Kolonietests, wie z.B. Erythroide Zellen, Monozyten-Makrophagen oder Granulozyten-Vorläuferzellen, zeigte, dass in transduzierten Zellen mit den Abstammungslinien-spezifischen Vektoren (MK-Vektoren), der Prozentanteil von GFP in allen Fällen niedriger war als bei der hPGK-Kontrolle (<10%). Die gleichen Ergebnisse wurden bei Transduktionen beobachtet, die in primären Maus-Fibroblasten- und gemischten kortikalen Kulturen durchgeführt wurden, welche die signifikant starke Präferenz der Expression in der Megakaryozyten-Linie und eine schwache Stärke der Promotor-Aktivität in Off-Target-Zellen bestätigen.

Zusätzlich zeigten die Ergebnisse aus peripherem Blut von transplantierten Mäusen, dass die GFP-Expression in Thrombozyten signifikant höher war in Vergleich zu den negativen Kontroll-Vektoren, mit einer geringeren Hintergrundpromotoraktivität in Leukozyten und Erythrozyten bei Mäusen, die mit den Abstammungslinien-spezifischen Vektoren transplantiert wurden. Der höchste GFP-Prozentsatz und die höchste-Stärke der GFP-Expression wurde mit dem mPf4-Vektor beobachtet, gefolgt vom hGP1BA-, hGP6- und hGP6s-Vektor. Jedoch war eine höhere Expressionsstärke von Pf4- und hGP1BA-Vektor auch mit höherer GFP-Expression Prozentsatz in Leukozyten assoziiert. Daraus ergab sich, dass der hGP6-Promotor der beste Megakaryozyten- und Plättchen-spezifische Promotor war. Diese Annahme wurde auch durch die *in vivo* Experimente nach Transplantation bestätigt, denn die Analyse der Knochenmarkszellen zeigte, dass der hGP6-Vektor die geringste Aktivität in den hämatopoetischen Stamm- und Vorläuferzellen (HSPC) mit weniger als 10% GFP-positiven Stammzellen aufwies. Dem Gegenüber zeigte der mPf4- und hGP1BA-Vektor in HSPC zwischen 20 und 70% GFP-positive Zellen. Dies wurde auch die RNA Expressionsuntersuchungen bestätigt. HCPCs zeigten eine wesentliche mRNA-Expression von mPf4, menschlichem Gp1ba und Gp9, wobei die mRNA Expression Spiegel von Gp1ba und Gp9 sehr ähnlich waren, wie es durch ihre Funktion als Untereinheiten desselben Rezeptorkomplexes erwartet werden konnte. Die mPf4-Expression war 2,5-fach höher als die Gp1ba-Expression, im Gegensatz zum murinen Gp6 in HSPC, dessen mRNA-Expression fast Null war. Die *in vivo* GFP-Expression in Kryoschnitten von Femora aus Mäusen, die mit den transduzierten BM-Zellen transplantiert worden waren, zeigte, dass in Megakaryozyten das GFP stark vom mPf4-Vektor und dem hGP1BA-Vektor exprimiert wurde, gefolgt von den hGP6s-Vektoren. Fluoreszenzsignale in Nicht-MK-Zellen wurden kaum nachgewiesen. Die GFP-

Expression von dem hGP9-Promotor war ähnlich zu dem hPGK-Kontrollvektor; entsrechend dem, was vorher im peripheren Blut beobachtet werden konnte.

Mpl-defiziente Mäuse haben einen bekannten Defekt in der Megakaryozyten Reifung und sind thrombozytopenisch. Über-expression des Thrombopoietin-Rezeptors Mpl, unter Kontrolle von MK-spezifischen Vektoren in $Mpl^{-/-}$ Linien-Marker-negativen Zellen, differenziert zu MKs, haben in in-vitro-Kulturen gezeigt, dass die Reifung und Polyploidisierung von Mks nach der Re-expression von Mpl deutlich erhöhte wurde, die durch signifikant mehr MKs mit hoher Polyploide (> 16n) in späteren Stadien der Reifung im Vergleich zu den $Mpl^{-/-}$ MKs angezeigt wurde; sowohl in analysierten zellulären Bildern von Cytospins als auch in Durchflusszytometrie-Experimenten. Diese Ergebnisse wurden durch *in-vivo* Analysen bestätigt, bei denen $Mpl^{-/-}$ transplantierte Mäuse, deren lin-BM-Zellen, mit mPf4- und hGP6.Mpl-Vektoren transduziert wurden, eine signifikant Erhöhung des Thrombozytenzahlen zeigten, im Vergleich zu den Kontrollmäusen, die mit dem PGK-Kontrollvektor (PGK-GFP) transduzierte-Zellen transplantiert wurden.

Intra-granuläre Proteinexpression

Unser Ziel, rekombinante Proteine in den Alpha-Granula von Megakaryozyten und Thrombozyten anzureichern, zeigte einen signifikanten Unterschied zwischen transduzierten Megakaryozyten mit dem RANTES.d2eGFP-Vektor und dem nicht-spezifischen SFFV.d2eGFP-Vektor. In den Kolokalisationstudien am LSM konnte gezeigt werden, dass der gewichtete Kolokalisationskoeffizient (WCC) mit dem RANTES.d2eGFP-Targeting-Vektor bei ca. 80% der Kolokalisation mit P-Selektin-gefärbten Granula und 70% mit von Willebrand-Faktor-gefärbten Granula war. Die Kontrollen mit dem SFFV.d2eGFP-Vektor zeigten dabei nur ca. 30% Kolokalisation mit P-Selektin oder von Willebrand-Faktor. Diese Daten stimmten mit den LSM-Digitalbildern von RANTES.d2eGFP-transduzierte-Mega-karyozyten überein. Mit den nicht gezielten SFFV.d2eGFP- und mPf4p.d2eGFP-Vektoren wurde eine einheitliche und homogene GFP-zelluläre Verteilung beobachtet, die sich auf die nukleären und zytosolische Räume erstreckte. Diese Ergebnisse wurden ebenfalls mit den Fluoreszenzintensitätsdiagrammen bestätigt. Es gab eine deutliche Überlappung der GFP-Signale, die aus den mit dem gezielten RANTES.d2eGFP Vektor transduzierten-Zellen emittiert wurden, sowohl mit dem P-selektin- als auch mit dem von Willebrand-Faktor-Signal, die aus dem gefärbtem Granulat emittiert ist.

Transmembrane Expression in Alpha-Granulae

Aufgrund der Bedeutung des P-Selektins für den zellulären Vesikeltransport für die granuläre Sekretion, fusionierten wir entweder die Transmembrandomäne und den zytoplasmatischen Schwanz von P-Selektin (TDCT) an den C-Terminus von d2eGFP, oder vom B-Domänen-deletierten FVIII. Beide Modellsysteme sollten uns erlauben, eine Kolokalisation des rekombinaten Proteins mit dem Gerinnungsfaktor VIII auf der Membran von Blutplättchen zu beobachten, sobald die Alphagranulate nach der Plättchen Aktivierung freigesetzt worden waren. Wir konnten zeigen, dass das GFP-Signal von MKs, die mit der P-selektin.d2eGFP-Fusion transduziert wurden, mit den emittierten Signalen von P-Selektin und

Willebrand-Faktor gefärbten Granulaten überlappte; diese konnte nicht nur in LSM-digitalisierten Bildern, sondern auch in der Fluoreszenz-Intensitätsanalyse gezeigt werden, was auf eine deutliche Kolokalisierung von dem d2eGFP in den Alpha-Granula hinwies. Gleichfalls konnte eine evidente Signal-Überlappung zwischen dem Signal des FVIII (FVIII TDCT) mit dem P-Selektin / von Willebrand-Marker wie erwartet beobachtet werden. Die Signal-Kolokalisation wurde durch das stark gepunktete GFP- und FVII_TDCT-Muster mit den Alpha-Granulat Markern P-selektin und von Willebrand Faktor bestätigt, welches in den Bildern der LSM-Analyse beobachtet wurde. Fluoreszenzintensitätsanalyse und WCC-Werte bestätigen dies. Andererseits deckt die subzelluläre Lokalisierung des d2eGFP, die vom nicht-gezielten mPF4p.d2eGFP Vektor emittiert wurde, Regionen ab, in denen keine spezifischen Signale für die Alpha-Granulat-Marker nachgewiesen wurden. Eine erwartete, aber trotzdem interessant Beobachtung, war eine spezifische Lokalisierung von FVIII (exprimiert von dem Nicht-Targeting-Vektor, mPF4p.hBDcoFVIII) hauptsächlich in den Alpha Granulat, die den von Willebrand-Faktor enthielten. Dies ist wichtig zu erwähnen, da kein Targeting-Signal in diesem Konstrukt enthalten war. Es wurde jedoch bereits in früheren Studien beschrieben, dass eine Überexpression von FVIII in verschiedenen Zelllinien in Gegenwart des von Willebrand Faktors zu einer Modifikation des FVIII führt, die den molekular Transport innerhalb der Zelle verändert, und deshalb in einer Speicherung des Gerinnungsfaktors in den sekretorischen Granula als lösliches Protein führt.

Genetisch modifizierte Thrombozyten

Knochenmarktransplantationen in Wildtyp-C57BL/6J-Empfängermäuse wurden durch Transfusion von Linienmarker-negativen Zellen durchgeführt, die in vitro mit den folgenden gezielte Vektoren transduziert wurden: mPf4.RANTES.d2eGFP P-sel.Sig.pep.d2eGFP.TDCT, mPf4.hBDcoFVIII_TDCT (FVIII_TDCT) und den nicht-gezielten-mPf4.hBDcoFVIII- (FVIII) und PF4.d2eGFP-Kontrollvektoren. Zwölf Wochen nach der Transplantation wurde ein Leukozyten-Chimärismus in transplantierten Mäusen zwischen 2 und 20 Prozent beobachtet, und die Transgen-Expression in Plättchen variierte im Bereich von 1 bis 3 Prozent. Obwohl das gesamte Anwachsen der Spenderzellen sehr gering war, war die Transgenexpression in einigen der transplantierten Mäuse ausreichend, um eine Einzelzellanalyse mittels LSM durchzuführen und vorläufige Daten über das gezielte Targeting in Thrombozyten *in vivo* zu erhalten. Die durchgeführte Kolokalisation- und Fluoreszenzintensitätsanalyse von aktivierten Blutplättchen bestätigte, was zuvor bei in-vitro differnezierten Megakaryozyten beobachtet wurde. Die fehlende Kolokalisation von dem P-sel.d2eGFP- und FVIII_TDCT-Vektor mit dem alpha-Granula-Marker von Willebrand-Faktor, die in aktivierten Plättchen beobachtet wurde, war ein wichtiger Hinweis der Protein-Translokation zur Zelloberfläche der Blutplättchen.

Schlussfolgerungen und Diskussion

In diesem Forschungsprojekt konnten wir 6 wichtige Megakaryozyten-spezifische Promotofragmente in lentiviralen Vektoren gründlich charakterisieren, in Bezug auf die

Spezifität und Aktivität in Megakaryozyten und nicht-megakaryocytischen Zellen, ebenso in den hämatopoetischen Stammzellen und Blutzellen-Vorläufern. In den Mausexperimenten konnten wir die Wichtigkeit der Verwendung von Linien-spezifischen Promotoren im Kontext einer Knochenmarktransplantation demonstrieren. Der signifikante Anstieg der Thrombozytenzahlen in thrombozytopenischen Mpl-Knockout-Mäusen zeigte, dass genetische Veränderungen in Zellen (in diesem speziellen Fall HSCs und Thrombozyten) verwendet werden können, um Funktionen zu modifizieren und sie möglicherweise für therapeutische Zwecke zu nutzen. In ähnlicher Weise zeigten Experimente, die mit Targeting-Vektoren durchgeführt wurden, dass der Einsatz von Signalpeptiden zur gewünschten Lokalisation in den Zielzellen führte, während die Kontrollvektoren die erwartete unspezifische Lokalisation zeigten. Dennoch sollten weitere Experimente, einschließlich funktioneller Gerinnungstests, ELISAs und Elektronenmikroskopie durchgeführt werden, um diese Ergebnisse weiter zu bestätigen und zu bekräftigen. Aus therapeutischer Sicht ist dieses Modell eine Verbesserung und Verfeinerung für eine gezielte, transgene Expression, die es ermöglichen sollte, in Zukunft auch therapeutische Proteine einzuführen, deren Lagerung und Freisetzung streng kontrolliert werden kann. Dies ist besonders wichtig in Fällen, in denen die Modulation von sehr spezifischen und punktuellen physiologischen Prozessen wie Gerinnung, Angiogenese, Gewebereparatur und Entzündung stattfindet. Aufgrund der zahlreichen physiologischen Prozesse, an denen Thrombozyten teilnehmen, sind experimentelle Therapien mit Megakaryozyten oder Thrombozyten nun möglich geworden. Dabei müssen nicht unbedingt Knochenmarktransplantationen eingesetzt werden, sondern es sind auch Ansätze durch die genetische Modifikation von MK-Vorläuferzellen denkbar, falls ein potentieller therapeutischer Effekt nur zeitlich begrenzt erwünscht ist. Weitere Anwendungen diesen Targeting-Modellen können auf die Korrektur von FVIII- Mängel bei Patienten mit FVIII-Inhibitoren fokussiert werden. Die genetische Modifikation von Megakaryozyten und Thrombozyten stellt einen interessanten und wichtigen zellmodellbasierten Ansatz dar, der nicht nur die Entwicklung einer neuen Generation von Zellen mit erweiterten Funktionen ermöglichen wird, sondern auch uns dabei helfen wird, neue Mechanismen und Wege wichtiger zellulärer Prozesse aufzuklären, indem wir Zellfunktionen und Zellinteraktionen modifizieren.

Chapter 1:

- Introduction
- Aims

1. Introduction

1.1. Hematopoietic Stem Cell (HSCs) and Hematopoiesis.

The blood is a fluidic tissue constituted by a cellular and a biochemical component, all immerse in a semi viscous liquid that freely circulate within the blood vessels in all the body. The cellular part is constituted by cells which differ in function, but share a common ancestor cell, also known as hematopoietic stem cell (HSCs), which differentiate itself into different hematopoietic blood progenitors in the bone marrow, by means of a process called haematopoiesis, to allow the constant and regulated blood production and reconstitution. The first experimental evidence that indicated the existence of hematopoietic stem cells (HSCs), was the discovery in 1961 by Till and McCulloch of a population of bone marrow (BM) cells capable of generating myeloid and erythroid colonies in the spleen of lethally irradiated hosts (Till and McCulloch, 1961). In the classical model of hematopoiesis, stem cells are defined as clonogenic cells capable of both self-renewal and multilineage differentiation, leading to the generation of all functionally mature effector cells in the blood. Hitherto, self-renewing progenitors have been isolated from the bone marrow in both humans and mice using multi-color fluorescence-activated cell sorting and monoclonal antibodies (Spangrude et al., 1988; Baum et al., 1992; Morrison and Weissman, 1994; Osawa et al., 1996). Spangrude and colleagues were the first ones to demonstrate that only the population of mouse HSCs with a surface marker phenotype of Thy-1^{low} Lin (Lineage-marker)^{neg} and stem cell antigen (Sca-1)$^{+}$, representing approximately just 0.05% of the mouse adult BM cells, were the only cells in mouse BM capable of transferring long-term reconstitution of the entire hematopoietic system, (defined as more than 4 months), after their transplantation into lethally irradiated mice. Later, in 1994 the cell population previously isolated by Spangrude et al, was shown to be constituted by at least 3 multipotent populations: the Long-Term (LT)-HSC shown as Lin$^{neg/low}$, Thy1.1^{low}, c-Kit^{high}, Sca-1^{+}, Flk2^{neg}, Short-Term (ST)-HSC, shown as Lin$^{neg/low}$, Thy1.1^{low}, c-Kit^{high}, Sca-1^{+}, Flk2^{+} and Multi-Potent Progenitor (MPP) (Lin$^{neg/low}$, c-Kit^{high}, and Sca-1^{neg}), a cell population that has lost the self-renewal capacity of HSC and with a mixed lineage progenitor committed (Morrison and Weissman, 1994). The loss of Thy-1.1 and gain of Flk-2 expression marks

the loss of self-renewal in HSC maturation (Christensen and Weissman, 2001). The cells surface molecule CD34 expression in the human HSCs (characterized as CD34+/CD38−/Lineage−) and it absences in the murine HSCs (CD34-/CD38+/Lineage−), represent the biggest difference between these 2 counterparts, in both cases representing <1% from the functional HSCs (Osawa et al., 1996; Dick, 2008).

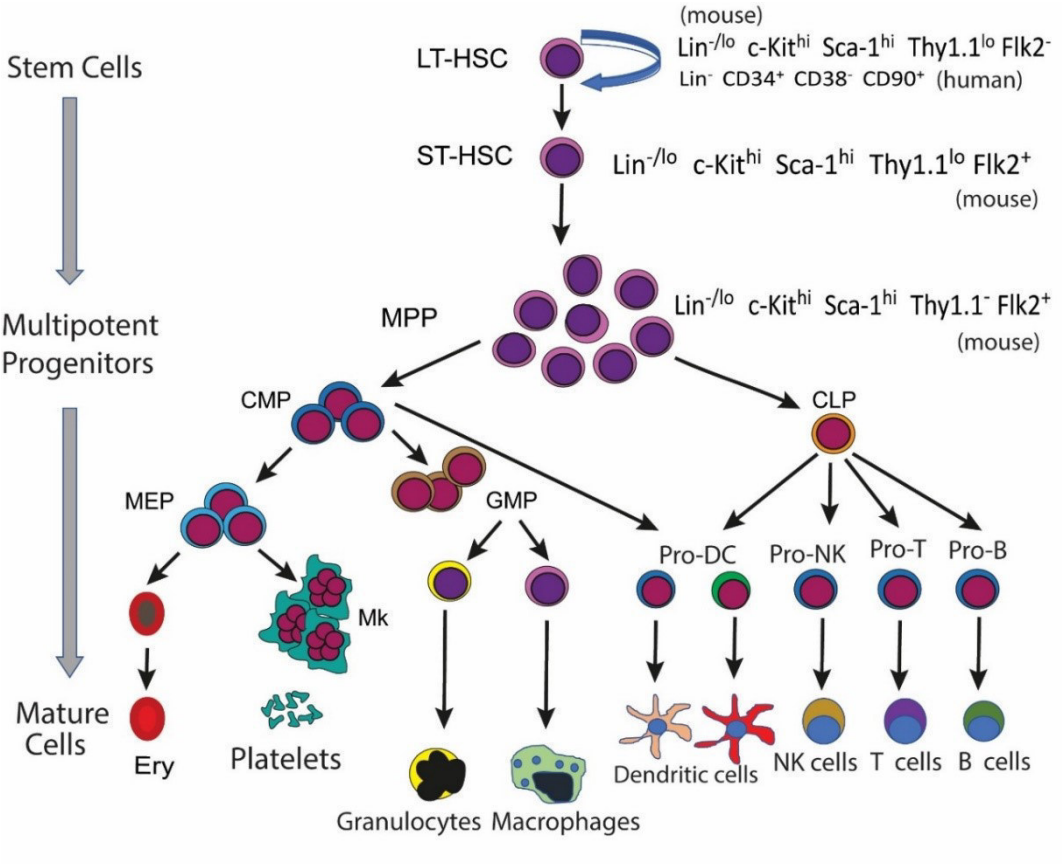


Figure 1.1. Hematopoietic stem and progenitor cells and mature blood cell lineages. HSCs can be divided into highly self-renewing LT-HSCs, and ST-HSCs, which reconstitute hematopoiesis for only a limited period. Both can differentiate into MPPs, and further to oligo-lineage-restricted progenitors: The CLPs, which give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells, and the CMPs, which give rise to GMPs that differentiate into monocytes/macrophages and granulocytes, and to megakaryocytic-erythroid progenitors (MEP), which generate megakaryocytes-platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells. All of these stem and progenitor cell populations can be separated as pure populations based on their cell surface marker expression. (picture was taken and modified from (Passegué et al., 2003).

So far, two kinds of oligolineage-restricted progenitors, derived from the multi-myeloid progenitors (MPP), have been identified in the mouse: The CLPs and the CMPs. The common lymphoid progenitors (CLPs), are restricted to give rise to T lymphocytes, B lymphocytes, and natural killer cells (Kondo et al., 1997). The common myeloid progenitors (CMPs), are committed for the myelo-erythroid lineages and give rise to granulocyte-monocytic progenitors (GMPs), which in turn produce monocytes-macrophages and granulocytes, and the megakaryocytic-erythroid progenitors (MEPs), which differentiate into megakaryocytes-platelets and erythrocyte, but still maintaining the potential for B cell lineage differentiation at an extremely low frequency (Akashi et al., 2000). Interestingly, both CMPs and CLPs can give rise to dendritic cells (Traver et al., 2000; Manz et al., 2001), suggesting the existence of alternative commitment pathways for myeloid and lymphoid lineages. The molecular mechanisms that regulates lineage commitment within the hematopoietic system, appear to be regulated by the alternate expression of specific transcriptional regulators, growth factors, and growth factor receptors, whose combination determines the lineage commitment and maturation (Orkin, 2000; Zhu and Emerson, 2002). In consequence, the mammalian blood system is a complex and multifunctional tissue constituted by more than ten distinct groups of mature cell types. (See figure 1.1). The main groups of cells that conform the blood are: the red cells (or erythrocytes) in charge of the transport of oxygen, white cells (or leukocytes) whose main functions are focused on the development and execution of the immune response and involve cells like basophils, eosinophils, neutrophils, lymphocytes and monocytes. The third group of cells are the platelets or thrombocytes, which are developed from megakaryocytes (MKs) and whose function are more related to the maintenance of the coagulation system. More recently, they have been associated in process like vessel development and maintenance, antigen presentation and immune response.

In a study performed by Velten and collaborators in 2017, by integrating flow cytometry analysis, transcriptomic and functional data at single-cell resolution, they were able to quantitatively map early differentiation of human HSCs towards lineage commitment. In this sense, they claimed that during homeostasis, individual HSCs can be

gradually biased into multiple directions without passing through hierarchically organized progenitor populations. Instead, lineage-restricted cells can emerge directly from a "Continuum of Low Primed Undifferentiated Hematopoietic Stem- and Progenitor Cells" (CLOUD-HSPCs). This process would be controlled by distinct gene expression sets which operate in a combinatorial manner to control stemness, early lineage priming and the subsequent progression into all major lineage of hematopoiesis (Velten et al., 2017). These results are supported by other previous studies where the new concept of "HSCs-biased" is suggested, showing by this, new alternative pathways of hematopoiesis (Challen et al., 2010; Gekas and Graf, 2013; Matatall et al., 2014; Miyawaki et al., 2015; Luchsinger et al., 2016) Based on this, it is important to highlight, that the model of hematopoietic differentiation hierarchy clearly only reflects current knowledge and will continue to change over time.

1.2. Origin of platelets: Megakaryopoiesis.

Megakaryocytes have been recognized as rare marrow cells for nearly 2 centuries, but it was just after the study with elegant *camera lucida* from Howell in 1890 that the term of "megakaryocyte" was for the first time established and identified as a cell entity (Howell, 1890). Later, in 1906 James Homer Wright suggested that blood platelets, at that time referred as "plates" are derived from the cytoplasm of megakaryocytes, (Wright, 1906; Brown, 1913). Since then much has been learned about the origin of platelets. Megakaryocytes, like all blood cells, derive from hematopoietic stem cells following a strict controlled process, regulated by the bone marrow surrounded microenvironment, expression of megakaryocyte-specific transcription factors, and the interaction of cytokines with their corresponding cell surface receptors, which modulate a complex cellular remodeling, that leads to the expansion, maturation of megakaryocytes and release of platelets.

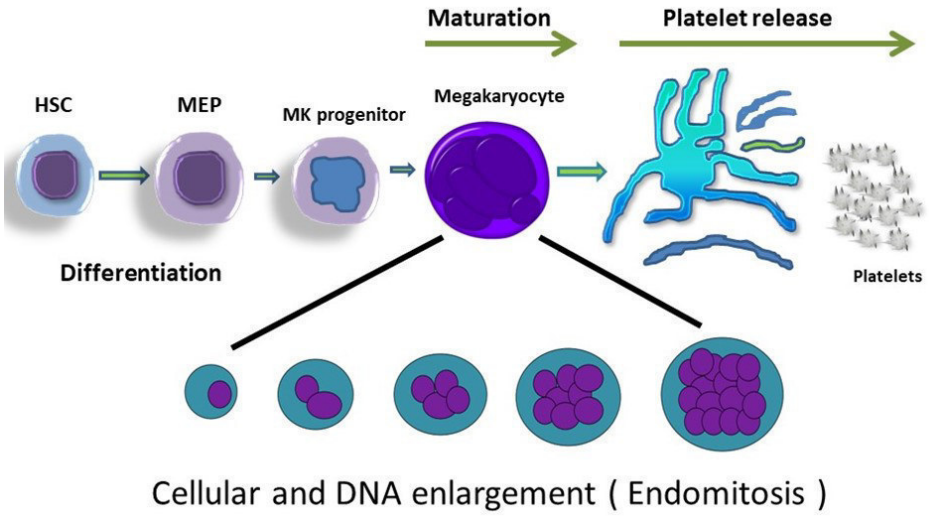


Figure 1.2. Platelets are developed from the megakaryocytes in the bone marrow. Megakaryopoiesis begins with the differentiation of the HSCs, leading the generation of the megakaryocytes and erythroid progenitors (MEPs). Megakaryocyte progenitors differentiate into megakaryocytes. To mature, megakaryocytes must increase in size and DNA content (polyploidy), by undergoing a remarkable number of cell cycles in a process called endomitosis.

1.3. Transcriptional control of megakaryopoiesis

Megakaryopoiesis is modulated by multiple extrinsic and intrinsic signals, as well as by complex network pathways that regulate the differentiation both positively and negatively. All these cellular changes are mostly controlled by transcription factors. Some of the reported transcription factors include GATA-1, Friend of GATA-1 (FOG-1), GATA-2, Fli-1, PU.1, NF-E2, and RUNX1. Function of transcription factors have been elucidated by in vivo and in vitro models. For instance, depletion of RUNX1 results in an up-regulated expression of megakaryocytic markers and polyploidization with a downregulation of cell proliferation. Its over expression decreased the activity of the megakaryocytic gene promoters, suggesting that RUNX1 down-regulates the terminal differentiation of MKs and promotes the proliferation of megakaryocytic progenitors (Nagai et al., 2006). On the other hand, it has also been shown that RUNX1 interacts with other transcription factors such as GATA-1 (Elagib et al., 2003; Xu et al., 2006) and FLI-1 (Huang et al., 2009). Another

transcription factor is GFI1B, identified as an important factor for erythropoiesis and megakaryopoiesis, due to the development of erythroid and megakaryocyte disorders (macrothrombocytopenia, red cell anisopoikilocytosis and platelet dysfunctions with almost absent α-granules and decreased platelet aggregation responses) in cases of autosomal dominant mutations in the GFI1B gene (Stevenson et al., 2013). Others transcription factors like TAL-1 or SCL, have been detected to interact with GATA-1 in a large complex involved in the regulation of erythroid genes. TAL-1 for example, is expressed in MKs (Mouthon et al., 1993) and plays a major role in platelet production during stress thrombopoiesis by regulating NF-E2 transcriptional activity (McCormack et al., 2006). So is the case of other members from the ETS family such as TEL-1 and ETS1 which also regulate MK maturation. Terminal MK maturation and platelet production are governed by NF-E2, which is regulated by GATA-1 and SCL (Takayama et al., 2010; Krumsiek et al., 2011). In this sense, overexpression of NF-E2 selectively enhances MK maturation, proplatelet formation, and platelet release. In addition, NF-E2 overexpression increases MK commitment during early megakaryopoiesis, while inhibiting white blood cell differentiation (Fock et al., 2008). Deletion of Nf-e2 in mice leads to profound thrombocytopenia (Shivdasani et al., 1995). Other factors like c-Myb, have been reported to inhibit megakaryopoiesis (Carpinelli et al., 2004; Metcalf et al., 2005), by acting in cooperation with GATA-1 to regulate megakaryocytic differentiation and production. Further, this proto-oncogene favors erythropoiesis, and its expression is down-regulated during megakaryopoiesis.

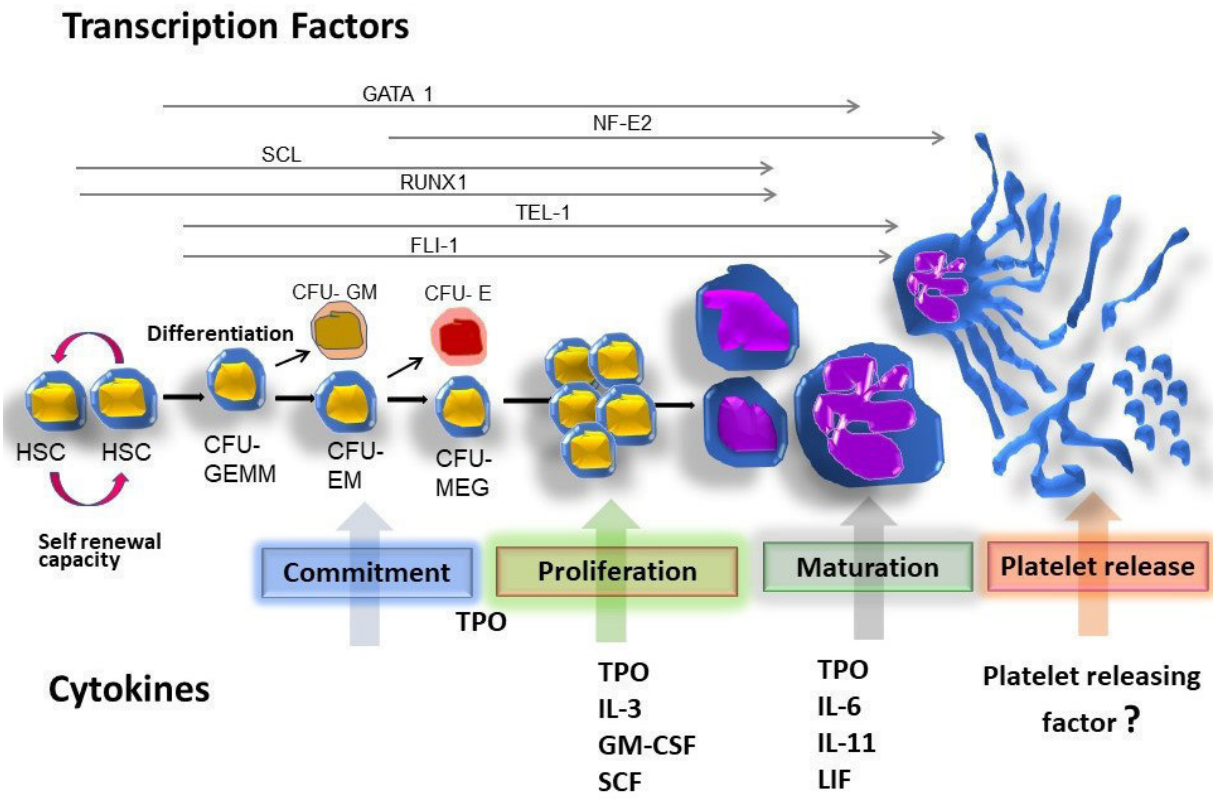


Figure 1.3. Regulatory factors during megakaryopoiesis. As it shown in the figure, numerous transcription factors and cytokines coordinate and modulate the process of differentiation and expansion of megakaryocytes, by interact during different stages in megakaryopiesis. Modified from (Chang et al., 2007; Deutsch and Tomer, 2013).

1.4. Cytokines that regulate platelet formation

The processes of megakaryopoiesis and platelet production occur in a highly specialized bone marrow microenvironment within osteoblastic and vascular niches (Avecilla et al., 2004). Gradients of chemokines, growth factors, calcium, oxygen and adhesive interactions regulate megakaryopoiesis and MK migration (Yoshihara et al., 2007; Deutsch et al., 2010). Thrombopoietin (THPO) is a primary cytokine for the regulation of thombopoiesis and megakaryopoiesis (Kaushansky et al., 1994; Kuter et al., 1994; Kato et al., 1995). Its binding to its surface receptor, c-Mpl, activates the downstream signaling PI-3 kinase-Akt (Pulikkan et al., 2012), MAPK (ERK1/ERK2) (Drachman et al., 1997; Kirito et al., 2003) and STAT pathways (Garcia et al., 2001; Reich and Liu, 2006).

However, non-THPO pathways, such as the SDF1/CXCR4 axis, Notch signaling, src family kinases, and Platelet Factor 4/low-density lipoprotein receptor-related protein 1, have more recently been recognized to also modulate megakaryopoiesis and thrombopoiesis in vitro and in vivo. THPO signaling pathways are therefore central, but not indispensable. In vivo, thrombopoietin stimulates platelet production without affecting the peripheral blood red or white cell counts (Kaushansky et al., 1994). Thrombopoietin contributes as well to the development of hematopoietic stem cells (HSC), supporting their survival and proliferation in vivo and vitro, promoting self-renewal and expansion of HSCs following marrow transplantation (Fox et al., 2002). Likewise, gene suppression c-Mpl or Thpo, leads to a severe thrombocytopenia in mice, reduction of megakaryocyte progenitors with impairment of polyploidization (Solar et al., 1998). A more severe phenotype is observed in humans, where patients displaying homozygous or heterozygous genetic mutations in condition in nonsense or missense mutations in the MPL gene; specifically those leading to a loss-of-function in the MPL gene, were found to be the cause of congenital amegakaryocytic thrombocytopenia (CAMT) (Ihara et al., 1999; Ballmaier et al., 2001); an inherited hematological disorder characterized by an initial severe thrombocytopenia and a hypomegakaryocytic bone marrow after birth of the patients, leading to a progressive multi-lineage pancytopenia or aplastic anemia which is lethal without bone a marrow transplant. The progression of the pancytopenia depends on the type of mutation of MPL (van den Oudenrijn, S et al., 2000; Ballmaier et al., 2001; Germeshausen et al., 2006). To dissect the cellular basis for megakaryopoiesis, culture conditions that support the proliferation of megakaryocytic progenitors in vitro were established in the late 1970s for both mouse and human, using several semisolid media. Since THPO has been reported, it has been used to generate enriched populations of MKs using in vitro differentiation systems. In this sense, MKs and platelets have been differentiated from lineage marker-BM cells, fetal liver cells (Mazharian et al., 2009), BM stroma cells (Matsubara et al., 2013) embryonic stem (ES) cells (Eto et al., 2002; Fujimoto et al., 2003; Gaur et al., 2006; Takayama et al., 2008) and induced pluripotent stem (iPS) cells (Nishimura et al., 2013; Feng et al., 2014; Nakamura et al., 2014; Liu et al., 2015).

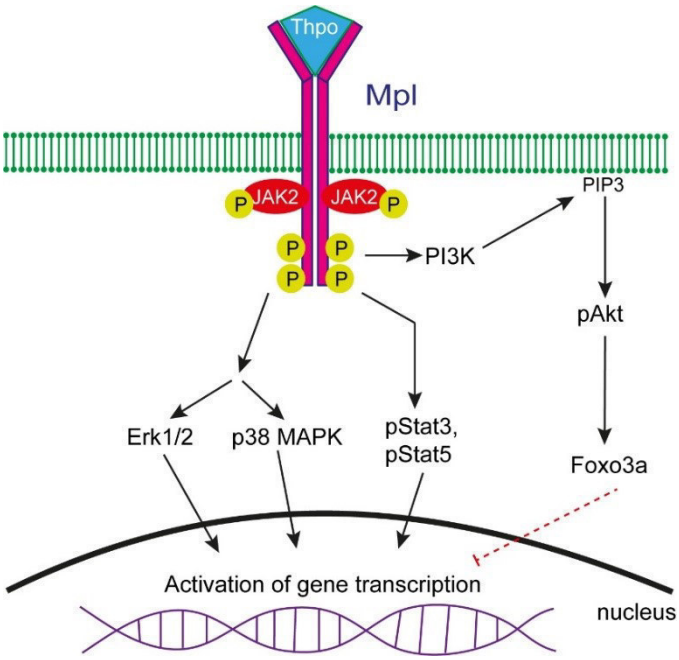


Figure 1.4. Thpo/Mpl pathway. Once Thpo binds and activates the Mpl on the stem cells and MKs, conformational changes initiate signal transduction via activation of the Janus kinases JAK2 phosphorylating the tyrosine residues on the Mpl cytoplasmic tail and activating multiple downstream signaling pathways, including (STAT3, STAT5), phosphoi- nositol-3-kinase (PI3K-pAkt), RAS/RAF/MEK, the mitogen-activated protein kinases (MAPKs like ERK1/2, p38 MAPK), and some isoforms of protein kinase (PK) C (Kaushansky, 2009). This leads to the initiation of transcription of numerous genes which are important for cell division, differentiation and survival. Negative regulation of the receptor complex occurs following stimulation with THPO. The receptor complex undergoes rapid clearance from the cell surface by clathrin-dependent endocytosis and Mpl can be recycled or subsequently ubiquinated and degraded by Cbl (CBL, Casitas B-lineage Lymphoma protein) through its E3 ubiquitin ligase activity. Figure extracted and modified from (Chotinantakul and Leeanansaksiri, 2012) and reviewed as well from (Saur et al., 2010; Teofili and Larocca, 2011).

1.5. Megakaryocyte maturation

During maturation, megakaryocytes undergo cellular remodeling including cell enlargement, multiplication of the DNA content and pseudopod development. One of the hallmarks for megakaryocyte maturation is the polyploidization. To achieve this, megakaryocytes undergo endomitosis without cell division (Ravid et al., 2002). In this case mitosis is aborted in late anaphase with failure of both karyokinesis and cytokinesis,

(Geddis and Kaushansky, 2006; Lordier et al., 2008). This allows the cell to produce large amounts of proteins and organelles necessary to assemble functional platelets (Deutsch and Tomer, 2006; Bluteau et al., 2009). Polyploid megakaryocytes in the bone marrow typically achieve a DNA content of up to 128N and each megakaryocyte may release between 2000 and 5000 platelets (Stenberg and Levin, 1989; Lordier et al., 2012). Small GTPase such as RhoA play in this regard a multifaceted role in MK maturation. Rho-associated protein kinase (ROCK) inhibition promoted demarcation membrane system (DMS) formation, and increased proplatelet formation and platelet release. Rho kinase inhibition also downregulated cMYC and NF-E2 and this down-regulation correlated with increased proplatelet formation (Gao et al., 2012; Avanzi et al., 2014). Moreover, cell cycle regulators play a role during MKs maturation and endomitosis regulation, such as D-type cyclins like CKD1 and CKD3, by promoting polyploidization when overexpressed (Sun et al., 2001). Cyclin E and cyclin A promotes progression to S phase and cell cycling increasing with this in the number of cell cycling cells and augmenting polyploidization (Baccini et al., 2001; Gilles et al., 2008; Eliades et al., 2010).

Cytoplasm remodeling is another hallmark for megakaryocytes maturation. In polyploid Mks, the cytoskeleton is the principal machinery for cytoplasm maturation and platelet production (Tablin et al., 1990; Hartwig and Italiano, 2006; Thon et al., 2010). In this sense, β1-Tubulin, the main tubulin isoform in MKs, is essential for proplatelet formation and elongation by using cytoplasmic dynein, a microtubule minus end–associated motor protein (Lecine et al., 2000; Patel et al., 2005b). Likewise, F-actin is required for the bending and bifurcation of proplatelet extension (Italiano et al., 1999; Patel et al., 2005b). During cytoplasm maturation, microtubules participate in the transportation of organelles and granules into the proplatelet extensions. In the figure 1.5 the process of subcellular remodeling of MK maturation is illustrated

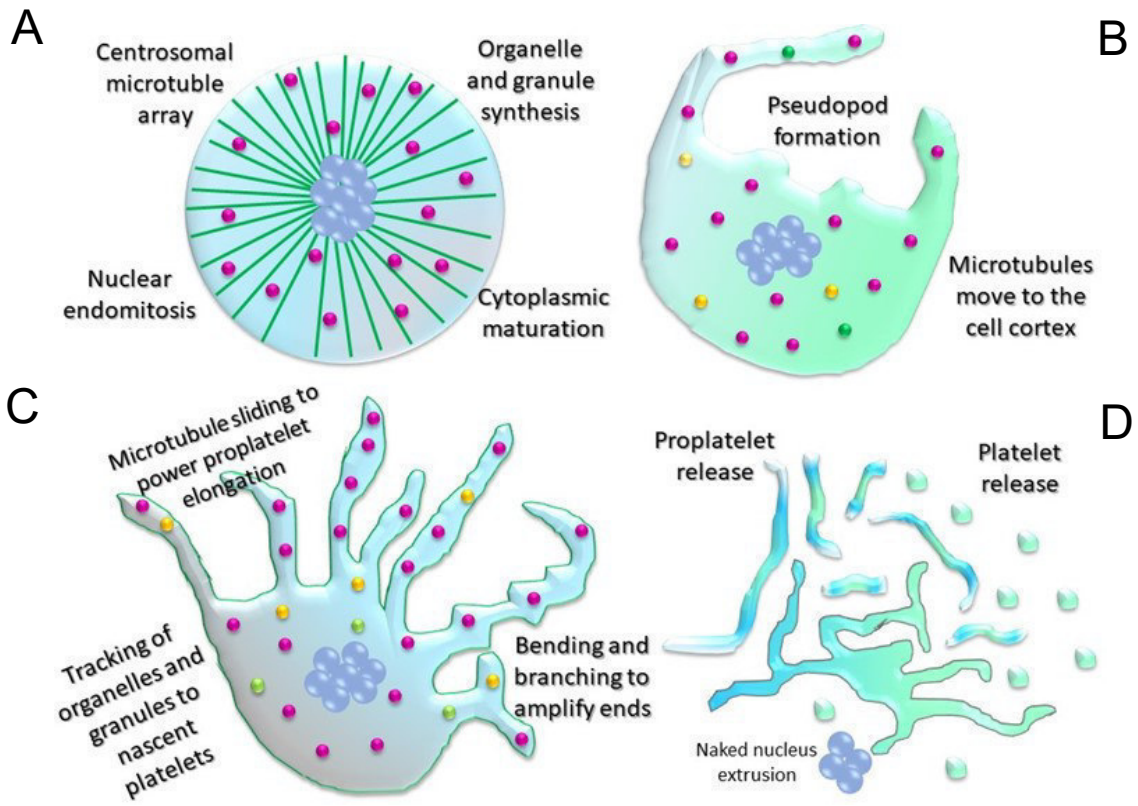


Figure 1.5. Overview of megakaryocyte maturation and platelet production. During maturation of megakaryocyte progenitors, centrosomes disassemble and microtubules extend to all the cellular space (A). Cytoplasm maturation begins with the cellular enlargement and development of thick pseudopods (B), which continues expanding throughout the cell, while bending and branching helps on amplifying proplatelet extension. This is possible, due to the movements of overlapping microtubules that powers proplatelet elongation, while simultaneously transporting and tracking organelles to proplatelet ends (C). Mature megakaryocytes move to the perivascular niche. The entire megakaryocyte cytoplasm is turned in its majority into an extended proplatelet elongations, which are released from the cell by budding from the proplatelets tips with the help of the bloodstream. Taken and modified from (Patel et al., 2005a).

1.6. Biogenesis of secretory granules

Megakaryocytic granules present a heterogenic granule population, which differ in number, structure and content. Initially, based on the characteristics observed with transmission electron microscopy (TEM) as well as by their content, 3 major granules have been described so far: alpha-granules (α-granules), dense-granules (δ-granules) and lysosomes, while more recently further granules such as T-granules were described.

The development of secretory granules begins in the megakaryocytes by biosynthetic pathways and continues later on during megakaryocyte maturation as in the circulating platelets as well (Zucker-Franklin, 1981; Blair and Flaumenhaft, 2009; Chen et al., 2017a; Hanby et al., 2017). Small vesicles budding from the trans-Golgi network (TGN) can be delivered directly to multivesicular bodies (MVB), where the cargo proteins are selected, sorted and eventually packaged into granules (Heijnen et al., 1998; Youssefian and Cramer, 2000). Previous studies suggest that MVBs represent an intermediate stage in the formation of secretory granules (Heijnen et al., 1998). it has been described that MVB contain molecules that are typically sorted to alpha granules (e.g. β-thromboglobulin, vWF), dense granules (CD63) and lysosomes (Youssefian and Cramer, 2000). Circulating proteins can be incorporated in secretory granules byendocytosis in platelets and megakaryocytes (Handagama et al., 1987). This process allows some plasma proteins to be temporarily stored in megakaryocytes and platelets before going back to the circulation (Banerjee and Whiteheart, 2017). Both alpha and dense granules develop from endocytic and synthetic pathways using MVBs as intermediaries but using different machinery.

Origen of alpha granules (α-granules)

Alpha granule biogenesis involves coat proteins such as clathrin (Miwako et al., 2003; Ohno, 2006; Ungewickell and Hinrichsen, 2007), adaptor proteins AP1 and AP2 (Nakatsu and Ohno, 2003; Ohno, 2006; Park and Guo, 2014), and proteins required for vesicle trafficking, including soluble *N*-ethylmaleimide-sensitive factor (NSF), attachment protein receptor (SNARE) proteins, SNARE regulators, particularly Sec1/Munc18 proteins like VPS33B and VPS16B (Lo et al., 2005; Urban et al., 2012; Bem et al., 2015) , small GTPases such as Rabs (Rab4) (Shirakawa et al., 2000), and BEACH domain containing proteins such as NBEAL-2 (Gunay-Aygun et al., 2011; Kahr et al., 2011; Kahr et al., 2013). The vesicles are coated with soluble clathrin molecules which are recruited to the TGN by interacting with coat proteins, such as the adaptor protein 1 (AP1) to form the coat. Emerging vesicles that are then directed to early endosomes and finally to MVB where they mature (pathway shown with black arrows in figure 1,6). This process requires VPS33B, VPS16B, and NBEAL2, (shown with green arrows in figure 1.6,) (Urban et al., 2012; Chen

et al., 2017a; Sharda and Flaumenhaft, 2018)In the endocytic pathway, proteins from the extracellular space are invaginated to form endocytic vesicles at the plasma membrane by means of adaptor protein 2 (AP2) and clathrin. Invaginated endocytic vesicles are transported to early endosomes and finally to MVBs.

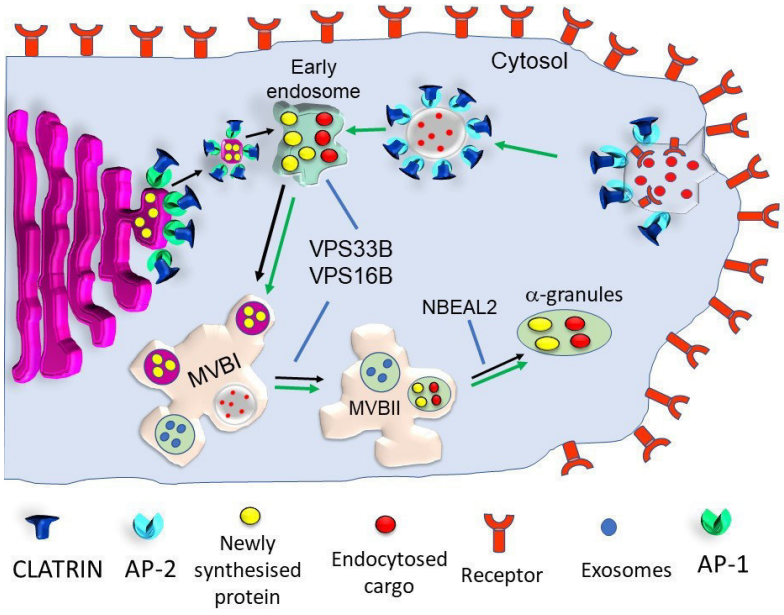


Figure 1.6. Model of alpha granule biogenesis. Biosynthetic pathway is indicated with a black arrow. Endocytic pathway is shown with a green arrow. Figure taken and adapted from (Sharda and Flaumenhaft, 2018).

In the alpha granules, endocytosis can be mediated by Adenosine 5'-diphosphate-ribosylation factor 6 (Arf6), IgG, albumin and receptor-mediated uptake (cellubrevin/vesicle-associated membrane protein-3 ,VAMP-3) for molecules such as fibrinogen (mediated by integrin αIIbβ3 or glycoprotein IIb–IIIa) (Handagama et al., 1987; Harrison et al., 1989; Huang et al., 2016; Banerjee et al., 2017), and von Willebrand Factor (vWF). This endocytosis would then also represent a platelet recycle mechanism which allows the alpha-granules membrane to acquire more similarity with the plasma membrane, exposing on their surface platelets-specific transmembrane proteins (Berger et al., 1996).

Origin of dense granules

Platelet dense granules (DGs) belong to the family of lysosome-related organelles (LROs), which are derived from the endosomal compartment (Meng et al., 2012). Most of the current knowledge about their development comes from extrapolatory studies in melanosomes and analyses of platelets in syndromes such as Hermansky-Pudlak syndrome (HPS, caused by mutation in several genes: HPS1, HPS3, HPS4, HPS5, HPS6 and HPS7, AP3B, DTNBP1 and BLOC1S3) and Chediak-Higashi syndrome (disorder that arises from a mutation in a lysosomal trafficking regulator, LYST, coded by the CHS1 gene) (Ward et al., 2000; Wei, 2006; Huizing et al., 2008). In DG development, MVB act as intermediate step in their biogenesis. In this regard maturation and differentiation into DG begins when the newly synthesized dense-granules specific proteins are delivered within immature vesicles from early endosomal compartments to the MVBs. (Ambrosio and Di Pietro, 2017). In analogy to what has been described in melanosomes, during dense granule development Rab38 and Rab32 interact with AP-3, AP-1, and BLOC-2 at early endosome membrane domains where the cargo is concentrated and packaged into intermediates or immature transport vesicles, see figure 1.7 (Bultema et al., 2012; Bultema and Di Pietro, 2013). Dense granule proteins such as bioactive amines and adenine nucleotides, are further transported into the maturing dense granules by specific membrane pumps, known as vesicular nucleotide transporter (VNUT), which has been proposed as a candidate for ADP and ATP accumulation in dense granules. Interaction of BLOC1 (biogenesis of lysosome-related organelles complex of HPS7, HPS8, HPS9, Muted, Cappuccino, Snapin, BLOS2, and BLOS3) with the AP-3 is required for the exit of maturing vesicles from endosomes by this tubular transport carriers (Sitaram et al., 2012). During this process, once the maturing vesicles leave the endosomal compartment, some components of the vesicle coats such as AP-3, AP-1, and clathrin dissociate from the budding vesicle, but some others like Rab32, Rab38, and possibly BLOC-2 (complex of HPS3, HPS5, and HPS6) remain to mediate further transport to maturing granules I, II and III stages, see figure 1.7 (Ambrosio et al., 2012; Sharda and Flaumenhaft, 2018).

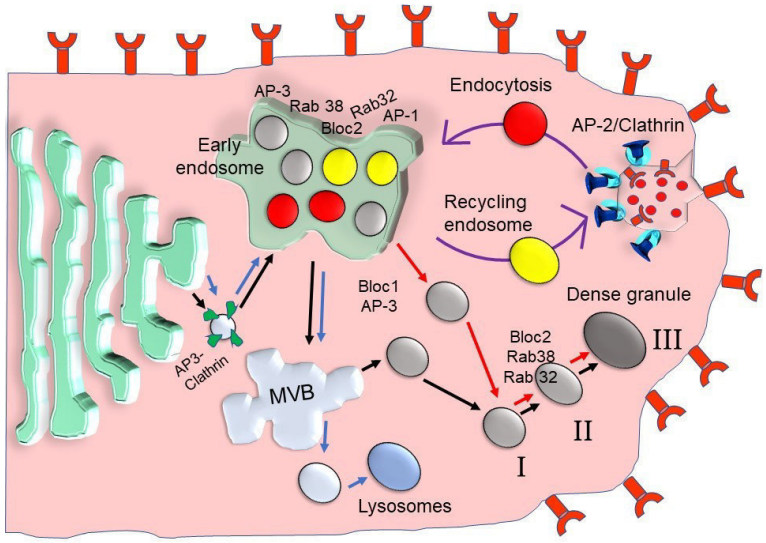


Figure 1.7. Dense granules development. Endocytic pathway of dense granules development is shown with purple arrows. Extracellular cargo is incorporated inside of AP-2/ clathrin coated vesicles and transport to early endosomes. In the synthetic pathway (in black arrows), AP-3 clathrin coated vesicles with newly synthesized cargo are transported to early endosomes and then to the MVBs. An alternative mechanism, highlighted in red arrows, has been also described where immature vesicles emerge directly from early endosomes without passing through MVBs as an intermediate step. Figure modified and adapted from (Ambrosio et al., 2012; Bultema and Di Pietro, 2013; Sharda and Flaumenhaft, 2018).

Transport of secretory granules

As megakaryocytes enlarge with maturation, a demarcating membrane system develops and granules are transported into the developing pro-platelet. Organelles and granules are transported and sent from the MK cell body to the proplatelet extensions, by means of bidirectionall movements mediated by the cellular cytoskeleton until they arrive to the proplatelet tips. Microtubules develop a bipolar arrangement within the proplatelet, which contributes to the bidirectional organelle movement. The plus end–directed microtubule motor kinesin participates as well in the transport of organelles and granules along the microtubules. These structures serve as tracks by which mitochondria and granules move at a rate of 0.1-0.2 μm/min (Richardson et al., 2005), Figure 1.8 (A).

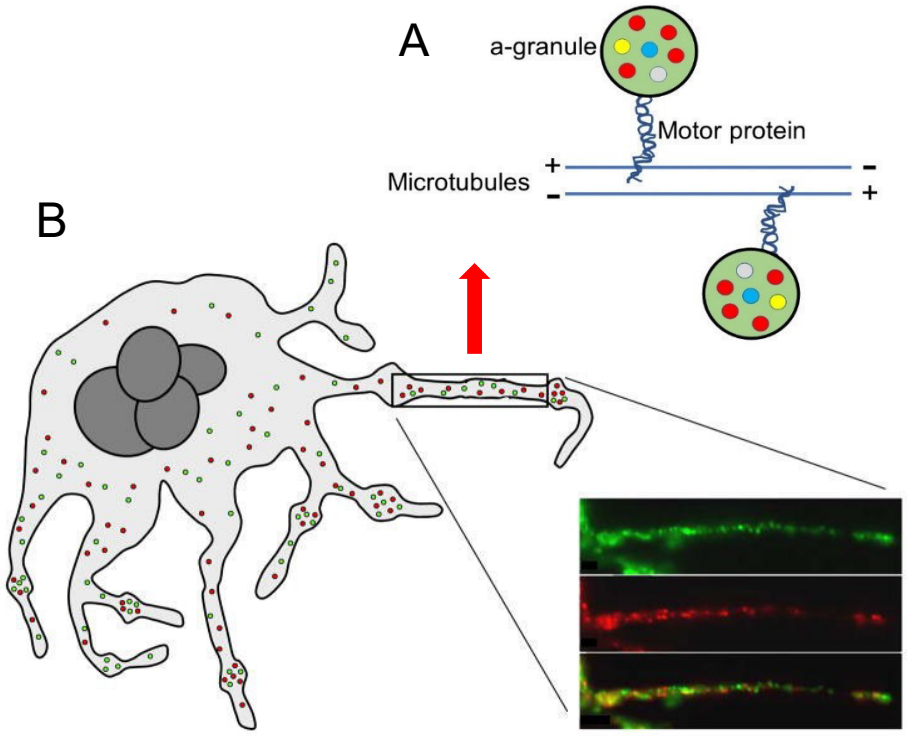


Figure 1.8. Model of transport of α–granules during platelet formation. Transport of secretory granules through the microtubular network (A). Platelet α–granules are transported along microtubules from the megakaryocyte cell body through long pseudopodal extensions called proplatelets with the help of Motor proteins. α–Granules are maintained in the nascent platelets by coiled microtubules. (B) Subpopulations of α–granules containing distinct cargos are transported along within proplatelet extensions. α–Granules containing fibrinogen are shown in green, while those containing vWf are shown in red, colocalization of both proteins is shown in yellow. Pictures taken and modified from (Patel et al., 2005a; Italiano et al., 2008; Goubau et al., 2013)

Although microtubule highways appear to transport granules and organelles long distances along proplatelets, the mechanism by which the necessary organelles and granules are packaged into platelets remains unclear. In addition, the idea that granules may be sorted into heterogeneous populations in platelets still remains under discussion (Italiano et al., 2008; Kamykowski et al., 2011; Jonnalagadda et al., 2012). In this regard, recent studies have identified heterogeneity in the α-granule morphology, membrane protein composition as well as granule motility during platelet activation (van Nispen tot Pannerden et al., 2010; Peters et al., 2012), see figure 1.8 B. A MK may extend 10–20 proplatelets, each of which

starts as a blunt protrusion that over time elongates, thins, and branches repeatedly. Platelets form selectively at the tips of proplatelets (Richardson et al., 2005), see figure 1.8. As platelets develop, they receive their granule and organelle content as streams of individual particles transported from the MK cell body. Platelets are finally released to the peripheral circulation by fragmentation of proplatelets tips with the help of sheer forces, once the mature megakaryocytes fenestrate with their proplatelets extensions to the vascular niche in the bone marrow (Blair and Flaumenhaft, 2009; Italiano and Battinelli, 2009).

PLATELETS

1.7. Platelets: more than a budded particle. Platelets as protein producers.

Platelets are the smallest cells in circulating blood, averaging only 2.0 to 5.0 μm in diameter and 0.5 μm in thickness and possessing a mean cell volume of 6 to 10 femtoliters, as described in their firsts reports (Bizzozero, 1882; TOCANTINS, 1938, 1938; Bessis, 1973).

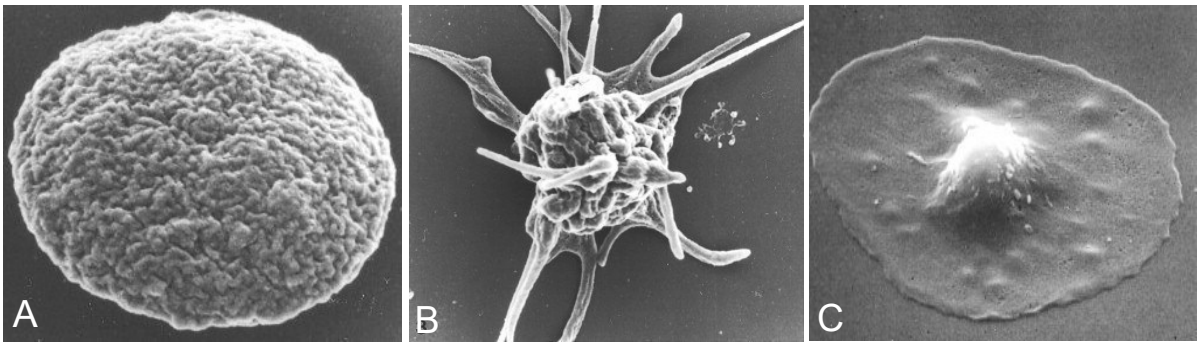


Figure 1.9. Platelets structure. A. Discoid platelet photographed in the low-voltage, high-resolution scanning electron microscope (LVHR-SEM). B. Early dendritic platelet viewed by LVHR-SEM. Fine processes extend in all directions. Surfaces of the pseudopods are smooth compared to the central body from which they extend. C. Spread platelet viewed by conventional SEM. Figures taken from (White, 2013).

Besides their cellular functions and regardless their absences of nucleus, platelets are able to synthesize proteins. Within their cytoplasm, they contain a complex translational machinery (such as ribosomes and spliceosomes) which allows them to initiate protein translation and post-translational modifications that allows them to express biologically relevant gene products in response of external signals, (Zimmerman and Weyrich, 2008; Weyrich et al., 2009; Rowley et al., 2012). To allow protein production in the platelets, the splicing components, along with specific pre-mRNAs, are transferred along with the other organelles from megakaryocytes to anucleate platelets. In the same way, stimulated platelets can activate their splicing machinery when required and generate mature mRNAs that are subsequently translated into proteins (Bugert et al., 2003; Gnatenko et al., 2003, 2003; Macaulay et al., 2005; Dittrich et al., 2006; Rowley et al., 2012).

1.8. DHL in the blood: Platelets as natural carrier and delivery cells.

Despite of their size, we see that platelets have a complex subcellular compartment characterized by the presence of organelles and other complex molecular machineries, needed to fulfil all the cellular functions and to response to the signals from the extracellular media. Within the vesicular compartment, platelets contain a heterogenic granule population, same as in megakaryocytes: alpha-granules (α-granules) dense-granules (δ-granules), lysosomes and T-granules.

Granular content

Table I: difference in secretory granules in the platelets

Type of granules	Number/platelet	Diameter (nm)	Surface Area (µm2)/Platelet	Common Markers	General Function
α-Granules	50–80	200–500	14	VWF	Haemostasis/thrombosis
				CXCL4 (PF4)	Inflammation
				P-selectin	Angiogenesis Host defense Mitogenesis

δ- granules	3–8	150	<1	CD63 Serotonin	Haemostasis/thrombosis Inflammation
T-granules	T granules have a mean cross-sectional area of 4,148 nm2, and a mean of eight T granules per PLT cross-sectional area (1,750,019 nm2) (Thon et al., 2012)			(TLR9) and (PDI)	Contribute to platelet secretion. Platelet mediated immune responses.
Lysosomes	<3	200–250	<1	Acid phosphatase Lamp-1 Lamp-2	Endosomal digestion provide selectins with carbohydrate ligands

- Table extracted and modified from (Flaumenhaft, 2013)

Lysosomes: Platelet lysosomes are typically small (175–250) nm and electron dense granules (Rendu and Brohard-Bohn, 2001). Platelet lysosomes contain the ubiquitous lysosomal membrane proteins LAMP-1, LAMP-2, and CD63 (LAMP-3) (Israels et al., 1996). Like in any other cells, they contain a number of acid hydrolases (at least 13), cathepsins D and E, and other proteins, which are also released after platelet activation (Dangelmaier and Holmsen, 1980; Leoncini et al., 1985; Ciferri et al., 2000).

T-granules: They are defined by the presence of Toll-like receptor 9 (TLR9) and protein disulfide isomerase (PDI),(Thon et al., 2012). The study by Thon and collaborators suggests that T-granules are recruited to the cell surface and contribute to secretion, possibly via the SNARE proteins VAMP-8 and VAMP-7. They arise as a novel type of electron-dense tubular system-related compartment (DTS), since TLR9 does not colocalize with any known platelets α-granule, δ-granule, lysosomal, or endosomal markers.

Dense-granules: δ-granules contain high concentrations of small molecules, giving these vesicles an electron-opaque appearance in ultrastructural studies. They contain cations like Ca2+, Mg2+, K+. likewise, phosphates Polyphosphate and pyrophosphate are also present at Millimolar levels (Ruiz et al., 2004). Bioactive amines like Serotonin, histamine have also been detected (Aikawa et al., 1971). Nucleotides such as ADP, ATP, UTP, GTP are also found, concentrated at approximately 653 mM in form of ADP and approximately 436 mM in form of ATP (Holmsen and Weiss, 1979). The presence of a vesicular H^+-ATPase proton pump maintains the dense granule lumen within an acidic pH of (5.4 -6) (Dean et al., 1984). Moreover, dense granule membrane proteins include those that are typically

sorted into lysosome-related organelles such as CD63 (known before as granulophysin) and LAMP-2. Some others platelet plasma membrane have also been identified in dense granule membranes, including some glycoproteins like GPIb and αIIbβ3 (Youssefian et al., 1997).

Apha granules: α-granules, are by far the largest and abundant secretion granules in the platelets. Micro-structurally, four distinguishable morphologic zones going from the outside to the inside of the granules can be distinguished (Sander et al., 1983; Suzuki et al., 1990). 1) The peripheral membrane of the granule, which contains P-selectin and αIIbβ3. 2) Tubular and vesicular structures, (up to 20 nm) containing multimeric forms of vWF (Cramer et al., 1985; Cramer et al., 1986), resembling structures also observed in the Weibel–Palade bodies from endothelial cells. 3) An electron-lucent area containing fibrinogen, thrombospondin and albumin. 4) An electron-dense nucleoid which is enriched with β-thromboglobulin, PF-4 and proteoglycans (Spicer et al., 1969). Some alpha granules also contain small vesicles or exosomes (40–100 nm), containing the lysosomal/dense granule protein CD63 that are released in response to thrombin (Denzer et al., 2000). They also contain microvesicles (100 nm–1 μm) that platelet membrane proteins (for example αIIbβ3 or P-selectin) and appear to be shredded from the plasma membrane after platelet activation. (Heijnen et al., 1999). Proteomic studies in platelets (Zufferey et al., 1998; Maynard et al., 2007; Senzel et al., 2009; Downing and Klement, 2012), demonstrated that more than 300 soluble proteins of bioactive proteins are stored and released from α–granules. These proteins are involved in a large variety of physiological and pathological process, allowing platelets to behave as a delivery cell of numerous biological effectors. In the following table (Table II), we show a small example of some of the proteins that have been isolated from the alpha-granules.

Table II: alpha-granular content

Type of cargo	Names
Integral membrane proteins	αIIbβ3, GPIbα-IX-V, GPVI, TLT-1, P-selectin
Coagulants, anticoagulants, and fibrinolytic proteins	Factor V, factor IX, factor XIII, antithrombin, protein S, tissue factor pathway inhibitor, plasminogen, plasminogen activator inhibitor 1, α_2-macroglobulin
Adhesion proteins	Fibrinogen, von Willebrand factor, thrombospondin
Chemokines	CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), and CCL5 (RANTES)
Growth factors	Epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factor β (TGF-β)
Angiogenic factors and inhibitors	Vascular endothelium growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), tissue inhibitors of metalloproteinases, angiostatin, endostatin
Microbicidal proteins	Thymosin-β4, thrombocidins 1 and 2 (from NAP-2)
Immune mediators	Complement C3 precursor, complement C4 precursor, β1H Globulin, factor D, factor H, C1 inhibitor, IgG

- Table extracted, reviewed and modified from (Blair and Flaumenhaft, 2009; Italiano and Battinelli, 2009; Flaumenhaft, 2013)

1.9. Platelets are multifunctional cells

Due to the wide range of proteins that platelets store and transport, by synthesis or by continuous uptake from the extracellular plasma through endocytic mechanisms; this allows platelets to participate in numerous physiological process by secreting its cargo to the extracellular space under control of external signals. In the figure 1.10 some examples of physiological and pathological cases are shown where platelets participate through protein secretion.

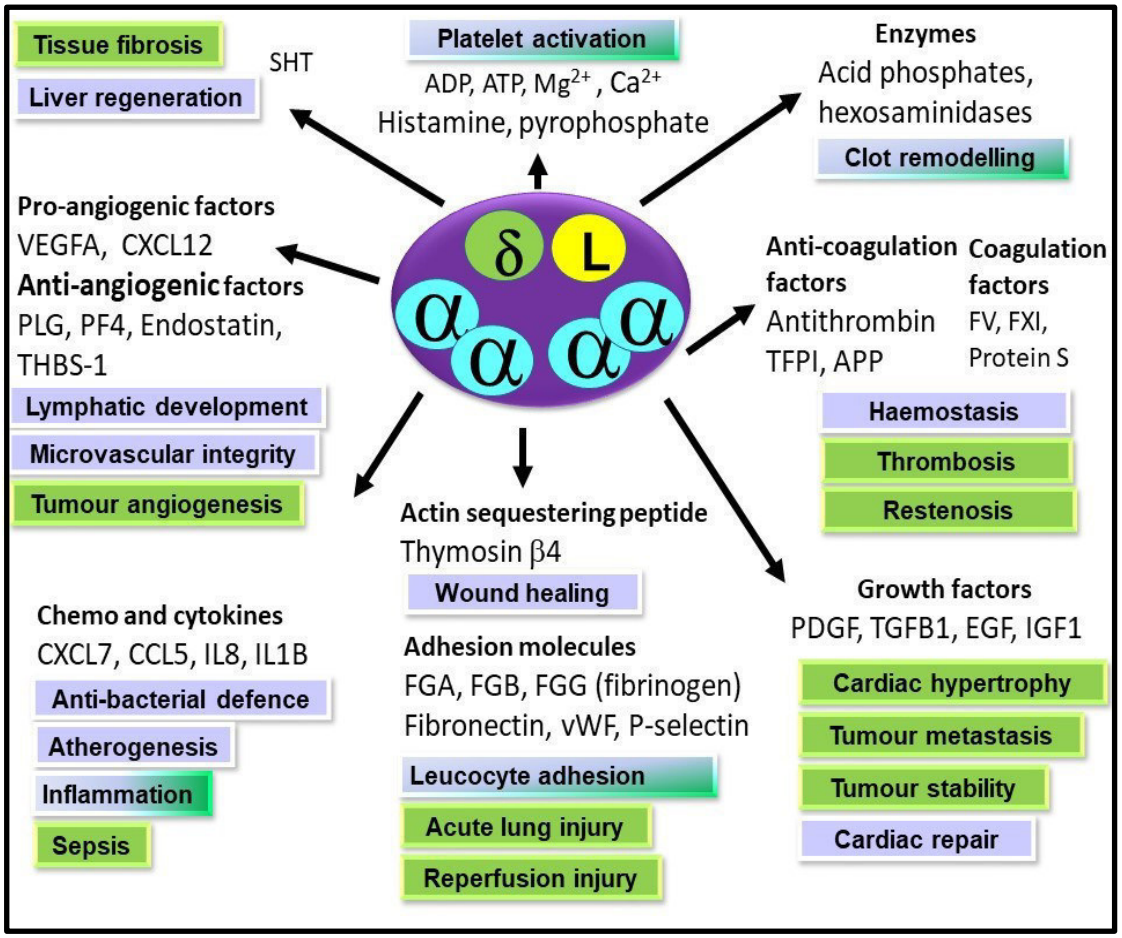


Figure 1.10. Platelet secretory granule cargoes. Physiological and pathological (shown in blue and green respectively) mechanisms. **(δ)** dense-granules, **(L)** lysosomes, **(α)** alpha-granules. α-granules contain protein cargoes with often opposing actions (like for example pro-angiogenic and anti-angiogenic factors). Cargoes are pointed out with a black arrow and black letters. Picture taken and adapted from (Golebiewska and Poole, 2013).

Platelets during coagulation

One of the most important and well-known function of platelets is their participation during haemostasis. In this sense, focusing more in the role of platelets rather than in the complete enzymatic reaction, the role of platelets begins when Factor V (FV) and Factor VIII (FVIII), are activated by traces of thrombin that are produced during initiation of coagulation. Activated factor IX (FIXa), together with activated factor VIII (FVIIIa), binds to a phospholipidic membrane, often from platelets, forming the tenase complex. The tenase complex activates factor X (FXa) and is composed of FIXa, FVIIIa, FX, and calcium. Activated FX (FXa) initiates assembly of the prothrombinase complex, composed by activated factor V (FVa), FXa, and calcium. Activation of prothrombinase complex, results in an explosive generation of thrombin with the subsequent fibrin clot formation. In the absence of FVIII (hemophilia A) and FIX (hemophilia B), the initiation of coagulation is normal (which is dependent on the Tissue Factor (TF) and Factor VII (FVII), TF-VIIa complex), but the subsequent propagation steps are severely diminished, leading to inadequate clot formation and the inability to maintain appropriate hemostasis when challenged (Hoffman, 2003; McMichael, 2012).

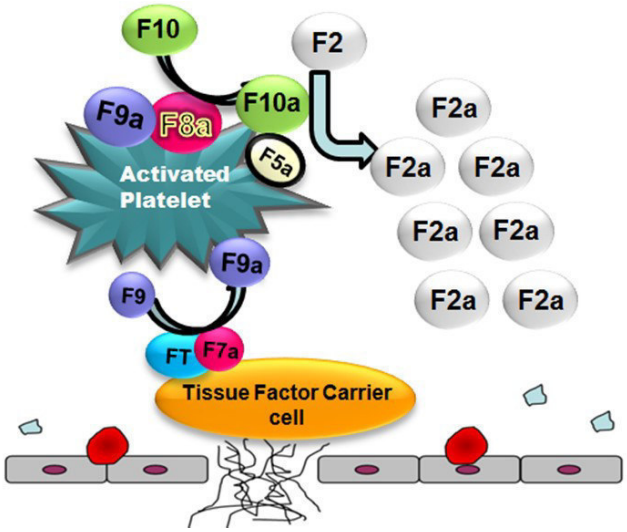


Figure 1.11 Cell based coagulation model. Figure modified from (Smith, 2009).9

The gene of the human coagulation Factor 8 (FVIII) is found at the long arm of the X chromosome (Xq28) (Harper et al., 1984). It is one of the largest genes known (spanning over 180 kb). Its transcription yields a 9-kb mRNA product (Gitschier et al., 1984). The factor VIII gene comprises 26 exons, which encode a polypeptide chain of 2351 amino acids (Vehar et al., 1984) This includes a signal peptide of 19 and a mature protein of 2332 amino acids precursor protein, that is organized in a distinct domain structure: A1-a1-A2-a2-B-a3-A3-C1-C2. Due to intracellular proteolytic processing, FVIII circulates in plasma as a heterodimer consisting of a 200 kDa heavy chain (A1-a1-A2-a2-B) and an 80 kDa light chain (a3-A3-C1-C2). The heavy chain and light chain of FVIII remain associated through a variety of interactions, some of which are metal-ion dependent (Thompson, 2003). In circulation, FVIII travels in complexes, associated with its carrier protein von Willebrand factor (VWF), preventing premature clearance and proteolytic degradation of FVIII (Lenting et al., 1998). For gene therapy purposes, due to its huge size, shorter variants have been generated to make possible the expression of FVIII from vectors. Early biochemical studies demonstrated that the FVIII B-domain was dispensable for FVIII cofactor activity (Toole et al., 1986). The B-domain-deleted (BDD)-FVIII, in our study referred as (hcoBDFIII), yields higher mRNA levels over full-length wild-type FVIII and an increase in the amount of synthesized primary translation product. Targeted point mutations within the A1 domain (Phe309Ser) have improved secretion efficiency as well (Pittman et al., 1993; Hoeben et al., 1995).

1.10. Physiology of platelets. Activation and secretion

Under physiological conditions, platelets circulate within the blood stream surrounded by the vascular walls and protected from activation by the healthy endothelial monolayer which provides a natural barrier. Platelets become activated when the endothelial layer is disrupted and the underlying subendothelial matrix is exposed, or when inflammatory process perturbs the endothelium. During its activation, platelets endure morphological changes in their cellular shape, leading to the release of the secretory granular content. Upon activation by contact with extracellular matrix components or soluble agonists, platelets

release more than 300 active molecules from intracellular granules. Those factors can both activate further platelets (for the enhancement of the reaction) and mediate a wide range of physiological process by interacting and modulating the function of other cells.

To activate platelet, mostly soluble agonists bind their receptors; these interactions lead to the activation of intracellular pathways that promote the cellular responses that allows platelets to secreteits intragranular content (Broos et al., 2012). The platelet receptors (such as P2X1, P2Y12, PAR1, PAR4, TPa, aIIbβ3, GPVI, α2β1, GPIb-IX-V between others) interact with collagen and von Willebrand Factor among other soluble molecules such as thromboxane, thrombin, ADP, ATP, Fibrinogen, collagen etc, as shown in figure 1.12. GPVI is thought to be the major signaling receptor involved in platelet activation on exposed collagen. (Jennings, 2009; Ozaki et al., 2013). Following GPVI interactions with collagen, platelets initiate strong activation and release the content of α- and dense granules. Binding of fibrinogen or vWF to GPαIIbβ3 have been describe to have central role in mediating platelet aggregation, formation of cross-linked platelets and contributes to thrombus stabilization (Jennings, 2009; Ozaki et al., 2013).

Most soluble agonists released by activated cells such as ADP, thromboxane A2 (TxA2), and thrombin trigger platelet activation through G-protein couple receptors GPCRs such as P2Y(1/12) and PAR (1/4) (Stegner and Nieswandt, 2011; Herter et al., 2014). For example, the ADP released from damaged endothelial cells and activated platelets binds to the P2Y1 and P2Y12 receptor in platelets, which lead to further platelet activation and release of more ADP. This increases the cytosolic calcium concentration and activates specific signaling pathways. One of the most important platelet agonists, thrombin, activates platelets through its binding with the protease-activated receptors (PAR) on the platelet surface. Signaling pathway of PAR1 mediates human platelet activation at low thrombin concentration, while PAR4 requires higher concentration of thrombin for platelet activation. Thrombin is the strongest platelet agonist and also mediates the conversion of fibrinogen into fibrin to stabilize the platelet plugs during coagulation.

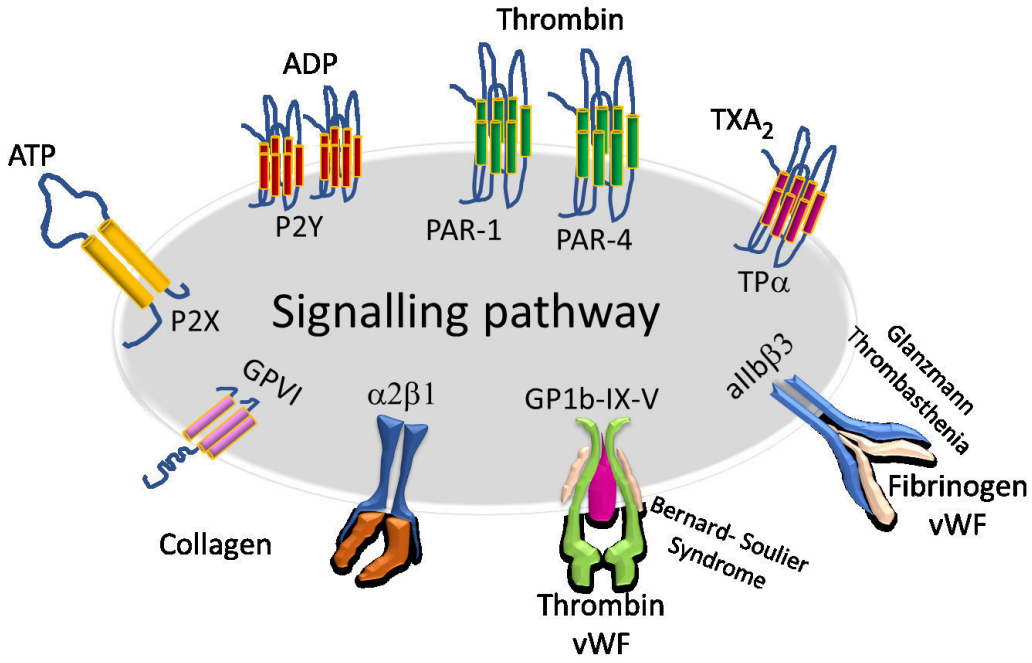


Figure 1.12. Major platelet receptor–ligand interactions. Mutations in GPIb-IX-V and aIIbβ3 genes give rise to Bernard–Soulier syndrome and Glanzmann thrombasthenia syndrome, respectively. Figure adapted from (Broos et al., 2012).

Due to the membrane fusion of granule and cellular membranes during platelet secretion, the plasmatic membrane of the platelets suffers modifications that leads to the presentation of proteins or receptors that were not exposed on the surface before. One of these proteins, considered as one the most important markers for platelet activation, is P-selectin, also known as CD62p. P-selectin was first identified in endothelial cells in 1989 (McEver et al., 1989). It is a type I integral membrane protein that is stored within alpha granules of unstimulated platelets (Stenberg et al., 1985; Berman et al., 1986) and unstimulated endothelial cells (Bonfanti et al., 1989; McEver et al., 1989). Although some studies have also shown that P-selectin it is also stored in dense granules (Israels et al., 1992), it is not consider a dense granule marker, but rather as a gold standard alpha-granules marker. Upon activation, platelets express large amount of P-selectin which is rapidly mobilized from α-granules and translocated to the platelet surface. P-selectin via its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), has a central role in the interactions of platelets

with leukocytes, and endothelial cells (Totani and Evangelista, 2010; Huang and Chang, 2012; Thomas and Storey, 2015). P-selectin is a major mediator of platelet-leukocyte aggregate formation, upregulating release of proinflammatory cytokines and the adhesion of platelets to endothelium (Jenne et al., 2013; Herter et al., 2014; Weyrich, 2014).

The protein structure is composed of a lectin domain, an epidermal growth factor domain, a series of complement repeats or sushi domains (9 repeats), a transmembrane domain and a cytoplasmic tail (Johnston et al., 1989). The extracellular domain is located within the lumen of the storage granule, the transmembrane domain is inserted through the granule membrane, and the short cytoplasmic tail always remains within the cytoplasm and is free to interact with platelet's cytoplasmic proteins. There is no evidence that P-selectin binds to any of the soluble components within the alpha granules.

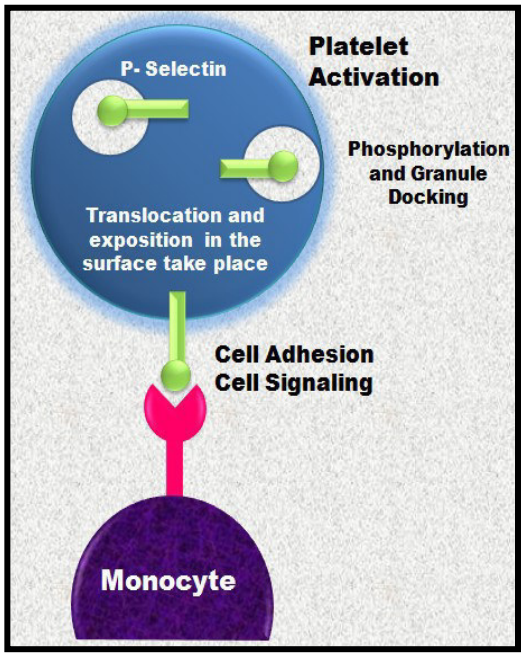


Figure 1.13. P-selectin subcellular localization and translocation. P-selectin resides in the alpha granule membrane in resting platelets. The extracellular domain is oriented inward (shown in green circle), into the lumen while the cytoplasmic tail resides in the cytosol. When the alpha granule membrane fuses with the plasma membrane, P-selectin is translocated from an internal storage granule to the external membrane where the extracellular domain can make contacts with adhesion molecules on other cells (P-selectin receptor shown in pink), particularly myeloid cells, monocytes and T-lymphocytes. Modified from (Furie et al., 2001).

Activated platelets also express CD40L (also known as CD154). Expression of CD40L on platelets has shown to affect dendritic cells as well as B and T lymphocytes, suggesting a link between innate and adaptive immunity. CD40L also interacts with CD40 on endothelial cells to promote secretion of chemokines and expression of adhesion molecules. Furthermore, platelets are a source of soluble CD40L (sCD40L), which can induce vascular cells to express E-selectin and P-selectin and release IL6 (Herter et al., 2014; Weyrich, 2014; Thomas and Storey, 2015).

During degranulation, activated platelets release various chemokines such as CXCL1, PF4 (CXCL4), CXCL5, CXCL7 (also known as NAP-2), IL-8 (also known as CXCL8), CXCL12, macrophage inflammatory protein- (MIP-) 1α (also known as CCL3), and RANTES (also known as CCL5). The major effect of these cytokines is to regulate leukocyte migration from the vasculature into the tissues, and other proinflammatory functions like phagocytosis and generation of reactive oxygen species (ROS) (Jenne et al., 2013; Herter et al., 2014; Thomas and Storey, 2015). PF4 promotes neutrophil granule release and adhesion to endothelial cells, mediated by L-selectin and leukocyte function-associated antigen-1 (LFA-1). PF4 prevents likewise monocyte apoptosis and promotes monocytic differentiation into macrophages. PF4 and RANTES (known as Regulated on Activation, Normal T cell Expressed and Secreted) form heterodimers, leading to promote monocyte recruitment to the endothelium (Jenne et al., 2013; Thomas and Storey, 2015).

All theis knowledge that have been acquired and collected so far, regarding the origin, biology and function, made possible to identify and recognize platelets as one of the most interesting and fascinating cells that can be used as a target for genetic modifications and therapeutic purposes. However, despite of all the steps forward given after their early discovery in 1882 by Bizzosero, nowadays, even 200 hundred years after their first description, important aspects regarding their function and physiology still remain to be described and understood.

1.11. Lentiviral vectors and their used in Gene Therapy.

The efficient delivery of therapeutic genes and appropriate gene expression are of critical importance for the development of clinically relevant gene therapy approaches. Retroviruses are naturally evolved vehicles which efficiently transfer and integrated their genomes into host cells. This ability made them desirable for gene transfer, and by engineering its viral genomes into transfer vectors, ensuring in this way a safer and reliable delivery of therapeutic genes with efficient gene expression. In the field of gene therapy, the stable transduction and gene expression in the hematopoietic stem cells is important to ensure and guarantee a long-lasting curative effect. This genetic engineering tool, together with the advances in stem cell transplantation, has transformed the gene therapy interventions as an important and innovative therapeutic option that can offers, in best cases, the cure for monogenetic hematological diseases, when no other treatment is available or possible. However, the fact that a transient gene expression can be achieved as well, by just introducing mutations in the integrase gene of their genome (integration-deficient vectors), made them an even more interesting tool (Bayer et al., 2008; Wanisch and Yáñez-Muñoz, 2009; Negri et al., 2011). Other viral vectors such as those developed from adenoviruses and adeno-associated virus (AAV), also follow the natural behavior of their ancestor virus and do not integrate their genomes (or at least very rarely <10%), but remain as episomes. Thereby, expression of the delivered genes is transient.

Gene therapy based on autologous transplantation offer the advantage of decreasing the immunological complications between graft and host cells, based on a potentially HLA mismatch. These therapies are based on the ex-vivo viral vector-mediated gene transfer and the transplantation of the genetically modified HSCSsor back into the patient. Differing from the classical allogenic bone marrow transplantation, where the procedure is subjected to the availability of a suitable donner, in autologous-based HSCs-therapies, the patient's own hematopoietic stem cells are used, usually obtained by mobilization from the BM into the peripheral blood through the use of the (G-CSF) cytokine (Molineux et al., 1990).

Retroviruses

Retroviruses are enveloped viruses, between 80–100 nm in diameter, and contain two copies of linear, positive-sense, single-stranded and non-segmented RNA of 7–12 kb of length. The principle feature of this family is its replicative strategy, where following entry into target cells, the RNA genome is retro-transcribed into double stranded DNA (dsDNA) and integrated into the genome of the host cell. This integrated state is known as provirus, which later on, undergoes transcription and translation in the same manner as the host cellular genes, producing in this way genomic viral RNA plus mRNAs that code for viral proteins. Subsequent assembly of new viruses will bud from the host cell to infect other cells (Kay et al., 2001; Zhang and Godbey, 2006; Maetzig et al., 2011; Nowrouzi et al., 2011).

Based on common patterns of pathogenesis, RNA-retroviruses (Group VI) initially were grouped into three basic subfamilies: Oncovirinae (including the mammalian and avian C-type retroviruses), Lentivirinae (such as HIV and other immunodeficiency viruses), and Spumavirinae (among them, the foamy virus). Nowadays however, Retrovirus are divided in two big families: Orthoretrovirinae (including the Genus Alpharetrovirus, Gammaretrovirus and Lentivirus) and the family Spumaretrovirinae. Most retroviruses belong to the simple retrovirus classification, while lentiviruses and spumaviruses (for example, human immunodeficiency virus and human foamy virus) are complex retroviruses (Zhang and Godbey, 2006; Merten et al., 2016). Both, simple and complex retroviruses contain three essential genes: the gag, which encodes the viral structural proteins (such as matrix [MA], capsid [CA] and nucleocapsid proteins [NC]); the pol, encoding part of a polyprotein (Gag-Pro-Pol), whose cleavage products release the reverse transcriptase (RT) and integrase (IN) and, in some lentiviruses, dUTPase (DU). In spumaviruses, pol is expressed via a spliced mRNA as Pol-Pol polyprotein. The env encodes an envelope polyprotein whose cleavage products SU (surface) and TM (transmembrane)are important for the tropism of the viral particles. There is an additional, small coding domain present in all retroviruses as part of the pol gene, called pro, which codes a viral protease (PR), responsible for facilitating the maturation of the virus. All retroviral genomes are flanked

by two long-terminal-repeats (LTR), which are important for the integration into the host genome and viral RNA expression, due to the presence of enhancer and promoter sequences capable of directing synthesis of the viral genomic RNA. Retroviruses classified as complex, like for example the lentivirus human immunodeficiency virus type 1 (HIV-1), also code for accessory proteins such as tat (transcriptional trans-activator), which is a low-molecular-weight protein that is essential for HIV-1 replication and activates transcription by binding to TAR (Tat-responsive region). Further accessory proteins are responsible for regulating viral replication and interact with the host cell immune response. These genes include rev (protein that binds to the RRE [Rev response element region] and facilitates the transport of unspliced and incompletely spliced mRNAs to the cytoplasm.), **nef** (negative factor), a myristylated intracellular protein that reduces the level of CD4 on the cell surface, and also stimulates some infected cells to divide; **vpr** (Viral Protein r), a protein, found in virions, that causes infected cells to arrest in G2 and may also promote transport of the pre-integration complex into the nucleus after reverse transcription; **vpu** (viral protein U), an intracellular protein, that causes degradation of newly synthesized CD4 and with this downmodulation of the viral entry receptor and promotes viral assembly and release; **vif** (Virion Infectivity Factor), which are dispensable for in vitro virus growth, but indispensable for in vivo replication (aids the production of infectious virions) and pathogenesis, however its not needed for retroviral vectors (Walther and Stein, 2000; Zhang and Godbey, 2006; Waehler et al., 2007; Maetzig et al., 2011; Matuskova and Durinikov, 2016; Merten et al., 2016) see in deep in figure 1.14 (A-C).

Retroviral vectors

For their application in experimental therapies, the generation of a replication deficient viral particle was crucial. This was only possible, once the functions of the different viral genes were defined and all the genetic information of the virus, important to replicate, were removed from the viral genome, leaving just a minimal viral genome that is only able to integrate into the host genome and to drive likewise the expression of the gene of interest (GOI). The genetic information removed is divided in three different plasmids, which need

to be co-transfected into the viral particle producer cell (Miller and Rosman, 1989), figure 1.14 (B).

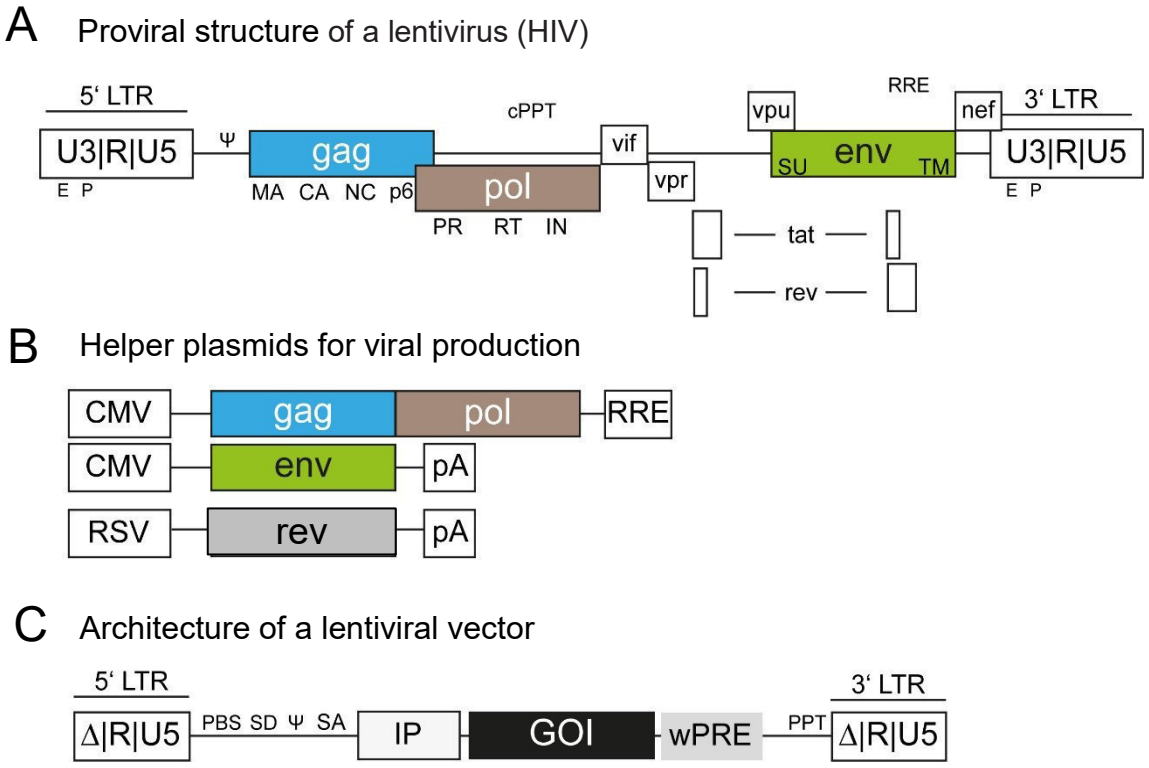


Figure 1.14. Viral genome editing for viral vector development. A) Proviral genome structure of the human immunodeficiency virus, HIV. The proviral genome is flanked by long terminal repeats (LTR) (shown in white boxes), that provide the transcriptional control (by enhancer-promoter elements), start and polyadenylation signals. The leader region, which follows the 5' LTR, contains the primer binding site (PBS, not shown) for the initiation of the reverse transcription, the splice donor site (SD, not shown) as well as the packaging signal Ψ. Downstream the viral coding sequence is differentiated into gag (coding for structural proteins matrix (MA), capsid (CA), nucleocaspid (NC) and P6 protein); the pol (coding for replication enzymes, PR: protease, RT: reverse transcriptase, IN: integrase), and env (envelope protein). The lentiviral genome contains accessory proteins (viral infectivity factor (vif), viral protein r (vpr), viral protein u (vpu), regulator of expression of virion proteins (rev), transactivator of transcription (tat), negative factor (nef)) that are generated by alternative splicing and promote the maintenance of the viral infectivity. **B) Helper plasmids for viral production**. For the generation of infectious viral particles, proteins like gag, pol, env, and rev are delivered in trans (from different expression plasmids). This allows the packaging and assembly of all structural proteins needed, but, because the RNA of these plasmids lack the packaging signal, just the RNA coding the transgene is packaged in the viral particles. **C) Architecture of a lentiviral vector.** For therapeutic purposes,

the genome of the virus is modified, in order to generate replication-deficient viral particles. The gag, pol and envelop genes are removed, and replaced by the coding sequence of the transgene. Elements like primer binding site (PBS), splice donor (SD), packaging signal (Ψ), splice acceptor (SA) are retained in the vector to guarantee packaging of the viral RNA (Ψ) and the initiation of reverse transcription (PBS).

One important step forward was the possibility of "pseudotyping", which modifies the viral particle tropism, expanding the number of target cells that can be transduced (tropism). Lentivirus infect human immune cells like CD4+ T-cells, macrophages and microglial cells. In the wild type virus, the entry into target cells is mediated by the interaction between HIV's gp120 and the CD4 molecules and chemokine co-receptors (CCR5 or CXCR4) present on the surface of the host cells. For gene therapy purposes, in order to infect cells without CD4/CCR4 or CD4/CCR5 expression and with this increase the tropism of the vector, pseudotyping of viral particles with other heterologous envelope proteins is routinely performed. On the other hand, native lentiviral envelope proteins make lentiviruses notoriously unstable and the virion's stability is a pre-requisite for the effective purification and concentration of viral vector preparations. The lentiviral envelope proteins are often replaced with stable heterologous viral envelope glycoproteins, most commonly G-protein of Vesicular Stomatitis Virus (VSV-G). VSV-G protein from the Vesicular Stomatitis Virus (VSV) binds the low density lipoprotein receptor (Finkelshtein et al., 2013) and has a broad tropism towards lipid membranes (suitable for murine and human cells), while rabies G-proteins has a distinct tropism to neural cells. Lentiviral vectors can be pseudotyped with many other proteins, including artificially designed proteins, to improve infectivity for a particular cell type or, alternatively, to restrict the viral tropism. This proteins are included in a helper plasmid, shown as "env" in the figure 1.14 (Verhoeyen and Cosset, 2004; Cronin et al., 2005; Frecha et al., 2008; Lévy et al., 2015).

Lentiviral vectors can be assembled through the transient co-transfection of the lentiviral vector backbone plasmid (transfer vector) together with the helper plasmids expressing the viral packaging functions, or by stably transfected packaging cell lines. One of the most often used cells are the HEK293T cells, a human kidney cell line transformed with DNA fragments of adenovirus type. These cells can be efficiently transfected by the

calcium phosphate method, protocols involving cationic lipids or by electroporation. HEK293T cells, which were derived from HEK293 cells and stably express large Simian Virus 40 (SV40) large T-antigen, are purported to generate retroviral and lentiviral vector preparations with particularly high viral particle titers (Walther and Stein, 2000; Thomas and Smart, 2005; Merten et al., 2016).

Adverse effects of gene therapy: Insertional mutagenesis and oncogenic effects.

The first studies using an engineered viral genome to transfer foreign genetic information to an eukaryotic cell, used retroviral vectors (Wei et al., 1981; Tabin et al., 1982; Cepko et al., 1984). In this sense, first attempts to use this gene transfer platform as an experimental therapy was advocated to cure monogenetic haematological disorders like severe immunodeficiencies such as Adenosine deaminase deficiency (ADA-SCID), X-linked severe combined immunodeficiency (SCID-X), Chronic Granulomatous Disease (CGD) and Wiskott–Aldrich syndrome (WAS) (Bordignon et al., 1995; Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002; Gaspar et al., 2004; Ott et al., 2006; Boztug et al., 2010; Aiuti et al., 2013; Hacein-Bey-Abina et al., 2014). However, later on during the follow up of the therapies, reports were published about severe adverse events in some of the patients who developed leukemia and myelodysplastic syndromes during the long term follow up of the studies, due to insertional mutagenesis (Hacein-Bey-Abina et al., 2003; Hacein-Bey-Abina et al., 2008; Howe et al., 2008; Stein et al., 2010; Braun et al., 2014). Further analysis on the cells of the patients revealed retroviral vector integration-mediated clonal expansion, because of the upregulation of different known oncogenes LMO2, CCND2, BMI1, PRDM16, SETBP, EVI1. Abnormal activation of these genes confers an uncontrolled cycling of the cells and growth advantage leading to further acquisition of mutations during cell division and genomic instability. Because of the random insertion of the vector, the possibility exist of adverse effects, given by the deregulation of neighboring genes, due to the possible cis / trans or long distance effects of vector elements on the surrounding genome, or even in more far away regions (Ivics et al., 2009; Singhal et al., 2011; Metzger et al., 2016). These effects can alter and modify the normal gene expression patterns on the cell, leading in worst cases to transformation. This process is called

insertional mutagenesis and occurs in most cases, when the enhancer / promoter region of the vector, after integration of the exogenous DNA, induces the deregulation of oncogenes or tumor suppressor genes (TSG). Exogenous DNA can enhance transcription or translation levels of oncogenes, generate chimeric or truncated transcripts, or inactivate TSG expression (Baum, 2007).

This cancerogenic predisposition mediated by retroviral vector integration and loss of genomic integrity was further in depth studied in murine bone marrow transplantation models (Li et al., 2002; Modlich et al., 2005). Likewise, further studies in the same direction identified the leukemia initiating mechanism, as the gammaretroviral vector integration preferences into promoter regions of transcriptionally active genes, as well as the vector intrinsic strong enhancer and promoter elements (Kustikova et al., 2005; Modlich et al., 2005; Cattoglio et al., 2007; Kustikova et al., 2007; Modlich et al., 2008). To some more details, mechanism by which insertional mutagenesis occur are explained in the following figure (figure 1.15).

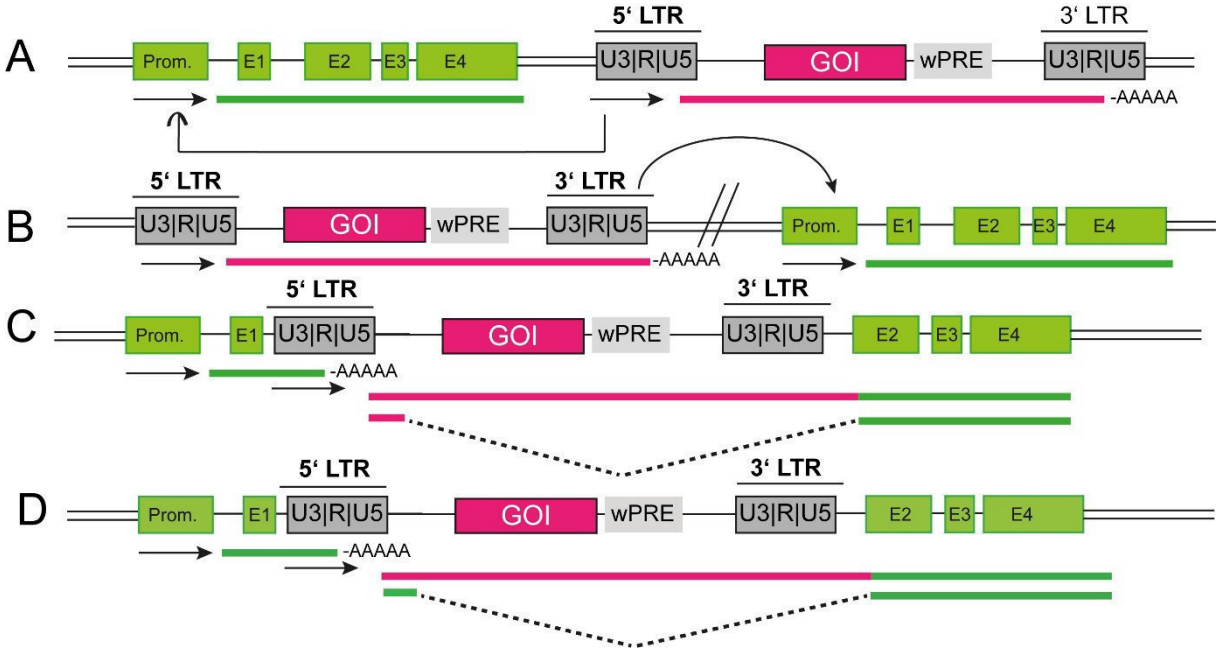


Figure 1.15. Mechanism of insertional mutagenesis. Enhancer insertions. Integration of enhancer/promoters of viral vectors close to or in promoters of cellular genes. this results in the up-regulation of endogenous gene expression. As regulatory elements may also be located distal to

genes, enhancer insertions can be located some distance from the proximal promoter and may affect the activity of elements via chromatin loops Although the effect can be independent from their orientation, they are classically downstream (A), or upstream (B), keeping however both viral and endogenous gene fully transcribed (represented in lines pink and green) (Selten et al., 1984; Uren et al., 2005). **Promoter insertions**. This occur when the vector integrates in sense orientation whether inside or close to the proximal promoter region of an endogenous gene, impairing this way the normal gene cellular transcription, and placing it under the control elements found within the insertional vector. In this case, the gene is transcribed as an RNA starting from the promoter of the insertional mutagen that reads-through the introns and exons of the host gene (C). It may be also possible, that splice signals from the integrating vector, induce the joining of the first vector-coded exon to those of the host gene, leading in the translation of high levels of chimeric transcripts. **Intragenic insertions.** Interferes with the splicing of genes into which they integrate (D). Many insertional vectors contain polyA signals, either engineered or endogenous, that may induce premature termination of gene transcription, or splice variants. Viral insertions in the 3′UTR region of a gene may remove the mRNA-destabilizing motifs such as AUUUA hairpins or miRNA target sequences, resulting in increased levels of a truncated, but wild-type protein-encoding mRNA. Alternatively, transcription may start from the integrated promoter, and in this case a 5′-truncated mRNA is transcribed. The resulting C-terminally or N-terminally truncated proteins may possess oncogenic properties and could induce eventually tumorigenesis. Picture reviewed and modified from (Nawijn et al., 2011; Ranzani et al., 2013)

Although many of the clinical trials were based on the use of MLV vectors, the general current tendency is towards the use of lentiviral vectors (LV). In comparison to γ-retroviral vectors, LVs are able to transduce nondividing cells because they can translocate across the nuclear membrane (Naldini et al., 1996a); their integration patterns are different from MLV vectors, lentiviruses preferentially integrate within the bodies of active genes, and intragenic regions, whereas the prototypical gammaretrovirus Moloney murine leukemia virus (MoMLV) favors integration along enhancers and active gene promoter regions. This fact makes the use of lentiviral vectors potentially less risky than gammaretroviral vectors with respect to insertional mutagenesis. The so called self-inactivating (SIN) configuration vectors were prepared in the 1980s (Yu et al., 1986). SIN vectors have a deletion in the 3’ U3 region, where the sequences of promotor and enhancer sequences is placed. During reverse transcription, this deletion is copied into 5’ LTR, and the viral vector becomes free of LTR-bound promotor activity. Transcriptional control is

therefore achieved under the promotor in the internal position. (Dull et al., 1998a; Kraunus et al., 2004; Schambach et al., 2006a; Schambach et al., 2006b)

1.12. Lineage specific vectors

When modifying megakaryopoiesis by transduction of HSC and avoiding transgene expression all along the hematopoietic cell lineage, the use of lineage- specific promoters becomes a potential option to restrict gene expression in megakaryocytes and to impair off target effects. This is a very powerful tool to study megakaryopoiesis or to modify platelets by lentiviral gene transfer, since it has been shown before, that the use of lineage specific promoters restrict the expression after vector integration, for example in the myeloid cells (Santilli et al., 2011), macrophages (Levin et al., 2012) or B-cells (Kerns et al., 2010). The most often employed MK-specific promoters are derived of the human alpha 2b (CD41), human GPIba (CD42b) or rat Pf4 genes (Ravid et al., 1991a). However, recent analyses of the Pf4 promoter activity in the Pf4-Cre mouse have raised concerns about the specificity of expression from this promoter (Calaminus et al., 2012). Furthermore, recent studies highlighted the close relationship between MK and HSC (Sanjuan-Pla et al., 2013; Haas et al., 2015) and the expression of CD41 in subset of the HSC as identified by their LSK cell surface phenotype (Nishikii et al., 2015) questioning the usefulness of the alpha 2b promoter to restrict expression to megakaryopoiesis and platelets. Mouse models are valuable tools to study gene function in vivo. Although gene knockout or transgenic approaches have been widely used for this purpose (Ravid et al., 1991a; Thompson et al., 1996; Yarovoi et al., 2003), the use of lineage specific promoters made a generational change in the development of mouse models, since this approached allowed the possibility of achieving site specific expression of the recombinases used for its generation.

In the field of megakaryocyte (MK) and platelet biology, the lineage-specific expression of Cre by the platelet factor 4 (Pf4, Cxcl4) promoter is often employed allowing the deletion of the gene of interest in megakaryocytes at early stages (Tiedt et al., 2007). Nowadays however, the use of lentiviral vector platforms represents a faster and practical approaches for the studies of gene function in vivo. To overcome the disadvantage of transgene size limitation in gene transfer by using lentiviral vectors, usually shorter forms

of the original promoters are used. However, this fact may drive side effects or difference on specificity and strength in transgene expression, when these "promoter fragments" are compared to the complete original promoter. These effects may be due to the absence of upstream and/ or downstream regulatory sequences that are needed, and which are inevitable excluded, when the promoter fragments are introduced into the lentiviral vector.

2. Concept of the study

Based on the key role of platelets in numerous physiological processes, as well as their properties to naturally carry proteins in their secretory granules, in this project we propose to generate genetically modified megakaryocyte and platelets as tools for cell and gene therapy, by targeting proteins to the alpha granules of megakaryocytes and platelets. These proteins would be stored and kept in the secretory granules until an external stimulus triggers platelet activation and platelet secretion takes place. During platelet activation, the therapeutic proteins would be then released to the extracellular space together with the natural intragranular proteins.

For long-term therapeutic effects, genetic modifications must be performed at the hematopoietic stem cell level; however, for gene therapy purposes, we must consider that any genetic modification introduced in the hematopoietic stem cells, will be inherited to all the hematopoietic lineages derived from the genetically modified stem cells. Therefore, to avoid transgene related toxicities and reduced the off-target expression, it is necessary first to ensure a proper transcriptional control, that restricts transgene expression as much as possible to the target cells, in this case megakaryocytes and platelets. One way to ensure transcriptional control is the use of lineage specific promoters

Therefore, as first approach, we aimed to compare and characterize the lineage-specificity of expression and activity of six MK-specific promoter fragments in the context of lentiviral vectors: the murine platelet factor 4 (mPf4) promoter (1222 bp,-1074 to +148), human glycoprotein Ib alpha (hGP1BA) promoter (595 bp,-265 to +330), a short and a longer promoter fragment of the human glycoprotein 6 (hGP6 / hGP6s:351 bp,-322 to +29)

/ 726 bp,-697 to +29), as well the human glycoprotein 9 (hGP9) promoter (794 bp, -782 to -12). These promoter fragments were included as internal cellular promoters into self-inactivating lentiviral vectors (SIN), using an enhanced green fluorescent protein (eGFP) as reporter gene and tested in vitro and in vivo. As ubiquitously expressing control vectors, we analyzed the expression from the hPGK promoter in the lentiviral vector or the viral spleen focus forming enhancer/promoter (SFFV) in the context of the LTR-driven gammaretroviral vector. Promoter fragments were tested in in-vitro cultures for GFP expression in blood cell progenitors as well as in in-vitro megakaryocytes. In vivo, promoter activity was evaluated in hematopoietic cells from spleens, blood and bone marrow cells in mice transplanted with BM cells transduced with the MK-specific lentiviral vectors.

As second level of targeting, we aimed to target protein expression to the secretory alpha granules of megakaryocytes and platelets. This subcellular targeting is achieved by using signals (small peptide sequences), which are important to the cell for directing the subcellular protein trafficking. For targeting of proteins to the secretory alpha granules of megakaryocytes and platelets, we followed two strategies:

The sorting signal of the cytokine RANTES (Regulated upon Activation Normal T cell Expressed and Secreted) was fused N-terminally to the destabilized GFP (RANTES. d2eGFP). This sorting domain is a small peptide sequence (VIATLKDGRK), which deliver the protein into the granules as soluble cargo.

In this case, the ectopic proteins would be stored in the secretory alpha granules until an external signal would trigger platelet activation, leading to granule secretion, and with this, the release of all intragranular content. The ectopic protein would be also then release to the extracellular space as a soluble protein (see figure 2.1).

The transmembrane granular targeting sequence of P-selectin (the transmembrane domain and cytoplasmic tail (referred as TDCT) was fused to d2eGFP or the B domain deleted codon optimized human coagulation Factor VIII cDNA (referred as BDcohFVIII_TDCT or FVIII_TDCT). The TDCT will deliver the protein into the membrane of alpha granules.

In this targeting model, when platelets get activated, due to the process of secretion, secretory granules are released and both membranes (the cellular membrane and the granular-membrane) are fused; as result from this fusion, some of the transmembrane-granular proteins are then translocated and exposed to the cellular membrane. In this case, our targeted protein would be also translocated and exposed as a transmembrane protein from activated platelets (See figure 2.2).

The targeting of ectopic proteins to the secretory granules of platelets would be a way of ensuring the controlled released of targeted proteins, preventing the development of inhibitors (antibodies) against the ectopic protein and avoiding with this the risk of possible therapeutic failures. For the expression of the protein fusions, the platelet factor 4 promoter was used as internal promoters in SIN-lentiviral vectors. These two strategies were tested in-vitro, from transduced differentiated megakaryocytes in liquid cultures, and in-vivo, by analysis of genetically modified platelets by means of Laser Scanning Confocal Microscopy (LSM) in colocalization and fluorescence intensity analysis. The weight colocalization coefficient (WCC) was determined, by comparing the von Willebrand Factor and/or P-selectin as alpha granule marker, with either GFP and/or FVIII / FVIII_TDCT as targeted protein. For the analysis, transduced cells with the targeting vectors were compared with the cells transduced with the non-targeting vectors. Colocalization analysis was performed at the single cell level, based on LSM- digital images.

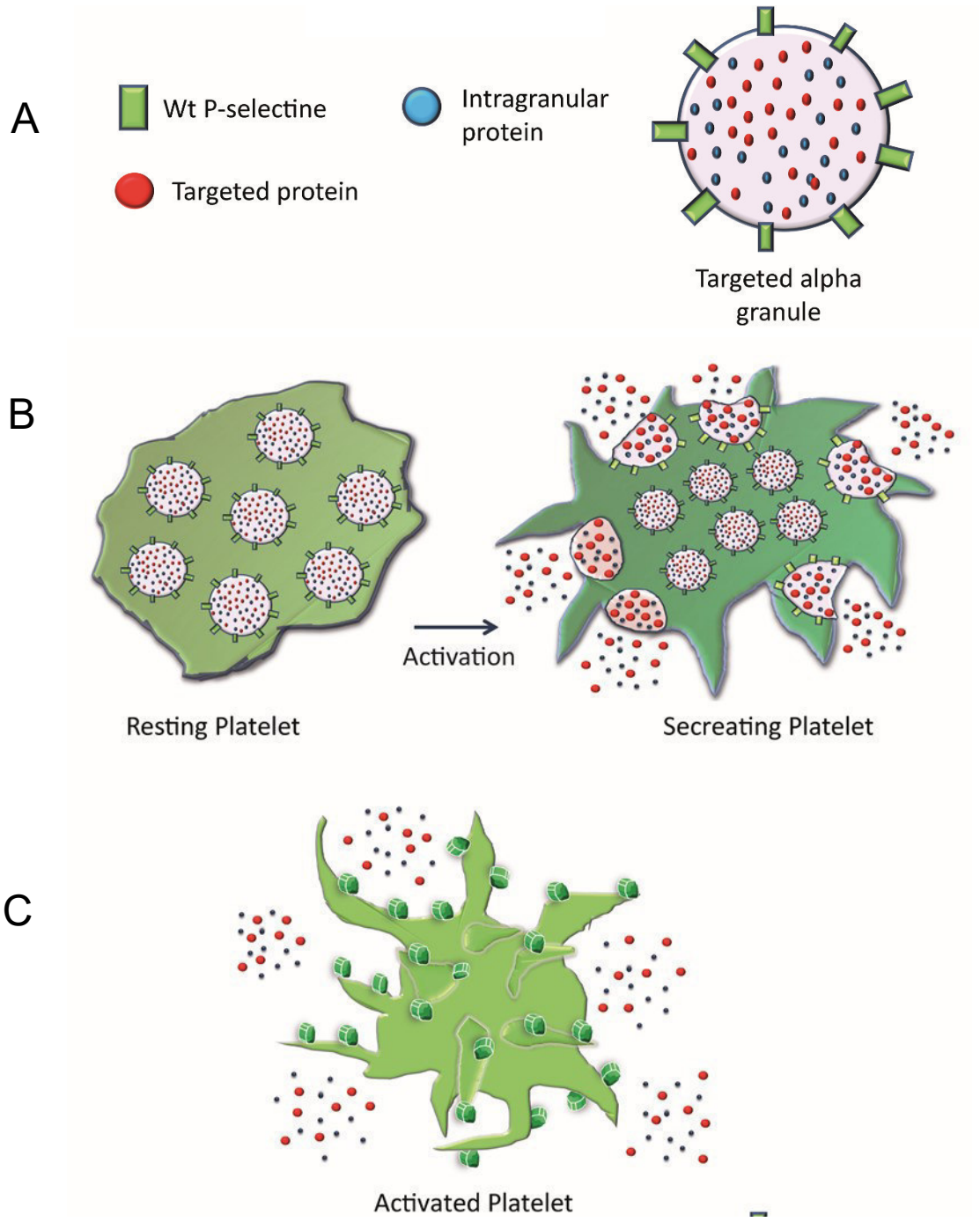


Figure 2.1. Intragranular targeting model. Alpha granule constitution (A). Secretory granules are constituted by transmembrane proteins (like P-selectin, shown in green) as well as by intragranular proteins, shown in blue. The targeted protein is shown in red. **Cellular changes during platelet activation (B)**, cells are spread, granules are secreted and intragranular content is released; during secretion, the membranes of both granules and surface are fused. **Activated platelets (C)**. Notes that intragranular content is released to the extracellular space

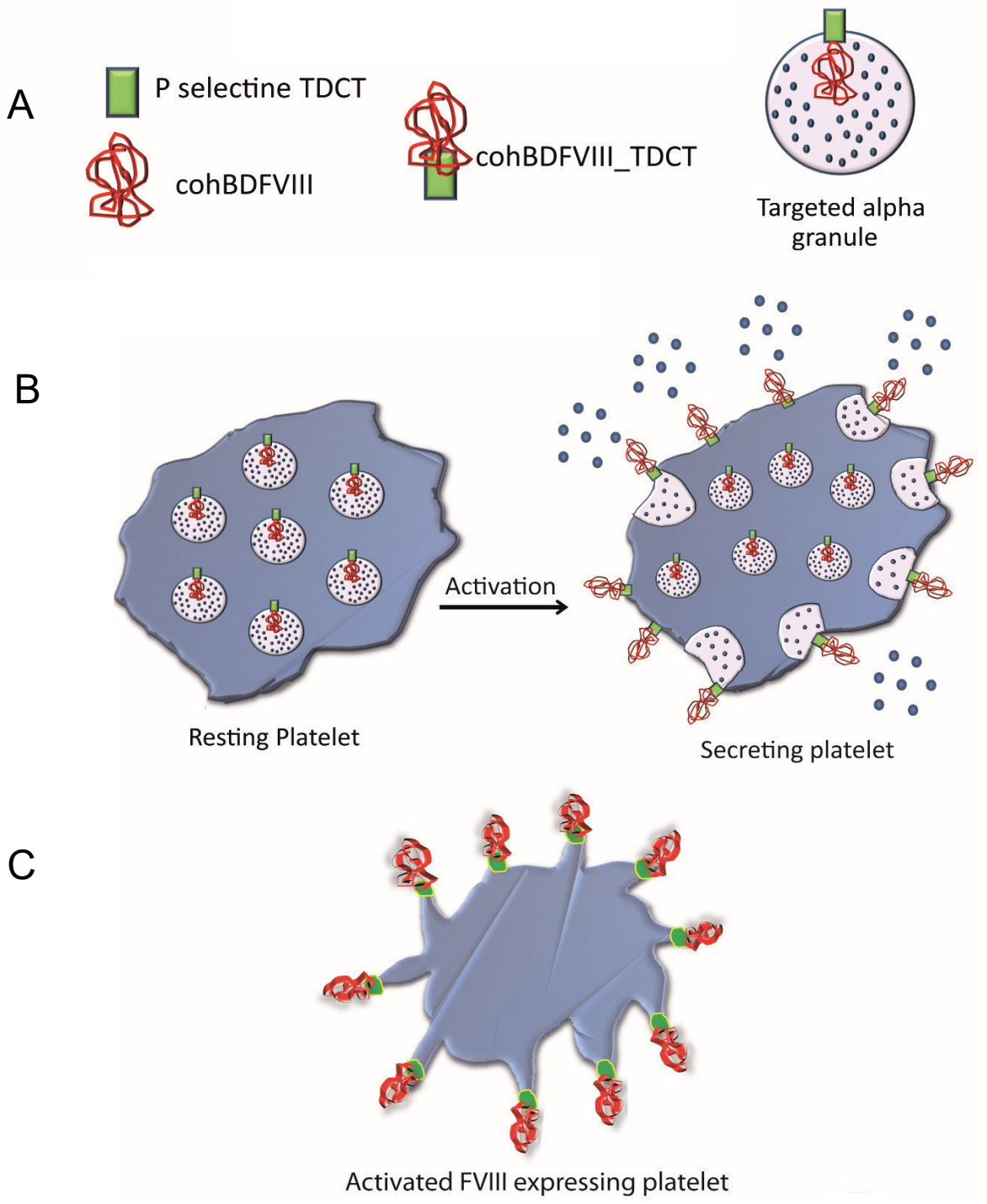


Figure 2.2. Transmembrane-granular model. The human codon optimized coagulation factor 8 (FVIII), shown in red (A), is fused to the transmembrane and cytoplasmic domain of P-selectin (TDCT), in **(A)** shown in green. Targeted protein is then stored as a transmembrane protein, in the membrane of the secretory alpha granules of platelets. During platelet activation **(B)**, intragranular content is released and as result of granular secretion, targeted FVIII is finally exposed at the membrane of the activated platelets **(C)**.

3. Specific aims of the project.

1) **Transcriptional targeting** of gene expression to megakaryocyte and platelets, by means of the use of lineage-specific promoters (mPf4p, mGP9p, hGP9p, hGP6p, hGP1BAp) in third generation SIN-lentiviral vectors constructs.
   The project includes: Generation of the lentiviral vectors:
   RRL.PPT.SFFV.GFP.pre, RRL.PPT.hPGK.eGFP.pre, RRL.PPT.mPf4.eGFP.pre
   RRL.PPT.hGP1BA.eGFP.pre, RRL.PPT.hGP6.eGFP.pre, RRL.PPT.hGP6s.eGFP.pre
   RRL.PPT.hGP9.eGFP.pre, RRL.PPT.mGP9.eGFP.pre.
   Production of viral particles via transfection in HEK 293T cells with the transient calcium phosphate co-transfection method, titration of viral particles supernatants by transduction in cell lines, q-PCRs for determination of the vector copy number in DNA from transduced cells, characterization of promoter activity by GFP measurements in flow cytometry analysis from blood cell progenitors and megakaryocytes differentiated from lineage-marker negative cells, as well in cell lines from in-vitro cultures; evaluation of GFP-transgene expression in platelets and leucocytes from peripheral blood and hematopoietic stem and progenitor cells (HSPCs) from transplanted C57Bl/6 mice, as well as in MKs, B and T cells from spleens of transplanted mice. Overexpression of the murine Thrombopoietin receptor Mpl in Mpl-/- cells, by transduction of cells with the RRL.PPT.mPf4.Mpl.pre, RRL.PPT.PGK.Mpl.pre, RRL.PPT.hGP6.Mpl.pre, RRL.PPT.hGP6s.Mpl.pre vectors. Subsequent evaluation of megakaryocyte maturation in polyploidization assays from in vitro cultures and analysis of platelet counts after transplantation of transduced-Mpl-/- HSCs in Mpl-/- mice, with the Mpl-lineage-specific vectors.

2) **Intragranular targeting.** Targeting of protein expression into the secretory alpha granules of platelets as soluble cargo, by using the sorting signals (small peptide sequences) of proteins specifically expressed and stored in megakaryocytes and platelets. This project includes: the generation of the GFP fusion-targeting construct, using the sorting sequence of the platelet cytokine RANTES (VIATLKDGRK). Generation of the RRL.PPT.SFFV.d2eGFP.pre, RRL.PPT.mPf4p.d2eGFP.pre and
   RRL.PPT.mPF4.Sig.pep.SortSigRANTES.d2eGFP.pre lentiviral vectors.
   Production of viral particles via transfection in HEK 293T cells with the transient calcium phosphate co-transfection method, titration of viral particles supernatants by transduction in cell lines, isolation and transduction of lineage marker negative cells for the generation of genetically modified in-vitro megakaryocytes and evaluation of the GFP targeting by means of laser scanning confocal microscopy. Transplantation of genetically modified hematopoietic stem cells with the RANTES-GFP targeting and the

non-targeting GFP vectors, using the platelet four promoter (mPf4p) as a transcriptional control.

3) **Transmembrane-granular targeting**. Targeting of the d2eGFP and the B-domain-deleted human coagulation factor 8 protein (FVIII) to the membrane of secretory alpha granules of megakaryocytes and platelets, by using the sorting signal (transmembrane and cytoplasmic domain, TDCT) of the P-selectin protein. This project includes**:** the generation of the P-selectin-GFP and the FVIII targeting fusions. Generation of the RRL.PPT.mPF4.hcoBDFVIII.pre,
RRL.PPT.mPF4.P-sel.sig.pep.HAtagd2eGFP.TDCT.pre and the
RRL.PPT.mPF4.hcoBDFVIII.TDCT.pre lentiviral vectors.
Production of viral particles via transfection in HEK 293T cells with the transient calcium phosphate co-transfection method, generation and titration of viral particles supernatants, isolation and transduction of lineage marker negative cells, generation of genetically modified in-vitro megakaryocytes, evaluation of the GFP and FVIII targeting by means of laser scanning confocal microscopy. Transplantation of Transduced HSCs with the GFP_TDCT, FVIII and FVII_TDCT vectors for the generation of genetically modified platelets. Isolation and analysis of genetically modified platelets from transplanted mice by means of laser scanning confocal microscopy

Chapter 2:

- Materials and Methods

2. Material and Methods

2.1 Materials

2.1.1 Technical Equipment

Table 1: Technical Equipment

Device	Label	Manufacturer, Headquarter
CO_2 incubator	Series C	Binder; Tuttlingen (GER)
Balance	Kern ABJ 320-4NM	Kern & Sohn GmbH; Balingen (GER)
Blood counter	Scil Vet abc	Scil Animal Care Company GmbH; Viernheim (GER)
Centrifuge	Fresco17 MultifugeX3R Cytospin 4 Avanti J-30I	Thermo Fischer Scientific; Waltham (MA USA) Beckman Coulter, Krefeld (GER)
Clean bench	MSC-Advantage	Thermo Fischer Scientific; Waltham (MA USA)
CO_2/O_2 Incubator	Series C	Binder; Tuttlingen (GER)
Confocal Microscope	LSM510	Zeiss, Jena, DE
Counting chamber	Neubauer	BLAUBRAND
Electrophorese power supply	EV245	Consort; Turnhout (BEL)
Electrophorese-chamber		neoLab; Hedelbert (GER)
Extraction System	Aspir8 4.4 CS	CellMedia; Gutenborn (GER)
Flow cytometer	Accuri C6	BD Biosciences; Heidelberg (GER)
Fridge-Freezing combination		Liebherr; Biberach an der Riss (GER)
Gel documentation		Intas; Göttingen (GER)
Heating block	Thermomixer basic	CellMedia; Gutenborn (GER)
Incubation shaker	MaxQ 6000	Thermo Fischer Scientific; Waltham (MA USA)

Device	Label	Manufacturer, Headquarter
Incubatior Microbiology	4360	Thermo Fischer Scientific; Waltham (MA USA)
Microscope	Primo Vert	Carl Zeiss; Oberkochen (GER)
UV-Microscope	HBO 50/AC	Carl Zeiss; Oberkochen (GER)
Microwave		Koenic; Ingolstadt (GER)
Device	Label	Manufacturer, Headquarter
Multipipette	Multipette M4	Eppendorf; Hamburg (GER)
NanoPhotometer	P 300	Implen; München (GER)
PCR devices	TPersonal Thermocycler TProfessional Thermocycler	Biometra; Göttingen (GER)
Pipet	accu-jet pro	Brand; Wertheim (GER)
0.5-1000 µl pipets	Pipetman	Gilson; Limburg Offheim (GER)
Real-time PCR system	StepOnePlus	Applied Biosystems; Thermo Scientific, Waltham (MA, USA)

2.1.2 Expendable items

Table 2: Expendable items

Material	Manufacturer
5ml round button tube with cell strainer cap	Falcon; Corning (NY, USA)
Cell culture dish; 3cm Ø	BD Biosciences, Heidelberg (GER)
Cell culture dish; 10cm Ø	Sarstedt; Nümbrecht (GER)
Cell strainer (70 µm, nylon)	BD Biosciences; Heidelberg (GER)
Cell scrapers	A.Hartenstein GmbH, Würzburg, DE
Cell culture flasks (T25, T75, T175)	Greiner, Frickenhausen, DE
Cell culture plates (6, 12, 24, 96 wells)	Greiner, Frickenhausen, DE
Microscope slide 76 x 26 x 1.1 mm	Marienfeld

Material	Manufacturer
Microscope coverslip 10-30 nm	Marienfeld
Multiwell plates (6-, 12-, 24, 48-well)	Sarstedt; Nümbrecht (GER)
Needles (20G, 23G, 30G)	B.Braun; Melsungen (GER)
PCR-Reaction tubes	Nerbe plus; Winsen/Luhe (GER)
Petri dishes (Molecular Biology)	Greiner Bio-One; Frickenhausen (GER)
Porcelain mortar	Neolab
Reaction tubes 1,5 ml	Sarstedt; Nümbrecht (GER)
Reaction tubes 15 ml, 50 ml	Greiner Bio-One; Frickenhausen (GER)
Serological pipettes	Greiner Bio-One; Frickenhausen (GER)
Sterile filter 0,2 µm	Merck Millipore; Billerica (MA, USA)
Syringes	B.Braun; Melsungen (GER)
Tissue culture dish 35x10 mm	BD Biosciences, Heidelberg (GER)

2.1.3 Fine Chemicals

Table 3: Chemicals

Label	Manufacturer
Agarose	Thermo Fisher Scientific; Waltham (MA, USA)
Ampicillin sodium salt	Sigma-Aldrich; Seelze (GER)
Apyrase	Sigma-Aldrich; Seelze (GER)
Calcium chloride ($CaCl_2$)	Sigma-Aldrich; Seelze (GER)
Chloroquine	Sigma Aldrich; Seelze (GER)
DAPI	Sigma-Aldrich; Seelze (GER)
dNTPs	Thermo Scientific; Waltham (MA, USA)
EDTA	Promega BioSciences, San Luis Obispo, (CA, USA)

Label	Manufacturer
Ethanol, absolute	Berkel AHK, Ludwigshafen (GER)
Fetal calf sera, Pharma Grade	Biochrom; Berlin (GER)
Giemsa modified solution	Sigma-Aldrich; Seelze (GER)
Glutamine	Biochrom; Berlin (GER)
Heparin	Sigma-Aldrich; Seelze (GER)
HEPES	Thermo Fischer Scientific; Waltham (MA, USA)
Histopaque-1083	Sigma Aldrich, Seelze (GER)
IPTG	Carl Roth; Karlsruhe (GER)
PGi-2	Sigma Aldrich; Seelze (GER)
Isopropyl alcohol	Berkel AHK, Ludwigshafen (GER)
May-Grünwald solution	Sigma-Aldrich; Seelze (GER)
Molwiol 4-88	Sigma Aldrich; Seelze (GER)
Triton X-100	Sigma Aldrich; Seelze (GER)
Thrombin	Sigma Aldrich; Seelze (GER)
Tween 20	Sigma Aldrich; Seelze (GER)
Penicillin	Calbiochem; Darmstadt (GER)
Propidium Iodide	Sigma-Aldrich; Seelze (GER)
Protamine sulfate	Sigma Aldirch; Seelze (GER)
Retronectin	Takara Bio; Shiga (JPN)
RNAzol	Sigma-Aldrich; Seelze (GER)
Sodium chloride (NaCl)	Promega BioSciences, San Luis Obispo, (CA, USA)
Streptomycin sulphate	Calbiochem; Darmstadt (GER)
TrisHCl pH 7,5 ultra-pure	Gibco; Carlsbad (CA, USA)
X-Gal	CarlRoth; Karlsruhe (GER)

2.1.4 Cytokines

Tabla 4: Cytokines

Name	Manufacturer
mFGF-1	PeproTech; Rocky Hill (NJ, USA)
mIL-3	PeproTech; Rocky Hill (NJ, USA)
mSCF	PeproTech; Rocky Hill (NJ, USA)
mTHPO	PeproTech; Rocky Hill (NJ, USA)
hIGF-2	PeproTech; Rocky Hill (NJ, USA)
mIL-11	PeproTech; Rocky Hill (NJ, USA)

2.1.5 Buffers and Solutions

Tabla 5: Buffers and Solutions

Label	Composition/Concentration
2 x HeBS	280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.05
20 x Tyrode's buffer (Maus)	2.73 M NaCl, 53.6 mM KCl, 238 mM $NaHCO_3$, 8 mM Na_2HPO_4, 0.5 M HEPES
20x TAE buffer	0.2 mM EDTA, 0.8 M TRIS, 22.84 ml/l glacial acetic acid,
Ampicillin	100 mg/ml dissolved in ddH_2O
Blocking buffer for Retronectin	PBS+2 % BSA
BSA	10%
Calcium chloride; $CaCl_2$	5 M, 0.1 M
Chloroquine	25 mM
FACS-buffer	136.89 mM NaCl, 2.86 mM KCl, 1.46 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 5 mM EDTA, pH 7,1
Glucose	10%
HEPES	1M, pH 7,9
IPTG	1mM

Label	Composition/Concentration
LB-Agar	LB-Medium, 1.5 % Agar
MACS-buffer	137 mMNaCL, ,1 mM Na_2HPO_4,2.7 mM KCl, 2 mM EDTA, 1.5 mM KH_2OP_4, 0.07 mM BSA (fraction V), pH 7.2
Magnesium Chloride; $MgCl_2$	0,1 M
P2	0,2 M NaOH, 1% SDS
P3	3 M KaAC, pH 5,5
PBS	136.89 mM NaCl, 2.86 mM KCl, 1.46 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7,1
Penicillin/Streptomycin	17 mM Streptomycin sulphate, 40.8 mM Penicillin G potassium
Protamine sulphate	4 mg/ml
X-Gal	20 ng/µl
Sodium hydroxide	5 M
Trypsin-EDTA	0.5 g/l Trypsin, 0.2 g/l EDTA dissolved in PBS
Washing buffer for Retronectin	PBS + 2.5 % HEPES

2.1.6. Kits

Table 6. Kits

Name	Manufacturer
NucleoSpin Tissue	MACHEREY-NAGEL; Düren, Germany
NucleoBond Xtra Maxi	MACHEREY-NAGEL; Düren, Germany
NucleoSpin Plasmid	MACHEREY-NAGEL; Düren, Germany
NucleoSpin Gel and PCR clean-up	MACHEREY-NAGEL; Düren, Germany
RNeasy Plus Mini Kit	Qiagen, Hilden, Germany
Lineage Cell Depletion Kit	Miltenyi Biotech, Bergisch Gladbach, Germany
QiAquick PCR purification Kit	Qiagen, Hilden, Germany
mouse-specific QuantiFast Probe Assays	Qiagen, Hilden, Germany
Direct-Zol RNA Kit	Zymo Research Europe, Freiburg, Germany

2.1.7. Enzymes

Enzymes were used during cloning procedures; all enzymes were obtained from Thermo Fisher scientific. Digestion reactions were performed in appropriate buffers recommended by the fabricant. Double digestions were performed in the buffers, recommended from http://www.thermoscientificbio.com/webtools/doubledigest/.

Table 1: Enzymes used for cloning and control digestions

Enzyme	Restriction site	Application
AgeI	A↓C C G G T T G G C C↑A	Cloning, control digestion
MreI	C G↓C C G G C G G C G G C C↑G C	Cloning, control digestion
XhoI	C↓T C G A G G A G C T↑C	cloning, control digestion
Bsp1407I	T↓G T A C A' A C A T G↑T	control digestion
NotI	G C↓G G C C G C C G C C G G↑C G	control digestion, control digestion
BamHI	G ↓ G A T C C C C T A G ↑ G	Control digestion
RsrII	C G↓G W C C G G C C W G ↑G C	Cloning, Control digestion
BsrGI	T↓G T A C A A C A T G↑ T	Cloning, Control digestion
EcoR1	G↓A A T T C C T T A A↑ G	Control digestion
SacII	C C↑G C ↓G G G G↑C G ↓C C	Control digestion
SalI	G↓T C G A C C A G C T↑G	control digestion

EcoRI	G↓A A T T C C T T A A↑G	control digestion
Acc65I	G↓G T A C C C C A T G↑G	control digestion

Taq polymerase; Thermo Scientific (Waltham, MA, USA)

Pfu polymerase; Thermo Scientific (Waltham, MA, USA)

T4-DNA Ligase; Thermo Scientific (Waltham, MA, USA), New England Biolabs (Ipswich, MA, USA)

Alkaline phosphatase; Thermo Scientific (Waltham, MA, USA)

RevertAid H Minus Reverse Transcriptase Thermo Scientific (Waltham, MA, USA)

2.1.8. Plasmids

pBlueScript II SK (+) (Addgene)

All lentiviral (LV) vector backbones used in this study were kindly provided by Prof. Dr. Axel Schambach (Institute of Experimental Hematology, MHH). The corresponding names of the generated vectors are shown as follow:

Table 8: Lentiviral vectors used

Name	Description / Usage
RRL.PPT.SFFV.GFP.pre	Used to drive the expression of GFP in blood cells, it carries the viral or non-specific control promoter (SFFV), (chapter 3)
RRL.PPT.SFFV.d2eGFP.pre	Used to drive the expression of the untargeted d2eGFP in megakaryocytes. It carries the viral or non-specific control promoter (SFFV), (Chapter 4)
RRL.PPT.mPf4p.d2eGFP.pre	Used to drive the expression of the untargeted d2eGFP in megakaryocytes. It carries the lineage specific platelet factor 4 promoter, (Chapter 4)
RRL.PPT.hPGK.eGFP.pre	Drives the eGFP expression, by using the ubiquitous control promoter of human PGK (Chapter 3)
RRL.PPT.mPf4.eGFP.pre	Lineage specific vector. Drives the eGFP expression, by using the lineage-specific platelet factor 4 promoter (Chapter 3)
RRL.PPT.hGP1BA.eGFP.pre	Lineage specific vector. Drives the eGFP expression, by using the lineage-specific human GP1BA promoter, (Chapter 3)
RRL.PPT.hGP6.eGFP.pre	Lineage specific vector. Drives the eGFP expression, by using the lineage-specific human GP6, 726 bp promoter fragment, (Chapter 3)

RRL.PPT.hGP6s.eGFP.pre	Lineage specific vector. Drives the eGFP expression, by using the lineage-specific human GP6 351 bp short promoter fragment, (Chapter 3)
RRL.PPT.hGP9.eGFP.pre	Lineage specific vector. Drives eGFP expression with the lineage specific human GP9 794 bp promoter fragment, (Chapter 3)
RRL.PPT.mGP9.eGFP.pre	Lineage specific vector. Drives eGFP expression with the lineage specific murine GP9 promoter fragment, (Chapter 3)
RRL.PPT.SFFV.Mpl.pre	Drives Mpl expression from the viral control SFFV promoter, (Chapter 3)
RRL.PPT.PGK.Mpl.pre	Drives Mpl expression from the ubiquitous control PGK promoter, (Chapter 3)
RRL.PPT.mPf4.Mpl.pre	Lineage specific vector. Drives Mpl expression under the control of the murine platelet factor 4 promoter fragment, (Chapter 3)
RRL.PPT.hGP6.Mpl.pre	Lineage specific vector. Drives Mpl expression under the control of the long human GP6 promoter fragment, (Chapter 3)
RRL.PPT.hGP6s.Mpl.pre	Lineage specific vector. Drives Mpl expression under the control of the short human GP6 promoter fragment, (Chapter 3)
RRL.PPT.mPF4.Sig.pep.SortSigRANTES.d2eGFP.pre	Targets the d2eGFP protein to the alpha granules of megakaryocytes and platelets. Contains the signal peptide and the sorting signal of the RANTES cytokine. Expression is driven by the murine platelet

	factor 4 promoter fragment. (Chapter 4)
RRL.PPT.mPF4. P-sel.sig.pep.HAtagd2eGFP.TDCT.pre	Targets d2eGFP to the alpha granules of megakaryocytes and platelets. d2eGFP is fused with the signal peptide of the P-selectin protein and with the HATag at the 5' end, as well to the transmembrane domain and cytoplasmic tail of P-selectin at the 3'end. Expression is driven by the murine platelet factor 4 promoter fragment, (Chapter 4)
RRL.PPT.mPF4.hcoBDFVIII.pre	Express the human B-domain deleted codon optimized coagulation factor 8 protein under the control of the murine platelet factor 4 promoter. It's a non-targeting control vector. (Chapter 4)
RRL.PPT.mPF4.hcoBDFVIII.TDCT.pre	Express the human B-domain deleted codon optimized coagulation factor 8 protein under the control of the murine platelet factor 4 promoter and target the protein to the alpha granules of the megakaryocytes and platelets. FVIII is fused to the transmembrane and cytoplasmic tail of P-selectin protein. It is a targeting vector. (Chapter 4)

2.1.9. Primers

Table 9: primers

Name	5' to 3' sequence	Dye
	Sequencing primers	
T7	TAATACGACTCACTATAGGG	-
T3	AATTAACCCTCACTAAAGGG	-
qPCR primers		
PTBP2 fw primer	TCTCCATTCCCTATGTTCATGC	-
PTBP2 rev primer	GTTCCCGCAGAATGGTGAGGTG	-
wPRE fw primer	GAGGAGTTGTGGCCCGTTGT	-
wPRE rev primer	TGACAGGTGGTGGCAATGCC	-
PTBP2 probe	ATGTTCCTCGGACCAACTTG	JOE
wPRE probe	CTGTGTTTGCTGACGCAAC	FAM
Sorting signal targeting vectors		
Name	**sequence**	**Nr.**
GFP Rev	TGTACAAGTAAAGCGGCCGC	01
RANTES_SP_SS_FWD	GGATCCACCGGTTGCTGGAAGCTCTGCCGCAGCATGAAGGTC TCCGCGGCAGCCCTCGCTGTCATCCTCATTGCTACTGCCCTCT GCGCTCCTGCATCTGCCCGCCGGCGTGTCGTCTTTGTCACCC GAAAGAACCGCCAAGGTGGCGGAGGCTCAGTGAGCAAGGGC GAGGAGCT	02
Short SortSig_FWD	GGATCCACCGGTTGCTGGAAGC	03
RsrII _GFP_Rev	TGCCAGCCCGGGTTTAACGGTCCGCTACACATTGATCCTAGC	04
P-selectin Sig.pep_FWD	GGATCCACCGGTTGCTGGAAGCTCTGCCGCAGCATGGCCAAC TGCCAAATAGCCATCTTGTACCAGAGATTCCAGAGAGTGGTCT TTGGAATTTCCCAACTCCTTTGCTTCAGTGCCCTGATCTCTGAA CTAACAAACCAGAAAGAAGTGGCAGCATTATCGCCGGCGGTG AGCAAGGGCGAGGAGCTGT	05
D2eGFP_TDCT_REV	TTACTTGTACAGTCTTAAGGACTCGGGTCAAATGCAGCGTTTG TAAAAACTCCATATGTTCCTAGGTGGCTGTGAGGATTCAAGGG GCATTTCCCATCATCTTTTTGTCTGAAACGCTTTCTTAGCAAAG CCAGGAGCGTCCCACCCATTATCAGACCTATCGTAGAAGCCAC CGCTCCACCAAAGTAAGTCAGTCGGTCCGGCTCGTCCATGCC GAGAGTGATC	06
BsrGI_TDCT_rev	GTGAGCAAGGGCGAGGAGCTGTTCA	07

2.1.10. Antibodies

Table 10: Antibodies for flow cytometry and immunofluorescences

Antibody	Application	Manufacturer
CD41-PerCpCy5.5	detection of GPIIb	Biolegend; San Diego (USA)
CD41-APC	detection of GPIIb	eBioscience; San Diego (USA)
CD42d-APC	detection of GPV	eBioscience; San Diego (USA)
CD42a-FITC	detection of GPIX	EmFret; Eibelstadt (GER)
CD62P-PE	detection of P-selectin	eBioscience; San Diego (USA)
CD62P-APC	detection of P-selectin	eBioscience; San Diego (USA)
CD41-PE	detection of GPIIb	Biolegend; San Diego (USA)
CD117 (c-Kit)	APC	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD11b	APC	Biolegend, San Diego (CA, USA)
CD16/32	Purified	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD16/32	PE-Cy-7	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD16/32	APC-Cy7	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD34	Biotin	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD45.1	APC	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD45.2	PE	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD45R (B220)	PE	eBioscience/Affymetrix, Santa Clara (CA, USA)
CD71	PE	Biolegend, San Diego (CA, USA)
Ly6A/E (Sca-1)	PerCP-Cy5.5	eBioscience/Affymetrix, Santa Clara (CA, USA)
Ly-6G/C (Gr-1)	PE	eBioscience/Affymetrix, Santa Clara (CA, USA)
TER-119	APC	eBioscience/Affymetrix, Santa Clara (CA, USA)

Antibody	Application	Manufacturer
Streptavidin	PE-Cy7	eBioscience/Affymetrix, Santa Clara (CA, USA)
Streptavidin	PE	eBioscience/Affymetrix, Santa Clara (CA, USA)
Anti-mouse lineage cocktail	Pacific Blue	Biolegend, San Diego (CA, USA)
Sheep anti human FVIII:C	FITC	Affinity biologicals IMC
Goat anti-mouse A10524	Cy5	Life Technologies
Donkey anti-sheep A11015	Alexa Fluor 488	Invitrogen
Goat anti-Rabbit	Alexa Fluor 647	Invitrogen
Goat anti-mouse	Cy3	Life technologies
Anti von Willebrand Factor Rabbit polyclonal	purified	Abcam
Phalloidin	Red 594	Biolegend; San Diego (USA)

2.1.11. Bacterial strains, Cells and Cell lines and culture media

The bacterial E.coli strain XL1 gold was used for amplification of plasmid DNA during cloning procedures. Cell lines and primary cells are listed below.

Table 11: Cells used in the study

Cells/ Cell line	Origin and Cell type	Organism	References
HEK 293T	Embryonic kidney carcinoma cells	*Homo sapiens*	(Graham et al., 1977) (Thomas and Smart, 2005)
SC1	Murine embryonic fibroblasts	*Mus musculus*	(Hartley and Rowe, 1975)J
HEL	Human erythroleukemia	*Homo sapiens*	(Martin and Papayannopoulou, 1982)
HT1080	human fibrosarcoma	*Homo sapiens*	(Rasheed et al., 1974)
HepG2	human hepatocarcinoma	*Homo sapiens*	(Morris et al., 1982)
SH-SY5Y	human neuroblastoma	*Homo sapiens*	(Biedler et al., 1973)

Primary Megakaryocytes (MKs)	Differentiated cells from lineage marker negative cell from bone marrow	*Mus musculus*
Primary HSPC	Bone marrow	*Mus musculus*

Table 13: Culture media

Strain/ Cell line	Medium	Manufacturer (Medium)
XL1 gold	LB-Medium, ampicillin (1:1000)	Paul-Ehrlich-Institute; Langen (GER)
HEK 293T	DMEM (10% FCS, 1% L-glutamine)	Lonza; Basel (CH)
SC1	DMEM (10% FCS, 1% L-glutamine)	Lonza; Basel (CH)
HEL	RPMI (10%FCS, 1% L-glutamine, 1% sodiumpyruvate)	Biowest SAS, Nuaillé (F)
HT1080	DMEM (10% FCS, 1% L-glutamine)	Lonza; Basel (CH)
HepG2	DMEM (10% FCS, 1% L-glutamine)	Lonza; Basel (CH)
SH-SY5Y	DMEM (10% FCS, 1% L-glutamine)	Lonza; Basel (CH)
Primary Megakaryocytes	StemSpan medium containing 1% L-glutamine, 1% Pen/Strep, 50 ng/ml mThpo	STEMCELL Technologies; Grenoble (FR)
primary bone marrow cells	StemSpan medium containing 1% L-glutamine, 1% Pen/Strep, 10 ng/ml mSCF, 20 ng/ml mThpo, 20 ng/ml hIGF-2, 10 ng/ml mFGF-1	STEMCELL Technologies; Grenoble (FR)

2.1.12. Mice

C57BL/6 [B6.Ly5.2] mice were purchased from JANVIER LABS (Le Genest Saint Isle, France). The Mpl-deficient ($Mpl^{-/-}$Ly5.2) mouse strain (B6;129S1-Mpl^{tm1Wsa}/ Mpl^{tm1Wsa}) was provided by Prof. Dr. W.S. Alexander (The Walter and Eliza Hall Institute for Medical Research, Royal Melbourne Hospital, Victoria, Australia).

2.2. Molecular biology methods

2.2.1. Generation of lentiviral vectors

Lineage-specific lentiviral vectors (chapter 3)

Lentiviral-vectors expressing GFP containing the lineage specific promoters (mPf4, hGP6, hGP6s, hGP9 and mGP9) were generated by S. Wintterle (Institute of Experimental Hematology, MHH). The LV vector containing the PGK and the SFFV enhancer and promoter (RRL.PPT.SFFV.GFP.pre) was kindly provided by Dr. T. Mätzig (Institute of Experimental Hematology, MHH). MK-specific promoters were amplified from human/mouse genomic DNA by PCR and inserted into the lentiviral vector RRL.PPT.SF.eGFP.pre (kindly provided by A. Schambach, Hannover Medical School (Schambach et al., 2006a)). 595 bp human glycoprotein 1b alpha promoter (*GPIba*, -265 to +330) (Hashimoto and Ware, 1995b; Ohmori et al., 2006; Heckl et al., 2011), 1222 bp murine platelet factor 4 promoter (*mPf4*,-1074 to +148) (Kufrin et al., 2003), 726 bp human glycoprotein 6 (*hGP6*, -697 to +29) (Holmes et al., 2002), 351 bp short version of the *hGP6* promoter (*hGP6s*, -322 to +29) (Ohmori et al., 2006), 794 bp human glycoprotein 9 promoter (*hGP9*, -782 to +12), 676 bp *mGP9* (-627 to +49) (Bastian et al., 1996b). As controls, a lentiviral vector with the human phosphoglycerate kinase promoter (*hPGK*, -436 to +76, vector RRL.PPT.PGK.eGFP.pre) and the SFFV enhancer and promoter were used. Vectors expressed GFP or the murine myeloproliferative leukemia oncogene (Mpl).

Alpha-granules targeting vectors (chapter 4)

The RRL.PPT.mPF4.Sig.pep.SortSigRANTES.d2eGFP.pre vector was generated by 2 PCR amplification rounds. In the first one, the d2eGFP was fused at it 3' end to the BamHI, AgeI restrictions site and signal peptide of RANTES followed by the sorting signal of Nap2, PF4 or RANTES. At its 5'end d2eGFP would be fused to the RsrII restriction site for cloning purposes (forward primer #2, reverse primer #4). The PCR product from the first amplification round was used as a template for a second PCR round. In this case, a shorter forward prime (prime #03 from table 9) was used, which would bind to the BamHI / AgeI sites and the Kosak sequence, together with the reverse primer #04 (RsrII _GFP_Rev from table 9) anchoring at the 5'end of the d2eGFP and including the RsrII and SmaI site. This

second PCR product was digested with AgeI and SmaI restriction enzymes, purified and ligated with the AgeI/SmaI RRL.PPT.mPf4.eGFP.pre digested/ purified vector backbone. P-selectin vector (RRL.PPT.mPF4.P-sel.sig.pep.HAtagd.2eGFP.TDCT.pre) was generated as well by the performance of two PCR amplification rounds, in which in the first round, very long forward and reverse primers were used in order to generate the GFP fusion at the 3 and 5'ends, and a second external round of PCR, using as a template the purified first PCR product with shorter forwards and reverse primers in order to amplify the signal and increment the number of molecules of the GFP fusion. For the first PCR round, forward primer #05 (P-selectin Sig.pep_FWD) and reverse primer #06 (D2eGFP_TDCT_REV) was used (see table 9), these primers would incorporate the signal peptide of p-selectin, and the HA-Tag to the 3'end of the d2eGFP and the transmembrane domain and cytoplasmic tail to the 5'end of the d2eGFP. This PCR product was used as a template for a second PCR where the shorter #03 and #07 primers were used for the amplification of the signal and incrementation of the DNA molecules.

The vector RRL.PPT.mPF4.hcoBDFVIII.TDCT.pre was generated in two subsequent steps. Due to the length of the FVIII, a PCR was inconvenient for the inclusion of the sorting signal, the transmembrane domain and cytoplasmic tail of P-selectin (TDCT). Therefore, in a first step, in a PUC57 cloning plasmid was send for synthesis the last 2000 base pairs from the C terminal end of the coagulation FVIII containing the transmembrane domain and cytoplasmic tail of P-selectin (TDCT), flanked by a SacII and SmaI cloning site linked, PUC57 with the recombinant fragment was digested with SacII/SmaI, insert was excised, purified and ligated with the SacII/SmaI digested purified RRL.PPT.mPF4.hcoBDFVIII.pre vector backbone. For all the cases final vectors were selected as single clones obtained from blue-white screening.

2.2.2. Bacteria transformation and cultivation

For the transformation 100 µl of chemically competent *E. coli* XL 10 Gold cells were thawed on ice and mixed with either 1 µl of plasmid DNA or 10 µl of ligation mixture (during cloning). Transformation was performed by heat shock at 42°C for 2 min followed by incubation in 1 ml LB medium for 30 min (37°C, 200 rpm). Bacteria were either plated

onto LB-agar-agar plates (100 mg/l ampicillin) for colony selection during cloning or inoculated into 500 µl of liquid LB medium, containing 100 mg/l ampicillin for the vector plasmid production, and incubated at 37°C overnight.

2.2.3. Blue white screen

The positive selection of inserted PCR products into the vector was performed with the beta-galactosidase encoding pBlueScript SKII (+) plasmid. Before bacteria inoculation, IPTG and X-Gal were additionally applied on ampicillin LB-agar-agar plates and finally incubated for 16-24 hours. In this vector system, the expression of the lacZ operon is triggered by the exposure to lactose. Hereby the galactose analogue IPTG which cannot be metabolized is used for triggering the expression of the operon. The lactose analogue Xgal will be metabolized by β-galactosidase into galactose and 5,5'-dibromo-4,4'-dichloro-indigo (blue). Those bacteria which are able to produce β-galactosidase, generate blue colonies whereas those who carry the gene of interest (GOI), and therefore lacking the active β-galactosidase, generate white colonies. For the identification of bacteria colonies containing the vectors carrying the gene of interest, just white colonies were selected and inoculated in liquid LB^{Amp} for further plasmid isolation and characterization.

2.2.4. Isolation of plasmid DNA from bacteria

Isolation of plasmid DNA from bacteria was performed using the NucleoSpin Plasmid Kit® and the NucleoBond® Xtra Maxiprep Kit (MACHERY-NAGEL) according to the manufacturer's instruction. For low amounts of plasmid DNA (1-3 µg) transformed bacteria were grown in 5ml of ampicillin (100 mg/l) containing LB medium at 37°C overnight. To obtain plasmid DNA amounts >400µg transformed bacteria were grown in 150-250 ml ampicillin (100 mg/l) containing LB medium at 37°C overnight. These isolation methods are based on Alkaline hydrolysis where harvested bacteria are resuspended in Tris-Cl-EDTA buffer followed by the addition of a NaOH/SDS buffer. SDS solubilizes the phospholipids and other membrane components which results in cellular hydrolysis

whereas the NaOH denatures chromosomal-and plasmid DNA. The addition of RNAse leads to RNA degradation. This is follow by a neutralization step, mediated by the addition of acidic potassium acetate for the precipitation of denatured proteins, cellular debris and chromosomal DNA, which are all removed from the preparation by centrifugation. Circular plasmid DNA is not denatured during this step because of its covalently closed conformation, staying in solution. Plasmid DNA is precipitated by washing with ethanol, and their isolation take place when it is added to a silica membrane under high salt conditions followed by washing and elution by low-salt condition buffer or water. Plasmid DNA concentration was determined by using the Nano Photometer (IMPLEN). For control digestion purposes, single colonies were inoculated in 3 ml LB^{Amp} (overnight, 37°C, 200 rpm). Plasmid DNA was isolated following same steps procedures, but using Home-made solutions provided by the chemical department of the Paul Ehrlich Institute.

2.2.5. Polymerase chain reaction (PCR)

DNA Fragments for cloning were amplified by PCR using the primers listed in table 9. Hereby important DNA restriction sites, or targeting domains were introduced in the amplification primers. To determine the optimal annealing temperature of the primers gradient PCRs were performed, in which the annealing temperatures varied between 55 and 67 °C. The composition of the PCR reaction and PCR temperature profile are listed in the following tables 14,15.

Table 24: PCR program

Preheating Lid	95°C	Pause	
Initial Denaturation	95°C	1 min	
Denaturation	95°C	1 min	35 cycles
Annealing	55 -67°C	1 min	
Elongation	72°C	2 min	
Final Extension	72°C	10 min	
	4°C	Pause	

Table 15: Composition of the reaction mixture

reagents	final conc./mass/vol.
Pfu-polymerase buffer (10x)	1X
DNA	20 ng
fw primer	0.5 µM
rev primer	0.5 µM
Pfu-polymerase	0.05 U/µL
dNTPs	0.2 mM
H_2O	20-50 µL

2.2.6. Separation of DNA by electrophoresis

PCR fragment were visualized in 0.8-2 % agarose gel (depending on the DNA fragment size) with TAE buffer and Midori Green for DNA staining. DNA intercalating dye was added to the gel to visualize DNA fragments under UV light. The dye interacts with DNA phosphate-residues and hereby tags DNA fragments. The DNA phosphate residues are also responsible for the negative charge of the DNA which can be used for separation in the electric field. Depending on the size of the fragments, DNA samples and 8 µL of the 1kb or 100bp DNA ladder (0.1 µg/µL) were loaded into the agarose gel and run at 80 V for 60 min.

2.2.7. Extraction of DNA from agarose

DNA extraction from agarose gels were performed with the QIAquick® Gel Extraction Kit according to the manufacturing manual. After electrophoretic separation, PCRs, digested PCRs or DNA fragments were cut under UV light exposition (366nm) and extracted gel slices were dissolved in the provided buffer containing a pH indicator to assure the optimal pH for DNA binding to the silica membrane. The further principle for DNA purification is described in 2.2.3.

2.2.8. DNA restriction digests

All enzymes for DNA restriction were obtained from Thermo Fischer Scientific and are listed in table 7. DNA restriction occurred at 37°C for at least 30 min. Concentration of plasmid DNA, restriction enzyme and appropriate buffers were used according to manufacturing recommendation. To prevent re-ligation of plasmid DNA without inserting the GOI by ligation, alkaline phosphatase (FastAP) was added to fragments which served as acceptors for DNA fragments (vectors/plasmids). This recombinant enzyme catalyzes the release of 5' phosphate group on DNA ends and thereby avoids a re-ligation of the vector without GOI. The ends of the GOI will carry the phosphate groups which are needed for the ligation process.

2.2.9. Ligation of DNA

DNA fragments were ligated by using 1 Unit of T4 DNA ligase per reaction. Ligation occurred at 16° overnight with a molar ratio of 1:3 of vector and insert. The vectors are the acceptor plasmids and inserts are the donor DNA. Hereby total amounts of 30 to 50 ng DNA were used. The amount of DNA per sample was calculated using the following equation.

$$ng\ (insert) = \frac{ng(vector) * kb(insert)}{kb(vector)} * \frac{x}{1}$$

X: ratio vector: insert

Formula 1: Determination of the amount of insert DNA needed for ligation

2.2.10. DNA concentration and purity

The concentration plasmid DNA was determined by UV absorption spectrometry using a NanoPhotometer. For determination of the DNA concentration, the optical density (OD) was measured at 260 nm, the absorption maximum of double stranded DNA (dsDNA).

Absorption (A260nm) corresponds to a concentration of 50 µg/ml of dsDNA. DNA purity can be concluded from the ratio A260nm to A280nm and A230nm.because proteins have an absorption maximum at 280 nm and salts at 230 nm. A A260nm/A280nm value of 1.8 and A260nm/A230nm value of 2.0-2.2 indicate high purity of the measured DNA. 2 µl of the sample was used for the measurement.

2.2.11. Sequencing

To ensure the fidelity of DNA sequences, the samples (vector constructs, plasmids) were sequenced via Sanger sequencing, performed by GATC Biotech. According to the manufacturer's description, samples were prepared (100ng of total DNA 0.5µM primer in 10ul ultrapure water), using the sequencing primers T3 and T7.

2.2.12. Real-time PCRs

Vector copy number (VCN) detection

VCN was determined on genomic DNA purified from leukocytes detecting the woodchuck hepatitis posttranscriptional regulatory element (wPRE) and the polypyrimidine tract-binding protein 2 (PTBP2) as an internal reference. VCN was also performed from transduced SC1 and MEGO1 cells for a precise viral titer detection. Specific primers and TaqMan probes directed against the PTBP2 gene and the wPRE-element. The average VCN was calculated as ratio between the PTBP2 housekeeping gene and the vector-internal wPRE-element using a plasmid standard as reference. The plasmid standard was kindly provided by Dr. Tobias Mätzig (Experimental Hematology, MHH). Real-time TaqMan PCR (Life Technologies GmBH, Frankfurt, Germany) was performed using the StepOnePlus system (Applied Biosystems; Thermo Scientific; Waltham; MA, USA) in triplicates. A plasmid standard harboring the sequences of the wPRE and PTPB2 was used for the quantification.

Cycling parameters

Number of cycles	Reaction step	Temperature	Duration	
1x	Preheating	50°C	2min	
1x	Initial denaturation	95°C	20sec	
40x	Denaturation	95°C	5sec	
	Annealing	56°C	20sec	
	Elongation	65°C	20sec	Data acquisition

Cell sorting and mRNA detection for gene expression profiling by q-PCR

The isolated bone marrow samples from C57BL/6 wildtype mice and experimental mice was stained with the anti-mouse Lineage Cocktail-Pacific Blue (CD3, Ly-6G/C, CD11b, CD45R, Ter-119), Sca-1-PerCP-Cy5.5, c-Kit-APC, CD16/32-APC-Cy7, CD34-Biotin-PE-Cy7, CD135-PE and analyzed on a BD AriaIII (BD Biosciences) for cell sorting. The different myeloid progenitor populations CMP (LK,CD34+,CD16/32-), GMP (LK,CD34+,CD16/32+), MEP (LK,CD34-,CD16/32-), and the LSK cell population were defined and sorted directly into RNAzol preloaded tubes for cell lysis and RNA stabilization. The RNA from the different cell populations of the experimental mice (positive control, negative control and mPf4, hGP6, hGP6s, GP1BA, mGP9 groups) was isolated with the Direct-Zol RNA Kit (Zymo Research) and reverse transcribed with the RevertAid H Minus Reverse Transcriptase (Thermo Scientific). Mouse specific QuantiFast Probe Assays (Qiagen) were used to measure the mRNA levels of mPf4, hGP6, hGP6s, GP1BA, mGP9 and Actin-beta as housekeeping gene. The TaqMan based qPCR was performed on the StepOnePlus Real-Time PCR System with the following cycling conditions: 95° - 5min// 40x 95°C - 30sec/ 60°C - 30sec.

2.3. Cell culture methods

2.3.1. Cultivation of cells

HEK293T, SC1 and MEGO1 cells were grown in 10 cm dishes using DMEM containing 10% FCS and 1% L-glutamine (culture medium). Cells were split every 2 to 3 days to avoid a confluence higher than 70 to 80%. For adhesion cells (293T and SC1) Passaging was performed by aspirating the medium followed by washing the cells once with PBS, detachment by incubation in trypsin-EDTA solution (7µM) and subsequent 1:2 dilution in appropriate medium followed by gentle resuspension. For culture maintenance cells were further diluted in the appropriate medium that a 70-80% confluence was reached after 2 to 3 days of incubation. Cells were kept in a humidified incubator at 37°C and 5% CO_2.

2.3.2. Viral particle production

Viral particles were produced using the human HEK 293T cell line, with the transient calcium phosphate co-transfection method, and the split packaging system as previously described (Dull et al., 1998b). Lentiviral vector particles were pseudotyped with the amphotropic VSVg glycoprotein (Naldini et al., 1996b). One-day prior transfection, 293T cells were counted and plated at a density of $5x10^6$ cells per 10 cm culture dish. On the following day for transfection, culture medium (DMEM medium, 10% fetal calf serum (FCS), 1% glutamine, 1% penicillin/streptomycin, 20 mM HEPES) was aspirated and 8 ml of transfection medium containing chloroquine (25µM) was added. Transfection mixture was prepared, containing the split packaging plasmids: 10µg of vector-plasmid, 10µg gag/pol plasmid (pCDA3.GP.4XC plasmid), 1.5µg plasmid encoding the VSV-G envelope (PMD.G plasmid), 5µg Rev-encoding plasmid. After preparation the mixture was dropped to 500 µl Hepes buffered saline (HeBS) using the “bubbling technique”. Hereby DNA mixture was added to the HeBS buffer under constant mixing by air bubbling to improve precipitation. After 10 min incubation solution was drop wise added to the medium of the individual plates, incubated on the cells for 6-8 hrs, and then replaced by fresh culture medium. Viral particle containing supernatant was harvested two times 36 and 48 hrs after

transfection. The supernatant was sterile filtered with a 0.22µm syringe filter (Merck-Millipore; Darmstadt, Germany) to remove cell debris of the 293T cells. Viral particles were then concentrated by ultracentrifugation (VSVg pseudotyped viruses at 83,000xg for 2-3hrs), resuspended in StemSpan (STEMCELL Technologies Inc.; Vancouver, Canada) and stored at -80° until usage.

2.3.3. Transduction of cell lines

Prior transduction, between $6x10^4$ to $1x10^5$ SC1 cells were seeded and plates were incubated (37°C, 5% CO2) until cell attachment to the dish surface was observed. For suspension cells, transduction was performed on the same day of the seeding. Before transduction with different volumes of viral supernatants, the medium was changed into culture medium containing protamine sulfate (4 µg/ml). After culture (37°C, 5%CO_2) overnight, protamine sulfate was removed from the cells by changing the medium. After 72h of culture (at 37°C, 5%CO_2) adherent cells were detached using PBS without Ca^{2+} containing EDTA (1M) and gentle pipetting. After 2-3 days the percentage of transduced cells was determined by flow cytometry to calculate the concentration of infectious viral particles per ml supernatant.

$$Titer = \frac{\%\text{pos cells } x \text{ seeded cell number}}{vol. of \text{ supernatant } [\mu l]}$$

Formula 2: Determination of the viral titer

2.3.4. Isolation and transduction of murine lineage marker-negative (lin-) BM cells

BM was flushed from femurs, tibias and hip bones of C57Bl/6 mice, by mechanically breaking up and disrupting the bones using a mortar and two repeated wash steps using IMDM with 2% FCS, 1% Pen/Strep, and 1% L-glutamine. To ensure a single cell suspension, the BM cells were flushed through a 70µm cell strainer. Mononuclear cells were purified by gradient-centrifugation (Histopaque® 1083, Sigma-Aldrich, Munich, Germany). For this, 5 ml of cell suspension was loaded on 5 ml of Histopaque1083 and

centrifugated at 800xg for 20 min. at RT without brakes. Mononuclear cells were isolated from the interface of the gradient, and washed with PBS. Lin- cells were selected by magnetic cell-sorting using lineage-specific antibodies: CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119 (Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min. at 4°C. Cells were then washed and the secondary staining with streptavidin coupled magnetic beads was performed for 30 min. at 4°C. After a further washing step with PBS the cell suspension was applied on LS separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany) fixed on a magnetic stand. The lineage negative (lin-) fraction was collected in a 15 ml reaction tube, spun down and counted. The lineage marker negative purified cells were either frozen as life cells in FCS containing 10%DMSO or seeded in a multi-well suspension plate in StemSpan-STIF medium (StemSpan containing 1% Pen/Strep, 1% L-glutamine, 10 ng/ml mSCF, 20 ng/ml mThpo, 20 ng/ml hIGF-2, 10 ng/ml mFGF-1) (Zhang, Lodish 2005).

2.3.5. Transduction of primary BM cells

Purified lineage marker negative cells were cultured in StemSpan-STIF medium at 37°C and 5% CO2 for 24hrs prior transduction for pre-stimulation. Before transduction, wells were pre-coated with RetroNectin (3.6µg/mm2) (Takara BIO Inc.; Otsu, Japan) and the necessary volume of the viral supernatant was added onto the pre-coated wells. Viral particles were attached to the RetroNectin by centrifugation at 2000rpm and 4°C for 30 min. Transduction of lin^- bone marrow cells were performed in two rounds with a MOI of 2.5 each, in a cell density ~$1x10^6$/ml, to a final MOI of 5. Plates were then incubated overnight at 37°C and 5% CO2. In the second round of transduction, approximately half of the medium was replaced with fresh medium and new virus supernatant. After additional 24-48 hrs. incubation time, the cells were harvested by gentle resuspension, washed off the plate with PBS and were prepared for further experiments.

2.3.6. Hematopoietic differentiation in-vitro

For megakaryocytes generation, one day after transductions were complete, cells were cultured in serum-free medium containing 50 ng/ml of Thrombopoietin (Thpo). Every 5 days during 3 weeks of culture, cells were analyzed for GFP expression in the CD61-PE/CD41-APC double-positive cells. For differentiation into non-MK-lineages, 1.5x104 cultured lin- BM cells were plated in semi solid-methylcellulose media, supplemented with 5 IU/ml human erythropoietin, 10 ng/ml murine IL3, 10 ng/ml murine IL6, 50 ng/ml murine SCF, 10 µg/mL recombinant human insulin, 200 µg/ml human transferrin, 2% L-glutamine (R&D Systems, Abingdon, UK, #HSC007) and cultured for 8 days.

2.3.7. Platelets isolation

For the analysis of the alpha granules targeting vectors (chapter 4), platelets from transplanted mice were isolated. For that, 50-100 µl of murine blood was mixed with 50 µl Heparin (20 units/ml). To prevent platelet activation, PGE1 (1 µM) and/or apyrase (0.2 U/ml final concentration) was added to the tube. Samples were then centrifugated at 200xg for 10 min (obtention of platelet rich plasma or PRP). PRP was aspirated, placed in a new tubed and centrifugated at 2000xg for 20 min. For inhibition of activation 0.2 U/ml of apyrase was added to the sample before centrifugation. Supernatant was aspirated and discarded, and the pellet was resuspended in 100 µl of tyrode's buffer carefully added containing 5 mM glucose and freshly added BSA (3 mg/ml). To prevent platelet activation, we added apyrase (0.2 U/ml final concentration). If the platelets needed to be activated in subsequent experiments, the addition of apyrase at this step was omitted and the tyrodes buffer contained 2 mM of CaCl2. Every step was handled at room temperature. In case that blood could not be processed immediately, PGE1 was added it to the sample and kept at 37 ^{0}C for a maximum period of 2 hours.

2.3.8. Flow cytometry analysis from in vitro experiments

In general, cells were stained with the respective antibodies in a final volume of 100µl FACS-buffer. In case of primary mouse cells, either from blood cells or bone marrow, the Fc receptor binding sites were blocked prior antibody staining with purified CD16/32 antibody.

Colony assays: the cells were washed from the semisolid media with PBS and stained for erythroid markers (Ter119/CD71, colony forming unit-erythroid (CFU-E)), monocyte-macrophage markers (CD11b/F4/80; colony forming unit-macrophages (CFU-M)) or granulocytic markers (CD11b/Gr1, colony forming unit-granulocytes (CFU-G)), all antibodies from eBioscience, California, USA). Flow cytometry was performed using the BD Accuri™ C6 flow cytometer (BD Bioscience, Heidelberg, Germany).

Megakaryocyte differentiation:

For flow cytometry analysis, cell cultures with transduced megakaryocytes were centrifugated (1000 rpm x7min) and pellet resuspended in 100 µl of PBS. For the staining, first the Fc-receptor was blocked with the purified CD16/32 antibody (incubation for 20 min on ice), followed by a washing step (1000 rpm x7min), resuspension in 100 of PBS and incubation for 30 min with 1 µl of one or more of the following antibodies (anti CD41, CD61, CD42b, CD42a, CD42d) on ice. Finally, cells were washed and resuspended in a final volume of 500 µl of FACS-buffer. For the analysis of the protein targeting vectors (chapter 4), transduced megakaryocytes were spun on glass cover slides (500 rpm and RT for 4 min), with the Shandon Cytospin 4 centrifuge (Thermo Scientific). Cells were then stained as indicated in 2.3.11.

GFP expression in polyploid megakaryocytes

For analysis of GFP signal transduction in polyploid megakaryocytes, cells were fixed in 4% PFA for 10 min., washed, and permeabilized on ice with 0.5% Triton X-100 in PBS for 45 min. Washed cells were then stained by the addition of PI, and analyzed with the flow cytometry for the measurement of DNA content.

FVIII staining in transduced megakaryocytes:

Transduced cells were fixed in 4% PFA for 10 min., washed twice with PBS and permeabilized on ice with 0.5% Triton X-100 in PBS for 30 min. Cells were then washed with PBS and blocked with 10% BSA solution and CD16/32 antibody overnight at 4^0C, followed by a washing steps with 0.05 % PBS-Tween20 (PBS-T) and incubation of a 1:100 solution of the primary antibody (anti-FVIII-FITC) and/or anti P-selectin-PE incubated for 1 hour on ice. Cells were then washed and resuspended in FACS-Buffer.

Platelets staining:

After platelet isolation (see 2.3.7), cells resuspended in 100 µl of FACS-buffer were incubated with 1µl of the CD16/32 antibody for Fc-blocking (20min), following by incubation with 1µl of one or more of the corresponding primary antibody (CD41, CD61, CD62P, CD42a, b, d). finally, cells were washed and resuspended with FACS-buffer. All steps were performed at room temperature.

Blood, spleen and BM cell analysis

For analysis of erythrocytes and platelets by flow cytometry, blood samples were diluted 1/2000 in PBS. Peripheral blood leukocytes were analyzed after erythrocyte lysis. Single cells from the spleen were isolated using a cell strainer. Antibody-staining was performed in PBS, 2% FCS, 2 mM EDTA using lineage-specific, conjugated-antibodies: platelets (CD41), granulocytes (CD11b, GR1), T-cells (CD3), B-cells (B220), erythroid cells (Ter119, CD71), HSPC (Sca-1, cKit, CD34, CD16/32). Antibodies were conjugated to FITC, PE, APC, e-fluor-450, Alexa700, PerCP-Cy5.5, or PE-Cy7 fluorochromes (Becton Dickinson, France, eBioscience, California, USA or Roche diagnostics, Mannheim, Germany). Dead cells were excluded by propidium iodide (PI) or 4',6-Diamidino-2-phenylindoledihydrochloride (DAPI) staining. Flow cytometry was carried out at the BD Accuri™ C6 or the LSRII flow cytometer (BD Biosciences, Franklin Lakes, USA. Flow cytometric analysis of total BM cells from transplanted mice was done with 5x106 cells. After erythrocyte lysis cells were stained in a volume of 100-150µl FACS-buffer for 30 min. on ice. The following mouse-specific antibodies were used in different combinations and with different fluorochromes: Anti-HA, anti-mouse Lineage Cocktail (CD3, Ly-6G/C,

CD11b, CD45R/B220, TER-119), Ly-6A/E (Sca-1), CD117, CD34, CD16/32, CD45.2, CD201, CD48, CD150, CD0202b (Tie2). Flow cytometric measurements were carried out by flow cytometry using the LSRII or AriaIII flow cytometers. Data analysis was performed using the BD FACSDiva, BD Accuri C6 (BD Biosciences, Heidelberg, Germany) or FlowJo software (TreeStar Inc.; Ashland, USA).

2.3.9. Fluorescence microscopy of cultivated cells.

Cells were placed or grown in µClear 96well-plates, fixed with 4% formaldehyde at 4°C for 12-48 hours, and subjected to imaging using an Operetta (Perkin Elmer) automated microscope in spinning disc–confocal mode. All pictures were acquired using the same settings and were adjusted similarly by the harmony software (Perkin Elmer) to a Gamma-value of 2 to enable visualization of low GFP signals.

2.3.10. Immunofluorescence staining on whole femora cryosections.

Femora of mice were isolated, fixed (4% PFA, A3813, AppliChem; 5 mM sucrose, S0389, Sigma-Aldrich), transferred into 10% sucrose-PBS and dehydrated using a graded sucrose series. Samples were then embedded in Cryo-Gel (39475237, Leica Biosystems) and shock-frozen in liquid nitrogen. Frozen samples were stored at -80°C. Seven-micrometer-thick cryosections were generated using the CryoJane tape transfer system (Leica Biosystems) and probed with Alexa488-conjugated anti-GPIX [30] antibodies (56F8, 1.0 µg/ml), for specific MK/platelet labelling, and Alexa647-conjugated anti-CD105 antibodies (3.33 µg/ml, 120402 (MJ7/18), Biolegend) to stain the endothelium. Nuclei were labeled with DAPI (1.0 µg/ml, D1306, Invitrogen). Samples were visualized with a Leica TCS SP5 confocal microscope (Leica Microsystems). ImageJ software (NIH) was used to determine mean cell fluorescence intensities.

2.3.11. Immunostaining for confocal laser scanning microscopy

In vitro transduced megakaryocytes were counted, spun down (1000rpm x 5 min) and cell pellets were resuspended with PBS in a cell density of $1x10^6$ cells/ml (1000 cells/µl). 200 µl of cell suspension was spun down in cover slips by cytospin (500rpm x 4 min) and let it to dry. Cells were then fixed with 4%PFA for 20min, and washed twice with PBS (if needed, cells were stored at this point at 4^0C up to 3 weeks). After fixation, cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min. Cells were then washed with PBS and blocked with 10% BSA solution and 1.5 µl CD16/32 antibody (Fc-block) overnight at 4^0C in case that primary anti FVIII antibody was to be used or just up to 1 hour at room temperature (RT) for any other antibody. This was followed by a two washing steps with 0.05 % PBS-Tween20 (PBS-T) and the subsequent incubation of a 1:100 blocking solution with the primary antibodies mixture (anti-FVIII-FITC/ anti P-selectin-PE, anti-FVIII-FITC /anti-von Willebrand Factor, or anti-P-selectin and/or anti-von Willebrand factor), this was incubated together with phalloidin, also in a 1:100 dilution, used for the F-actin staining (cellular demarcation). Primary antibody mixture was incubated for 1 hour at RT. Cells were then washed twice with PBS-T and incubated for 1 hour at RT with a 1:000 dilution of the secondary antibodies, together with a final concentration of 1 µg/µl DAPI (4,6-diamidino-2-phenylindol) for DNA staining. Finally, unbound dyes and antibodies were then removed from cell preparation by washing twice in PBS and coverslips were then sealed on top of microscope slides with 15 µl of Mowiol. For the settings and analysis of the samples, positive (stained) and negative (unstained) controls for every antibody was employed. LSM was used to detect fluorescent-labeled proteins in a defined layer within a cell. This method offers the possibility to investigate the colocalization of proteins. The analysis was performed using a LSM 510 microscope and LSM image browser software. 63x and 100x objectives were utilized.

Colocalization analysis of the samples by confocal laser scanning microscopy

Having in consideration that one picture is constituted by hundreds of pixels, colocalization analysis is then a final or summation interpretation of a pixel-based analysis, in which the total frequency and the intensity of the signal from two different channels (or colors) are measured and compared to addresses the question, whether the signals of these 2 selected channels, are detected together in all the pixels that conform one picture. For the analysis, every pixel in the image is plotted in a scatter plot diagram based on its intensity level from each channel (see figure 2.1).

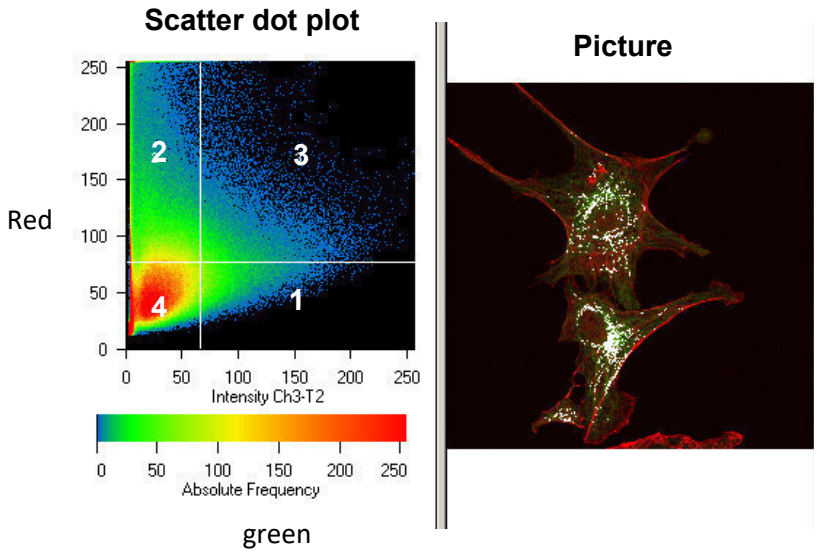


Figure 2.1. scatter dot plot diagram

Four quadrants are designated in the software by use of the crosshairs. The lower left, (figure 2.1 Quadrant 4) in the scatterplot represents pixels that have very low intensity levels in both channels; it is therefore referred to as background and not taken into consideration for colocalization analysis. In this example, Quadrant 1 show pixels that have high green intensities and low red intensities and Quadrant 2 represents pixels that have high red intensities and low green intensities. Quadrant 3 represents pixels with high

intensity levels in both green and red. In these pixels both signals are considered to be colocalized.

For the quantitative analysis, the colocalization coefficients are measured for each channel. They are calculated (by the software), by summing the pixels in the colocalized region (Quadrant 3) and then dividing by the sum of pixels either in Channel 1 (Quadrant 1 + Quadrant 3) or in Channel 2 (Quadrant 2 + Quadrant 3), having each pixel a value of 1. In the calculation for the colocalization coefficients, every pixel has the same value in the equations. The weighted colocalization coefficient (WCC) takes into account the intensity value of the summed pixels. The weighted colocalization coefficients use the same equation as the colocalization coefficients, but the value for each pixel is equal to its intensity value. Their values will range from 0 to 1, where 1 means an absolute colocalization.

2.3.12. Mouse experiments

Animal experiments were approved by the local ethical committees (Lower Saxony State Office for consumer protection and Food Safety, Hessen; Regierungspräsidium Darmstadt) and performed according to their guidelines. During the first three weeks after lethal irradiation and transplantation, mice were monitored once or twice per day for their state of health. To circumvent further infection complications, 0.1mg/ml of Ciprofloxacin were supplied to the mice in drinking water. In case mice were found to be ill (hunched posture, limited mobility, ruffled fur, anemic) or at the end of the observation time of the experiment, mice were euthanized with CO2 and subsequent cervical dislocation. Transplantation in wild type C57Bl/6 mice were performed by transfusion of 5x105 cells transduced bone marrow cells in 8-10 weeks lethally irradiated mice (10Gy) approximately 12hrs after irradiation. The cultured and transduced donor cells were resuspended in PBS and transplanted via tail-vein-injection in a volume of 150µl per mouse. Starting from 6-8 weeks post Tx, blood samples were taken by retro-orbital bleeding in intervals of 3 weeks. Mice were sacrificed at 16-24 weeks or in a moribund state and subjected to pathological examination including histopathology, blood counts, flow cytometry, DNA and RNA isolation, blood smears, and bone marrow cytospins. A 70µm cell strainer was used to

isolate cells from the spleen. Bone marrow cells were flushed from femurs, tibias, and hips. Remaining cells were live frozen in FCS with 10% DMSO and kept in liquid nitrogen. For the Mpl-/- to Mpl-/- transplantation model, the bone marrow lin- cells of Mpl-/-Ly5.2 mice were isolated as described in 2.3.4, cultured, transduced with the different lineage specific lentiviral vectors, and finally transfused into 3-6 months lethally irradiated mice (8Gy).

2.3.13. Histopathology

Organs of transplanted mice were fixed in 4 % formalin for at least 24h and embedded in paraffin. The bones were decalcified by ethylene-diamine tetra-acetic acid (Decal; SERVA) prior paraffin embedding. 1-3 µm sections of paraffin-embedded organs and bones were cut and stained with Hematoxylin/Eosin.

2.3.14. Blood counts and flow cytometry

A volume of 100-150µl peripheral blood (PB) was obtained by retro-orbital bleeding into EDTA-solution containing tubes. Blood counts were measured with the Scil Vet Abc (Scil Animal Care Company GmbH; Viernheim, Germany). Prior to staining of PB leucocytes, erythrocytes of 50µl blood were lysed at RT using Red Blood Cell Lysis buffer (Sigma-Aldrich) and the Fc-receptor was blocked with the CD16/32 antibody. Antibody staining was always carried out for 30 min on ice in FACS-buffer. The following murine-specific antibodies were used for leucocyte staining: CD45, CD45.2, CD45.1, CD11b, Gr-1 (Ly-6G/C), CD3, B220 (CD45R), anti-HA. Measurements were carried out by flow cytometry using the Accuri C6 (BD Biosciences).

2.3.15. Cytospins

For cytospin preparations $1x10^5$ total BM or in vitro differentiated lin- cells were resuspended in 150µl of PBS. Cells were then spread onto glass slides by spinning at 300rpm and RT for 8 min. with the Shandon Cytospin 4 centrifuge (Thermo Scientific). The cell spot was air dried for at least 24hrs prior May-Grünwald-Giemsa staining to distinguish the different blood cell types.

2.3.16. May Grünwald-Giemsa staining

For the morphological analysis of the different blood cell types, blood smears and cytospin glass slides May-Grünwlad-Giemsa staining were performed. The glass slides were incubated in fresh May-Grünwald staining solution (Sigma-Aldrich) for 5 min and subsequently washed with PBS. Giemsa staining solution (Sigma-Aldrich) was freshly diluted 1:20 in ddH2O and applied for 20 min., followed by another washing step, and finally air-dried o/n. The stained cells were analyzed with the Leica DMRBE microscope (Leitz).

2.3.17. Statistical analysis

For comparison of two experimental groups, the two-tailed unpaired t-test with Welch's correction or the unpaired Mann-Whitney test was used. For multiple comparisons, we used the Kruskal-Wallis One-way ANOVA Test with Dunn´s correction. The level of significance was set at $p<0.05$. Calculations were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA USA).

Chapter 3

- "Targeting expression to megakaryocytes and platelets by lineage-specific lentiviral vectors"

Original Article:

1. **Targeting expression to megakaryocytes and platelets by lineage-specific lentiviral vectors.** **Latorre-Rey LJ**, Wintterle S, Dütting S, Kohlscheen S, Abel T, Schenk F, Wingert S, Rieger MA, Nieswandt B, Heinz N, Modlich U. J Thromb Haemost. 2017 Feb;15(2):341-355.

3.1 Generation of in vitro megakaryocytes

For the evaluation of expression from the lineage-specific vectors, lineage marker-negative cells were isolated from whole bone marrow of C57BL/6J donor mice. Cells were cultured in vitro and differentiated into megakaryocytes by the addition of 50ul/ml thrombopoietin (THPO). During differentiation, megakaryocytes undergo phenotypic changes, characterized by an enlargement of size (Figure 3.1.2), due to polyploidization and cytoplasmic remodeling. As shown in figure 3.1.1, this phenotypic change also involves a cell surface modification, given by the expression of the lineage specific cell surface markers CD61/CD41, as well as others like CD42a, and CD42d, characteristic of the megakaryocyte lineage.

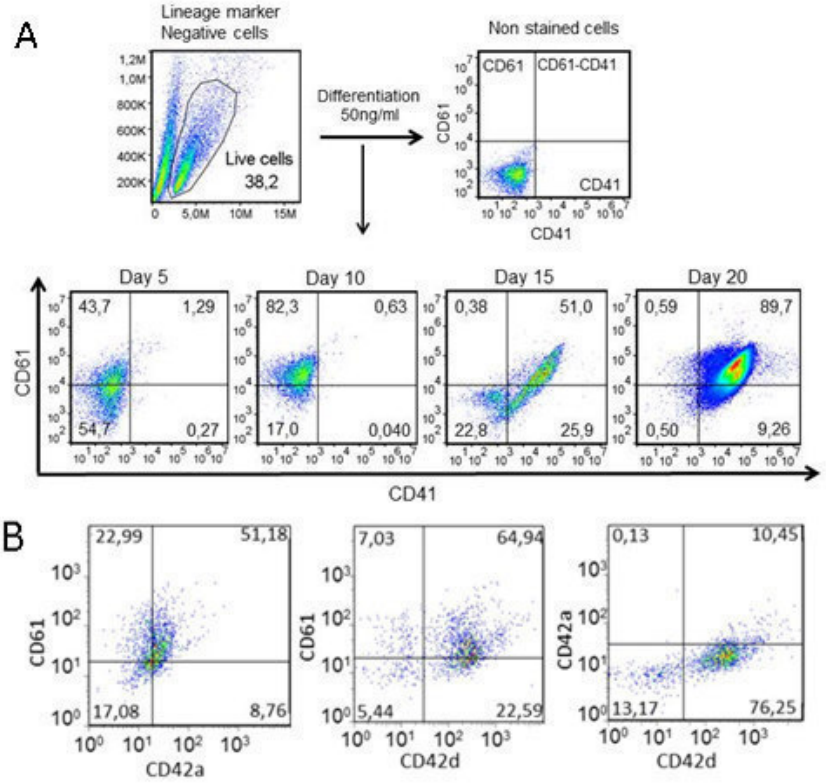


Figure 3.1.1 Differentiation into megakaryocytes from lineage marker-negative cells *in vitro*. For the analysis of the lineage-specific vectors, lineage marker-negative cells were isolated from the bone marrow, transduced with the respective vectors and induced into megakaryocyte (MK) differentiation by the addition of THPO (50ng/ml). Dot plots showing CD61 and CD41 expression in in vitro differentiated megakaryocytes with thrombopoietin **(A)**. Dot plots showing expression of cell surface markers CD42a, CD42d and CD61 at final week of differentiation **(B).**

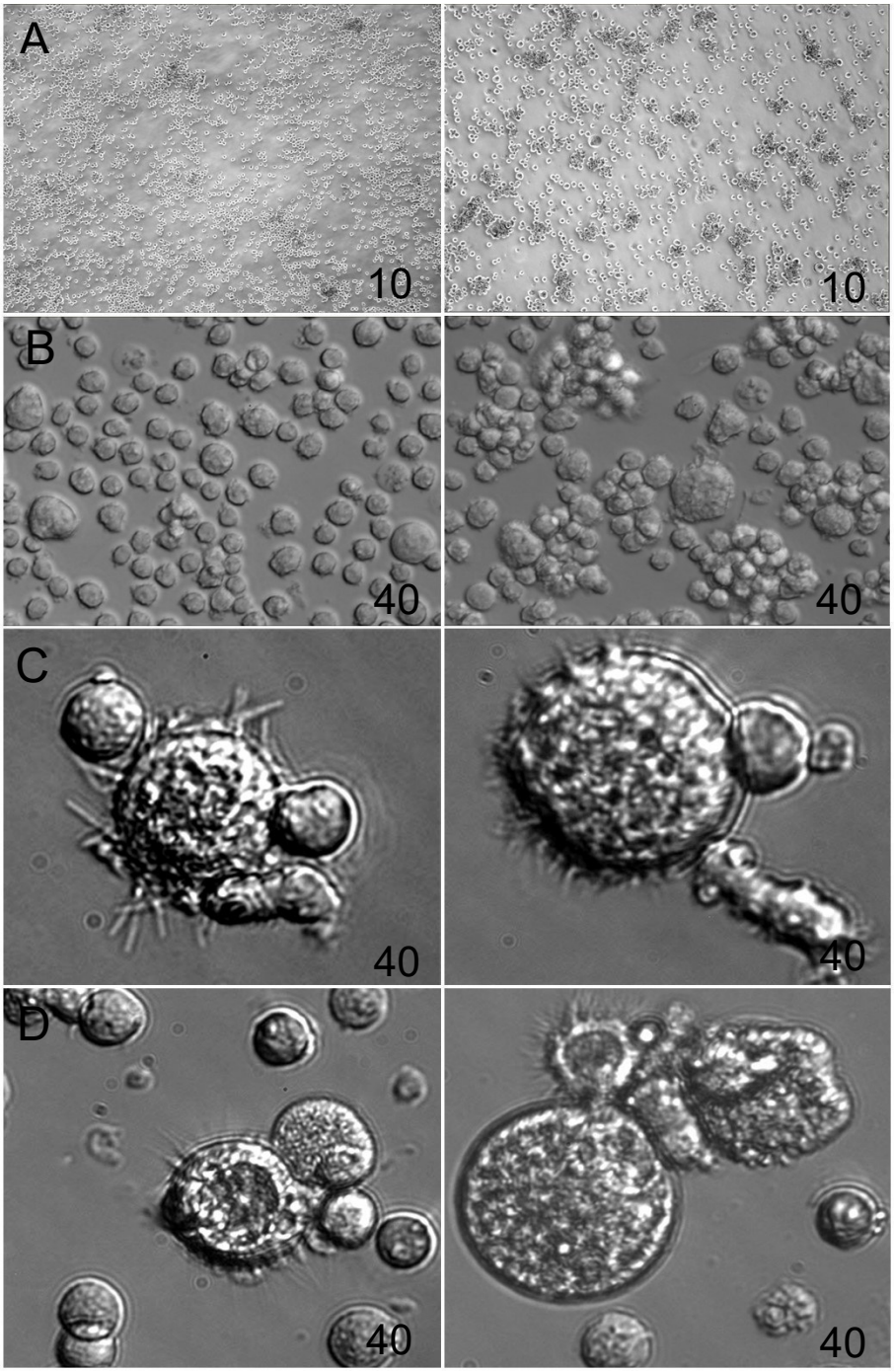


Figure 3.1.2. *In vitro* megakaryocytes. Lineage marker-negative cells (A) were isolated from whole bone marrow (BM) and differentiated into megakaryocytes by the addition of 50ul/ml THPO. Pictures taken with inverted microscopy, 10 and 40x. Notes the cellular enlargement during megakaryocyte differentiation (B, C, D).

3.2. Design of lentiviral vectors for megakaryocyte-specific expression.

With the aim to develop lentiviral vectors that restrict expression to MKs and platelets, we introduced promoter fragments of MK-specific genes into third-generation, self-inactivating (SIN) lentiviral vectors in the internal position (Figure 3.2.1). We selected a 794bp/676bp fragment of the *h/mGP9* promoter (Bastian et al., 1996a), a 726bp fragment of the *hGP6* promoter (Holmes et al., 2002) and a shorter fragment of the *hGP6* promoter of 351bp (Ohmori et al., 2006), a 1.2kb of the murine *(m)Pf4* (Kufrin et al., 2003) and 595bp of the *hGP1BA* promoter (Heckl et al., 2011). As ubiquitously expressing control vectors, we analyzed the expression from the *hPGK* promoter in the lentiviral vector or the viral spleen focus forming enhancer/promoter (*SFFV*) in the context of the LTR-driven gammaretroviral vector. The latter is considered to have strong activity in hematopoietic cells (Wahlers et al., 2002), and was included in our *in-vivo* experiments, while the PGK promoter is known for achieving sufficient expression levels for correcting genetic hematological diseases (González-Murillo et al., 2010; Huston et al., 2011). As a reporter, the enhanced green fluorescent protein (GFP) was expressed. All vectors were produced with unconcentrated titers of $1x10^6$ - $1x10^7$ transducing particles/ml. To investigate the specificity of expression, we transduced murine lin^- BM cells which were further differentiated into MK and other hematopoietic cells *in-vitro* or transplanted into mice to assess *in-vivo* specificity of expression in blood cells and HSPC (Figure 3.2.2).

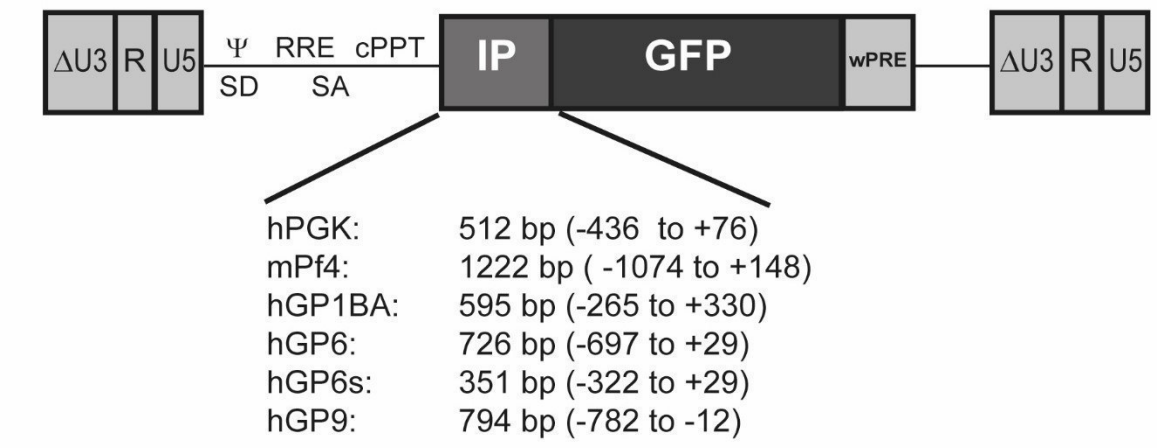


Figure 3.2.1. Lentiviral vector design. Third generation self-inactivating lentiviral vectors were generated, expressing the reporter GFP from different MK-specific promoters of indicated sizes in the internal position. Murine platelet factor 4 promoter (mPf4), human glycoprotein Ib alpha promoter (hGP1BA), human glycoprotein 6 (hGP6) or the short version of hGP6, referred as hGP6s, as well the human glycoprotein 9 (GP9) promoter. As ubiquitously expressing control promoter, the human phosphoglycerate kinase promoter (hPGK) was used. U3 and U5: unique region 3' and 5', respectively, R: repeat region, ψ: packaging signal, SD: splice donor, SA: splice acceptor, RRE: rev responsive element, cPPT: central polypurine tract, IP: internal promoter, wPre: woodchuck hepatitis virus posttranscriptional regulatory element. Promoters were verified by sequencing after insertion into the vector.

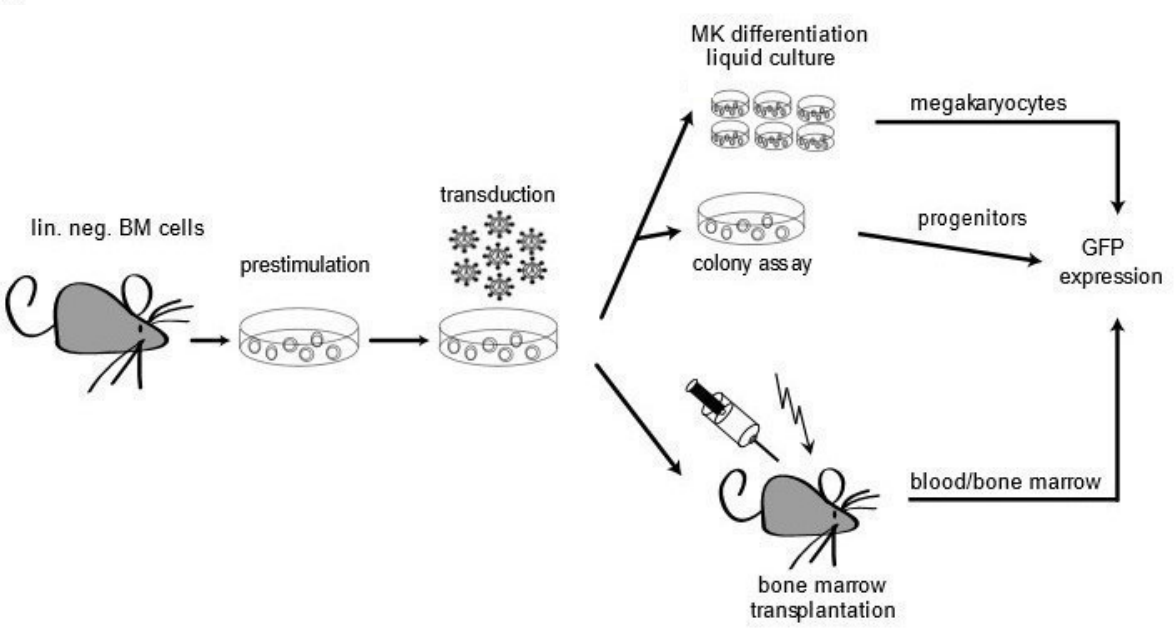


Figure 3.2.2. Work-flow of in-vitro and in-vivo experiments. Lineage marker-negative cells from the BM of C57BL/6 wild type mice were isolated, pre-stimulated for 24 hours and transduced twice with lentiviral vectors. Cells were then either differentiated to MK in liquid cultures, plated into semi-solid differentiated medium for colony assays, or transplanted into conditioned C57BL/**6** recipient mice. Reporter gene expression was measured by flow cytometry in the respective cell populations.

3.3. Activity of lineage-specific promoters in in-vitro megakaryocytes.

Murine lin$^-$ BM cells were transduced with MK-specific vectors, differentiated to MK in vitro (Fig.3.1.1) and analyzed for percentage of GFP expression and fluorescence intensity. Transduction efficiency was set to be below 30% to avoid multiple vector integrations per genome (Kustikova et al., 2003), therefore not all MK were transduced and GFP positive. The percentage of GFP expressing cells transduced with the MK-specific lentiviral vectors in undifferentiated lin$^-$ BM cells was 4 to 8-fold lower compared to the hPGK vector control group, although the same MOI was applied for all vectors ($p=0.0065$, Kruskal-Wallis-test, Figure 3.3.1C). After differentiation into MK cells, the percentage of GFP-positive cells in the CD61/CD41+ cells (Fig.3.3.1 B), increased between 2.4 and 5-fold in the cultures transduced with the MK-specific vectors (Figure 3.3.1 D, Kruskal-Wallis-test $p=0.0058$), while expression from the hPGK-vector was only slightly increased (mean 1.6-fold), indicating that MK-specific lentiviral vectors became more active in MKs compared to the control, (see gating strategy in figure 3.3.1 A and B).

The strength of expression could also be estimated by the mean fluorescence intensity (MFI) of the GFP signal in transduced cells from liquid cultures. In MKs the mPf4-vector was the strongest followed by the hGP6 and hGP1BA-vectors, while the m/hGP9-vectors were the weakest (Figure 3.3.2 A, B). Among the vectors, the mPF4 vector was the strongest, about 2.5-times more intense, measured by the mean fluorescent intensity (MFI), than the hPGK control vector followed by the hGP6 and hGP1BA-vectors, while the m/hGP9-vectors were the weakest (Figure 3.3.2 B). GFP intensity increased with polyploidization, a measure of MK maturation, in cultures transduced with the MK-specific vectors, except for the hGP9-vector and the hPGK control vector (Figure 3.3.2 C).

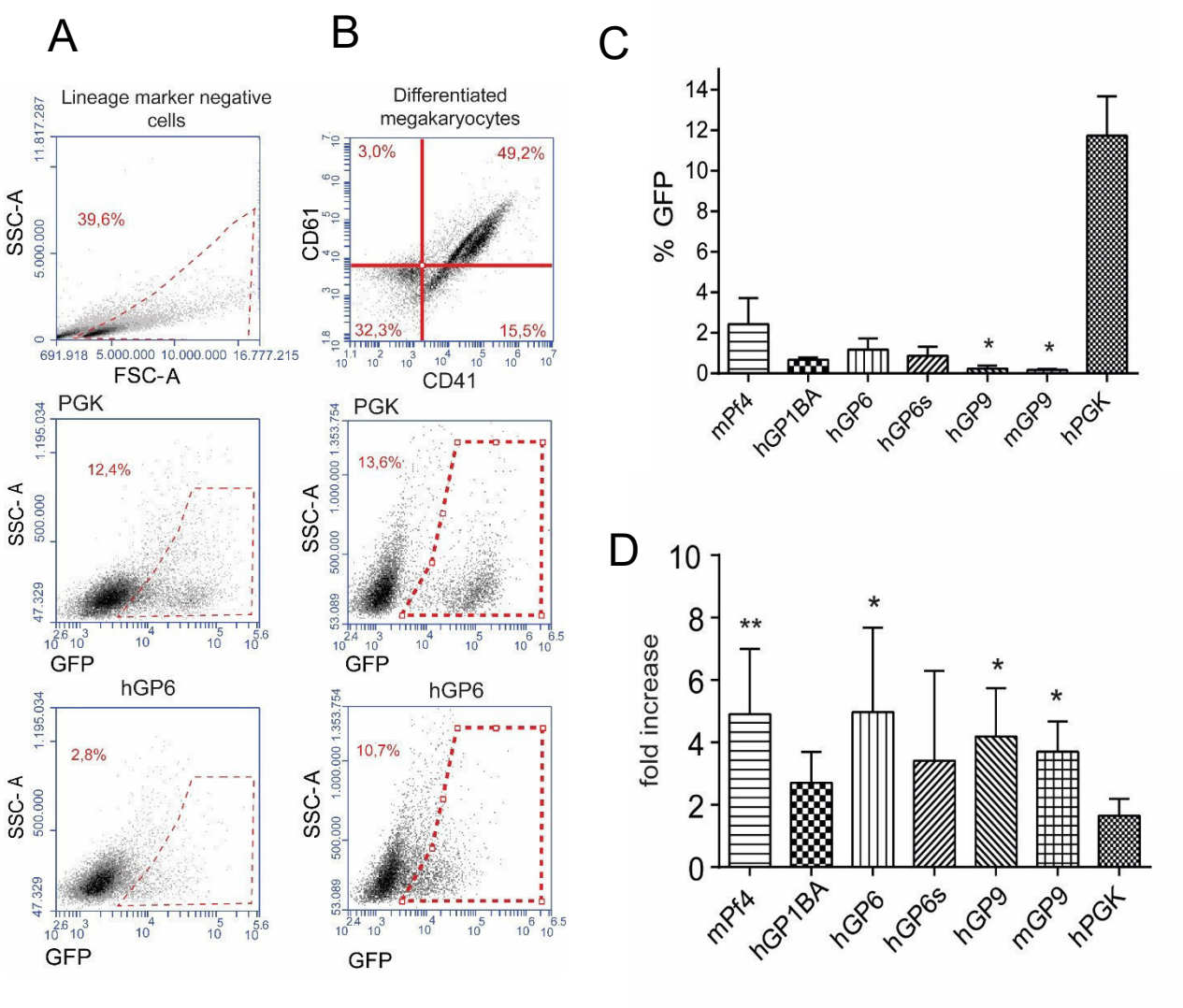


Figure 3.3.1. Expression from the MK-specific lentiviral vectors in hematopoietic cells after *in-vitro* differentiation. (A) Gating strategy for undifferentiated cells. Whole lineage marker negative cells were gated for the live cell population. Subsequently, live cells were analyzed for GFP expression. (B) Gating strategy for differentiated cells. After culture in differentiation medium (50ul/ml THPO), megakaryocytes were defined as the CD61/CD41 double positive cells. For the analysis of the vectors CD61/CD41 double positive cells were finally gated for GFP expression. (C). Percentage of GFP-positive cells in lin- BM cells one day after transduction (non-differentiated cells) (n=3, significant differences between the groups p=0.0065, Kruskal Wallis test, *significant difference by Dunn´s multiples comparison test) (D) Fold increase of GFP expression in MKs compared to expression in lin- cells. Fold increase was calculated as the ratio of %GFP positive CD61/CD41+ megakaryocytes divided by % GFP positive cells in the transduced-non-differentiated lin- cells. (n=6, Kruskal Wallis test p=0.0026, *significant difference by Dunn´s multiples comparison test).

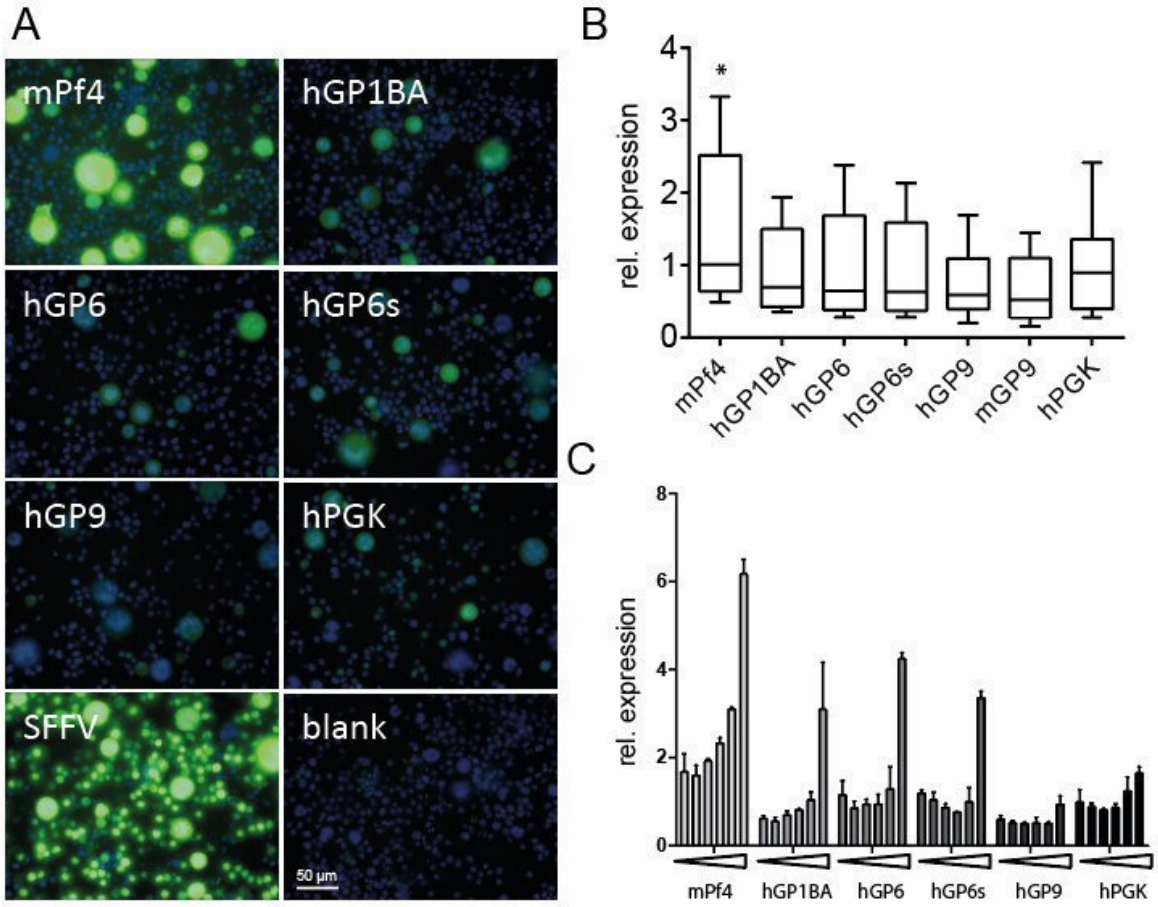


Figure 3.3.2. Expression from the MK-specific lentiviral vectors in hematopoietic cells after in-vitro differentiation. (A). GFP expression in MK differentiated in vitro. GFP expression (green) and Hoechst-staining for nuclei (blue) was acquired by an automated confocal fluorescence microscopy. (B) Relative expression in differentiated MK in-vitro, MFI in GFP-positive cells from MK-specific vectors, hPGK set to 1. (whisker blot, line indicates the median). (C) Relative intensity of expression along the polyploidization. The columns present the mean expression in 2n, 4n, 8n, 16n, 32n and >64n cells, increase of DNA content indicated by the Triangulum.

3.4. Activity of lineage specific promoters in in-vitro non-megakaryocytic cells.

To assess the activity of the MK-specific lentiviral vectors in non-megakaryocytic cells, transduced lin$^-$ BM cells were plated into semi-solid medium for eight days. Cells were then washed, stained and analyzed for cell surface markers and GFP expression, identifying granulocytic (CD11b+/Gr1+), macrophage/monocytic (CD11b+/F4/80+) and erythroid (CD71/Ter119) cells. The percentage of GFP-expressing cells transduced and differentiated with the MK-vectors was slightly increased, however, in all cases lower than in the hPGK control (Figure 3.4.1 A, Fig.3.4.2 A, B, C, D). Promoter strength, as estimated by the MFI of GFP in positive cells, was always lower in cells transduced with MK-specific vectors than in cells transduced with the hPGK-vector, indicating the lower activity of MK-vectors in non-megakaryocytic cells (Figure 3.4.1 B).

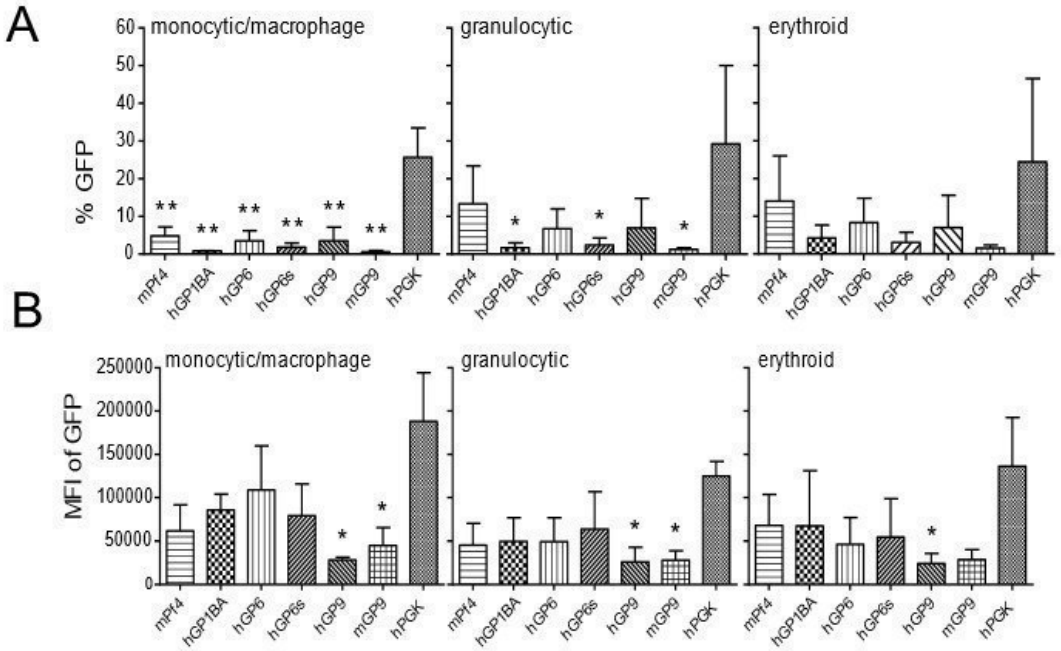


Figure 3.4.1. Percentage of GFP positive cells in monocytic/macrophages, granulocytic and erythroid progenitors differentiated in colony assays. (A) % of GFP expression (Kruskal Wallis test, *significant difference by Dunn´s multiples comparison test). (B) Mean fluorescence intensity of GFP expression in the GFP positive cells in colony assays (Kruskal Wallis test, *significant difference by Dunn´s multiples comparison test). (*<0.5, **<0.01). For the experiment, transduced lineage marker-negative cells were cultured in semisolid medium for 7 days. Cells were defined as Granulocytes (CD11b/GR1), monocytes/macrophages (CD11b/F4/80), erythrocytes progenitors (Ter119 /CD71).

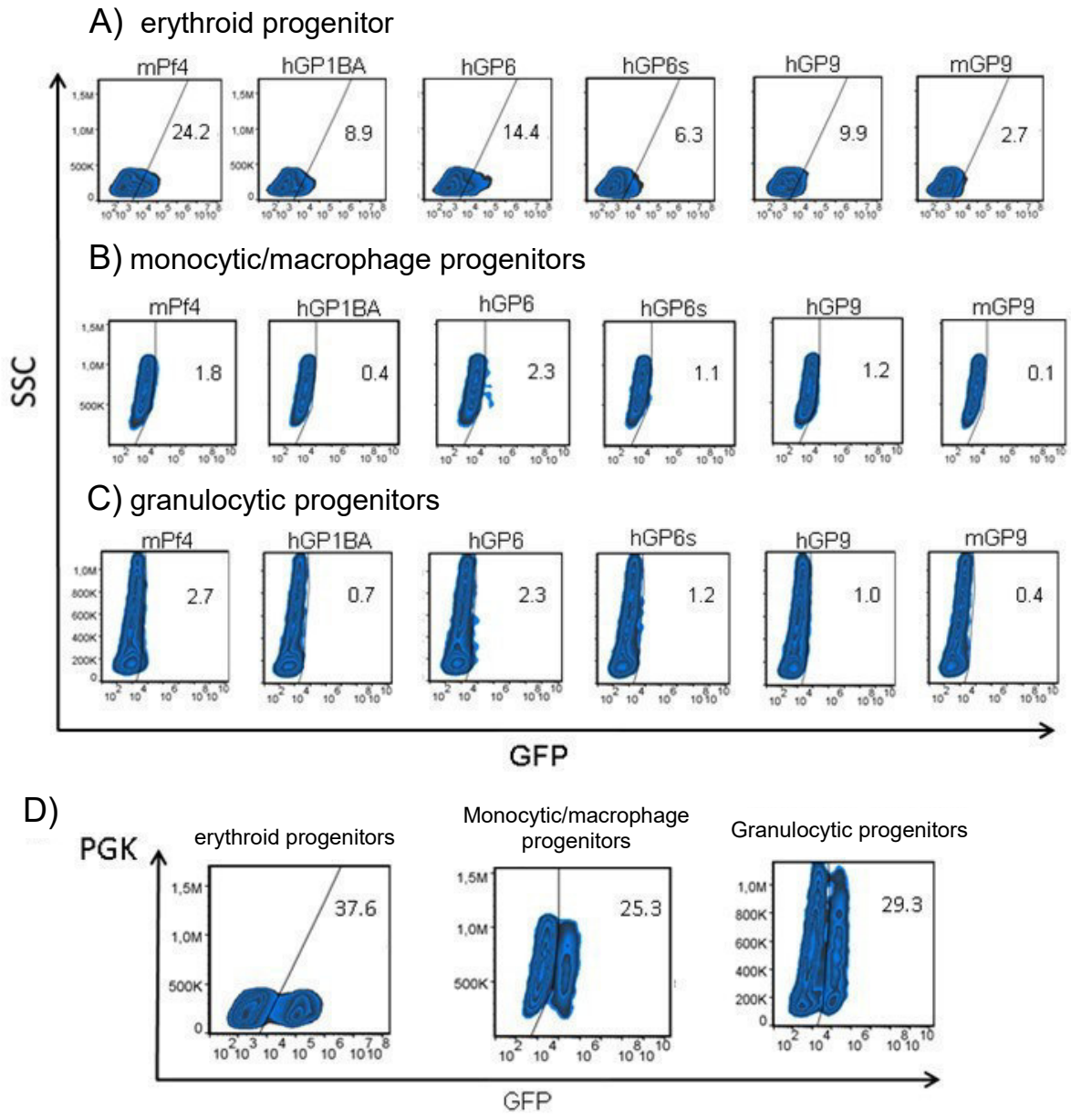


Figure 3.4.2 GFP expression from MK-specific lentiviral vectors in hematopoietic cells differentiated in semisolid medium. $1.5x10^5$ lineage marker-negative were transduced and plated into semisolid medium for differentiation. After one week, cells were flushed from the colony assays and analyzed by flow cytometry. Percentages (%) of GFP in (A) erythroid progenitors (B) monocytic/macrophages progenitors, (C) granulocyte progenitors. (D) Expression from the PGK promoter in the respective lineages.

To further study the promoter activity, we evaluated the strength of expression of our vectors in the context of non-hematopoietic cells. For that, the human fibrosarcoma (HT1080), the human hepatocarcinoma (HepG2) and the human neuroblastoma (SH-SY5Y) cell lines were transduced with the lineage-specific vectors (MOI:1) and analyzed for the mean fluorescence intensity, settled as relative MFI, by using as reference, the values obtained with the transduced megakaryoblastic cell line MegO1. In this experiment, the MFI for the mPf4, hGP1BA and hGP6 (long and short) was clearly lower than with the hGP9 and PGK vectors. Same results were observed with transductions performed in primary mouse fibroblast and a mixed cortical culture, see in (Latorre-Rey et al., 2017). These results, confirm the significant strong preference of expression in the megakaryocyte lineage and a week strength of promoter activity in off-target cells. The inefficient and non-specific expression from the human and murine GP9 promoter was unexpected, nevertheless, experiments with the human GP9 promoter were continued for further analysis.

3.5. MK-specific lentiviral vectors confer high GFP expression in platelets

To analyze expression from the MK-specific lentiviral vectors in platelets, we next performed BM transplantations with transduced lin^- BM cells into wildtype C57Bl/*6* mice (cumulative MOI 10, 4-6 recipients each). As the murine and human GP9 vectors conferred the weakest expression levels between the MK-vectors with the lowest specificity, we selected only the hGP9 vector to be tested *in-vivo*. GFP expression was analyzed in the peripheral blood cells 20 weeks post-transplantation. Promoter strength, measured as the MFI of GFP positive cells, was correlated to the expression from the hPGK-vector, which was set to 1, referred to as relative expression. The highest expression in platelets was observed from the mPf4-vector, both in respect of percentage (Figure 3.5.2 and 3.5.3 A) and intensity (5.2+/-3.4-fold higher than the hPGK-vector, p=0.009, Figure 3.5.3 B). In platelets, the mPf4-vector was even stronger than the SFFV LTR-vector (Figure 3.5.3 B). Furthermore, expression from the hGP1BA, the hGP6 and hGP6s vectors was reasonably

strong in platelets with the same relative expression as from the hPGK control vector (Figure 3.5.3 B). Expression levels from the hGP9-vector, however, was low and non-specific (Figure 3.5.3 A, B) even though the median VCN in the transplanted mice was the highest between the MK-specific vector groups (Figure 3.5.3 C). As previously observed, the expression of this vector was neither strong nor restricted to platelets (Figure 3.5.2 and 3.5.3 A, B).

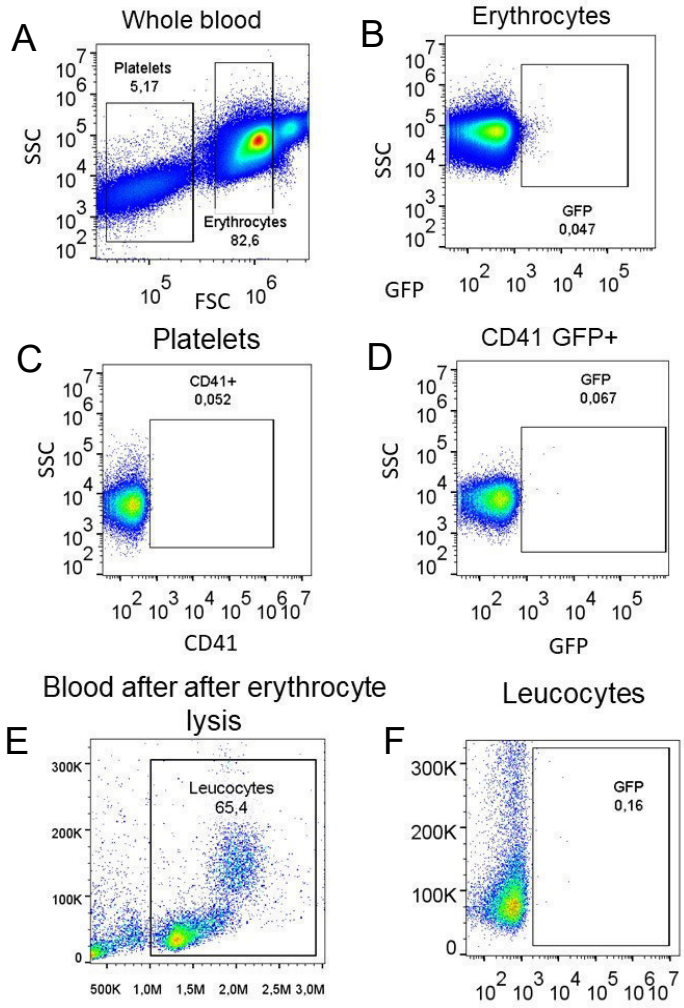


Figure 3.5.1. Gating strategy for the analysis of GFP expression in leucocytes, erythrocytes and platelets from Blood cells of transplanted mice. Whole blood cells (1/1000dil) was analyzed by flow cytometry and gated for erythrocytes and platelets population (A). Erythrocytes were gate for GFP (B). Within platelets population the gaiting of CD41 positive cells was based on unstained sample (C). CD41 positive cells from stained samples were selected and gated for GFP expression (D). Leucocytes were selected by erythrocyte lysis (E) and further gated for GFP expression (F).

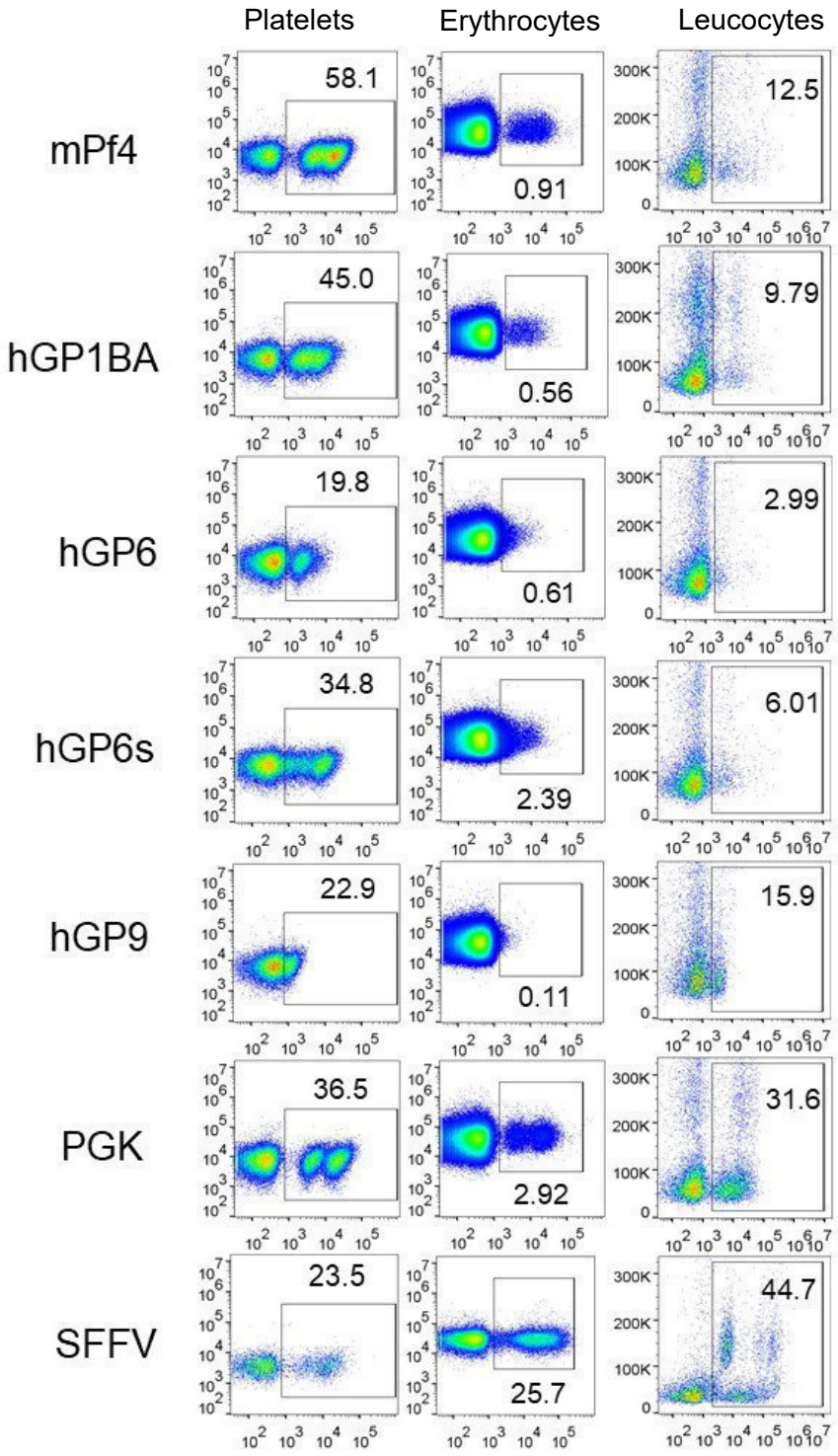


Figure 3.5.2 Expression from MK-specific lentiviral vectors in blood cells of transplanted mice. Representative sample of GFP expression in platelets, erythrocytes and leukocytes of one mouse of each group 20 weeks after transplantation. Platelets were defined as the CD41+ cells in the blood. Leukocytes were obtained after erythrocyte lysis.

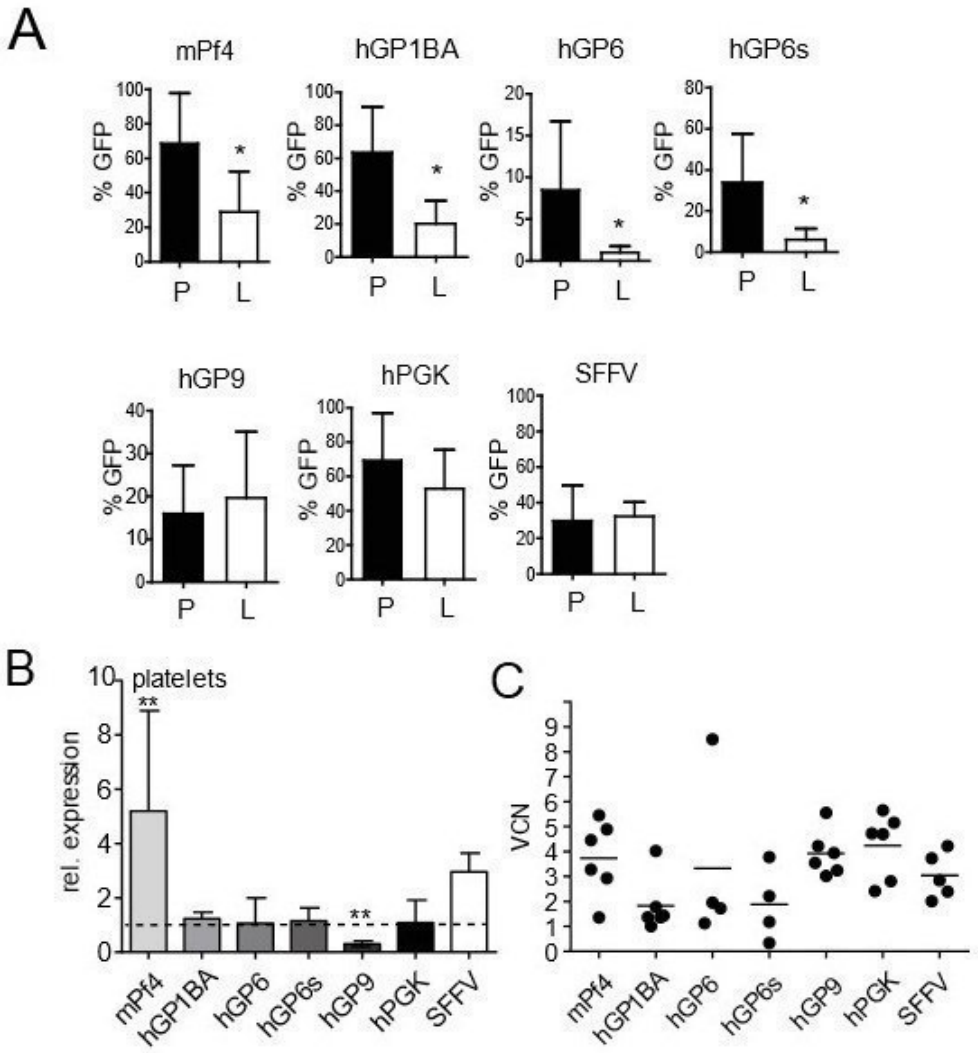


Figure 3.5.3 (A) Percentage of GFP positive cells in the platelets (P) and the leukocytes (L) from the blood of transplanted mice. Statistical analysis compared percentages of expression between platelets versus leukocytes in the respective groups (mPf4 p=0.06; hGPIBA p=0.04; hGP6 p= 0.05; hGP6s p=0.016). Note the different scales of the y-axes. (B) Relative GFP expression (MFI) in the platelets. The relative intensity of expression in platelets was calculated by correlating the mean MFI of each group by the mean MFI of the hPGK control group, which was set to 1. (C) Vector copy number of transplanted mice, each dot represents one mouse. The vector copy number was detected by quantitative PCR in DNA isolated from peripheral blood. The horizontal line indicates the median. (All data are shown as mean +/-SD of n=4-6 mice, for statistical analysis Mann-Whitney test was performed, compared to hPGK if not stated otherwise, *p<0.5, **p<0.01.).

3.6. MK-specific lentiviral vectors show lower activity in hematopoietic non-target cells

When we analyzed in detail the leukocytes, a smaller background promoter activity was observed in leukocytes from the MK-specific vectors compared to the SFFV promoter, especially from the mPf4-vector (29+/-22% GFP-positive leukocytes, Figure 3.5.3 A). Among the MK-specific vectors, the hGP6-vector was most restricted to platelets, with 8+/-4.2-fold more GFP-positive platelets than leukocytes (Figure 3.5.3 A, figure 3.6.1 B) and this specificity was maintained also with the shorter promoter-fragment hGP6s. Furthermore, no advantage or difference in the ratio of percentage from the expression in platelets/leukocytes was evidenced with the hGP9, hPGK and SFFV promoters, as observed in our previous experiments. Confirming with this the non-specificity of the hGP9 vector used in this study.

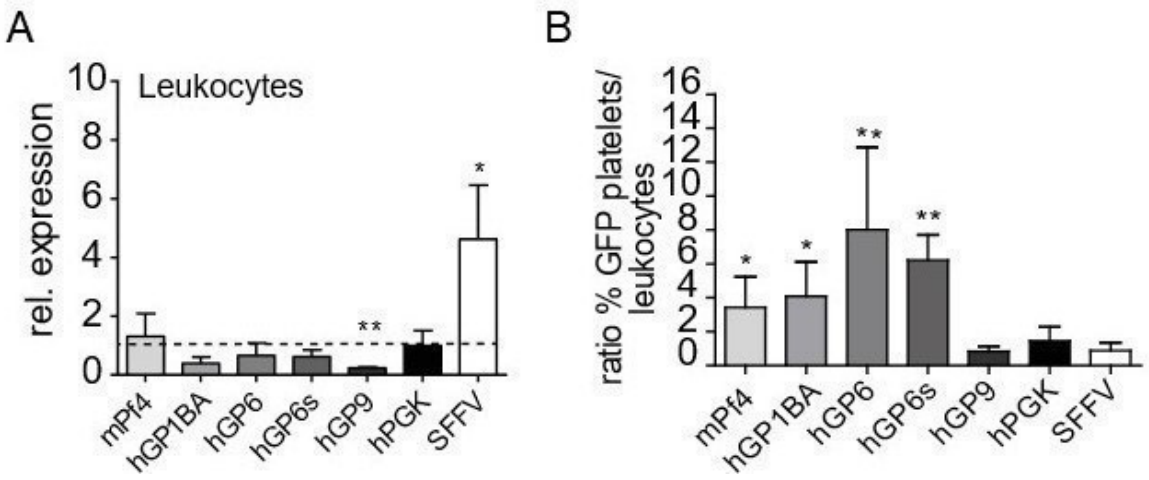


Figure 3.6.1. Expression from MK-specific lentiviral vectors in blood cells of transplanted mice. Relative expression of the Lineage-specific vectors in the leukocytes (A). Ratio of % GFP-positive cells in platelets/leukocytes (B). Relative GFP expression (MFI) in the leukocytes. The relative intensity of expression in the leukocytes was calculated by correlating the mean MFI of each group by the mean MFI of the hPGK control group, which was set to 1.

On the other hand, within the leukocyte-fraction, most of the background activity of the MK-specific vectors resided in granulocytes, followed by B-cells and T-cells (Fig.3.6.2 A). More importantly, the intensity of expression from the lineage specific vectors remained significantly lower compared to the SFFV vector (Fig.3.6.2 B).

Expression in erythrocytes was almost undetectable from the MK-specific vectors, but also from the hPGK-vector, possibly due to the long-life span of erythrocytes, which may have lost the GFP protein (Figure 3.5.1 A). Expression in erythroid progenitors however, isolated from the spleen demonstrated expression from the MK-specific vectors at the erythroblast stage which significantly declined with maturation. Among the vectors, expression in the erythroid lineage was the most pronounced from the hGP6 vector (Fig.3.6.4).

To evaluate the activity of the promoters in other hematopoietic tissues besides the bone marrow, we analyzed the GFP expression in megakaryocytes, B cells and T cells from spleens of transplanted mice. As previously observed, a smaller background activity was observed in splenic B and T cell from the lineage-specific vectors, when these values were compared with the activity of the hPGK ubiquitous vector. In the megakaryocyte population, all the vectors expressed to similar percentage, detecting no significant differences neither in percentage nor in intensity of expression. The strength of expression was, however, significantly higher in the megakaryocytes than in the other cell populations from the lineage-specific vectors. As expected, no difference was observed with the PGK control vector, figure 3.6.3.

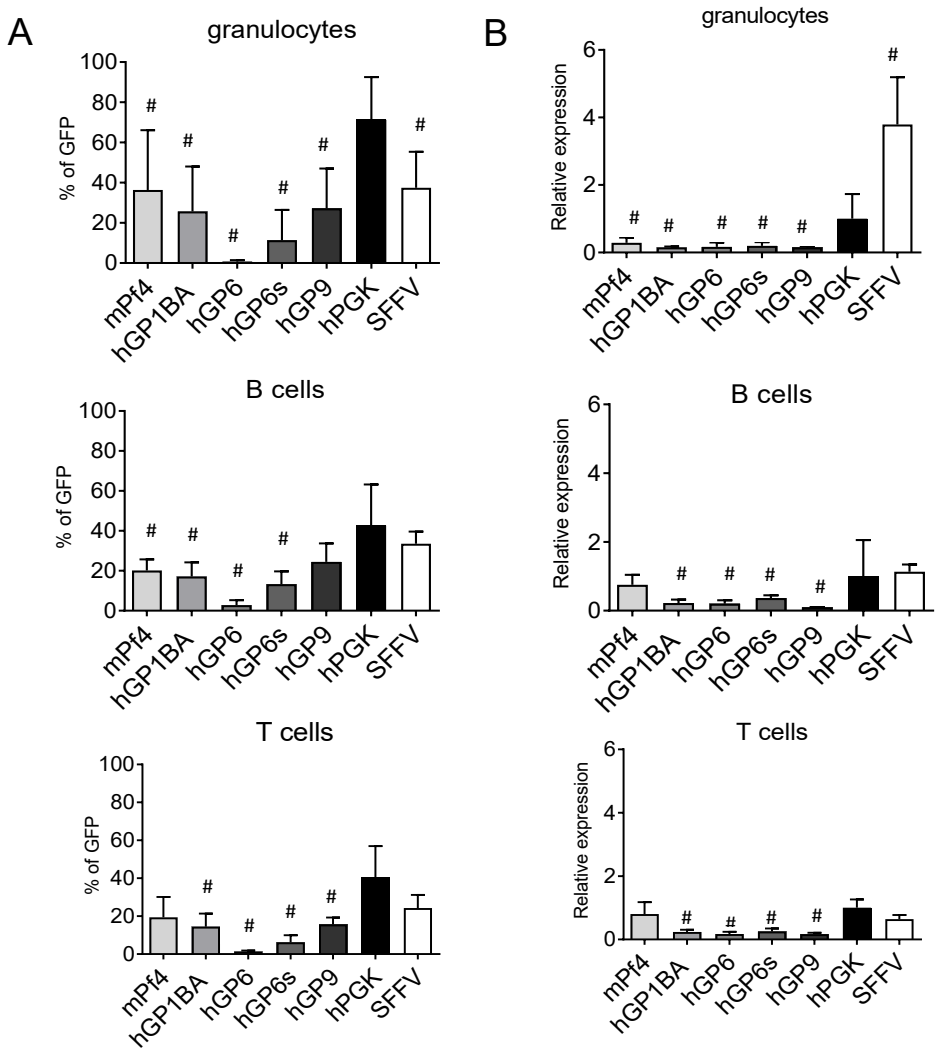


Figure 3.6.2. Column (A) Percentages (%) of GFP positive cells in the leukocytes (granulocytes, B and T cells). Leukocytes were analysed in the whole blood of transplanted mice after erythrocyte lysis. Leukocyte lineages were stained with specific antibodies marking granulocytes (CD11b/ Gr1), B cells (B220 or CD19) and T cells (CD3e). Columns show the mean, the whiskers (error bars) represents the standard deviation**.** For statistical analysis Mann Whitney test was employed (shown as #). In parallel, for every graph, Kruskal Wallis test (statistical significance in the granulocytes p=0.0009, B-cells p=0.0003, and T-cells p=0.0001) as well as multiple comparison test with Dunn´s correction was utilized; using PGK as a mean control: Granulocytes (hGP6***, hGP9**), B-cells (hGP6***, hGP6s*),T cells (hGP6***, hGP6s**). **Column (B) Relative expression of GFP in the leukocyte subpopulations (granulocytes, B- and T-cells)**. The mean fluorescence intensity of GFP was normalized to the MFI of GFP from the PGK promoter (relative expression). Analysis was performed at 20 weeks post transplantation. Mann Whitney test (#) it is shown in the Graphs. Kruskal-Wallis test in the granulocytes (p=0.0003), B-cells (p=0.0001) and T-cells (p=0.0001). Multiple comparison test with Dunn´s correction in the granulocytes (hGP6*), B-cells (hGP9**), T-cells (hGP1BA*, hGP6**, hGP9**). All comparisons are referred to PGK control promoter.

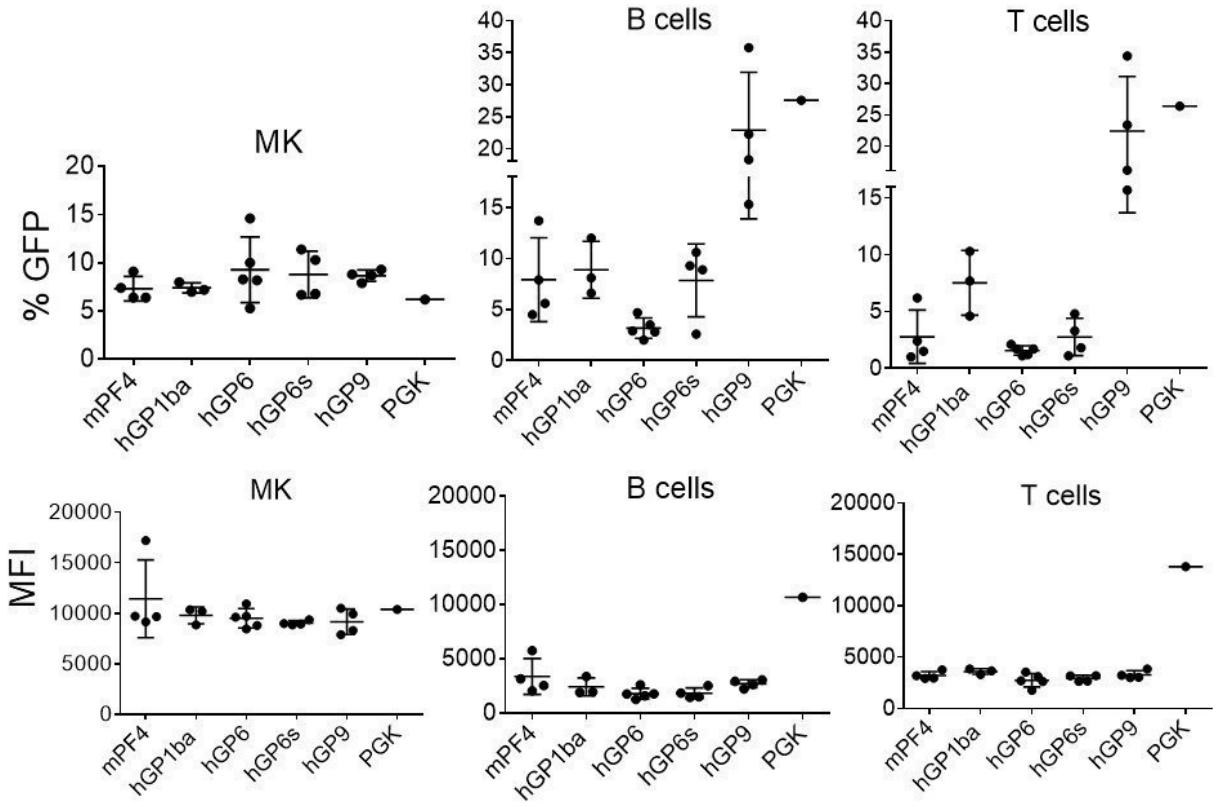


Figure 3.6.3. GFP expression from MK-specific lentiviral vectors in hematopoietic cells isolated from the spleen. Spleen cells were isolated, erythrocytes were lysed and remaining cells were stained with specific antibodies for megakaryocytes (CD41/CD42b), B cells (B220) and T cells (CD3e). **(A)** Percentages (%) of GFP expressing cells **(B)** Mean fluorescence intensity of GFP expression. In the graphs, whiskers (error bars) represents the standard deviation**,** and the dots indicate the values of each mouse analysed.

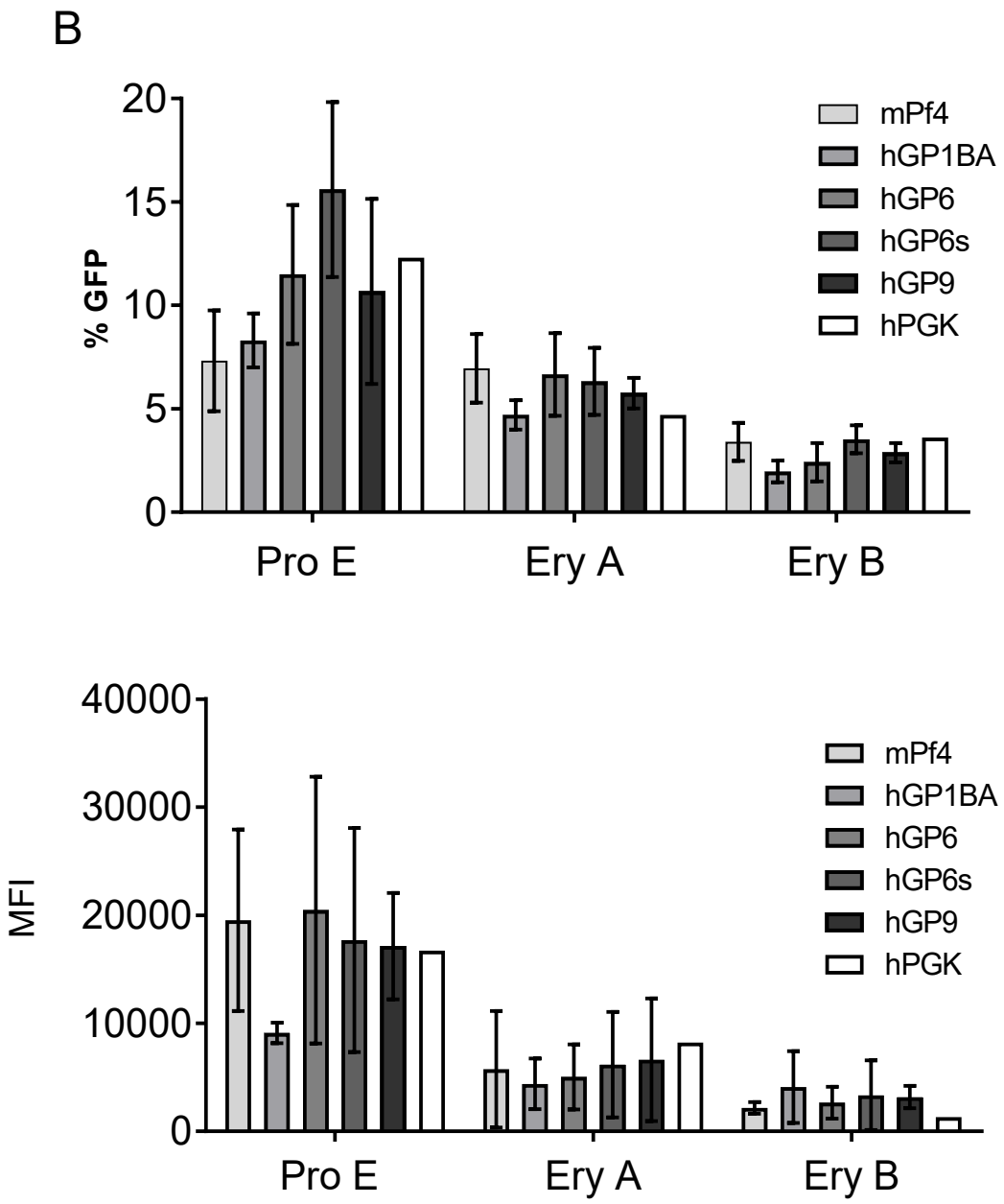


Figure 3.6.4. GFP expression in percentage (%) from MK-specific lentiviral vectors in erythroid progenitors. Erythroid progenitors were identified using CD71 and Ter119 specific antibodies (proerythroblast: Pro E (Ter119 low, CD71 high), erythroblast maturation increases from Ery A (Ter119 high, CD71 high) to Ery B (Ter119 high, CD71 med) following the classification of progenitors classification from (Socolovsky et al., 2001; Koulnis et al., 2011), (A). Mean fluorescence intensity of GFP in erythroid progenitors (B).

3.7. Expression from MK-specific lentiviral vectors in HSPC

mPf4 promoter activity in HSC was previously reported in the transgenic Pf4-Cre mouse (Calaminus et al., 2012), however, this mouse contains a much longer 5' region of the rat *Pf4* gene. In our own previous studies, we demonstrated the correction of HSC defects in Mpl-/- mice by the use of the *hGP1BA* promoter in the context of lentiviral Mpl expression, highlighting *hGP1BA* promoter activity also in HSC (Heckl et al., 2011). To investigate whether the new *hGP6*, *hGP6s*, *hGP9* or the *mPf4* promoter fragments used in our lentiviral vectors were more specific with lower activity in HSPC, we analyzed the percentage of GFP-positive cells in the HSC-enriched cells (lin^-, Sca+, ckit+ (LSK); LSK/CD34-) and progenitors (CMP, GMP, MEP) by flow cytometry (Figure 3.7.1). As shown in the figure 3.7.2 and 3.7.3, the hGP6-vector showed the lowest activity in the HSPC with <10% GFP-positive cells and ~20% activity in the LSK-cells compared to the intensity of the hPGK-control vector (Figure 3.7.2 B, 3.7.3 B and Fig.3.7.4). The hGP6s-vector was only slightly more active (<15% positive LSK cells). In contrast, the mPf4 and hGP1BA vectors were both highly active in the HSPC, (10-70% GFP-positive cells in the respective HSPC, Figure 3.7.2 B, 3.7.3 B and Fig.3.7.4. When comparing the promoter strength (MFI) of the MK-specific promoters to the hPGK, the relative expression was in all cases lower, while the relative expression from the SFFV promoter was 4.5+/-2.3 –fold higher compared to the *hPGK* promoters (Fig.3.7.3 B and 9).

Table 3.1 Kruskal-Wallis test (A) with Dunn's multiple comparison (B) for the statistical analysis of the percentage of GFP expressing cells among the hematopoietic stem and progenitor cells for the comparison of promoter activity. Analysis was performed in comparison to hGP6.

A

Kruskal Wallis test	P value
GFP in LSK	0.0198
GFP in CMPs	0.0007
GFP in GMPs	0.0004
GFP in MEPs	0.0073

B

Promotors	LSK	CMP	GMP	MEP
	P value	P value	P value	P value
mPf4	0.0261	0.0453	0.0147	0.4302
hGP1BA	0.0242	0.003	0.0147	0.0991
hGP6s	>0.9999	>0.9999	>0.9999	>0.9999
hGP9	0.1126	0.1406	0.0453	0.128
hPGK	0.0094	0.0014	0.0013	0.0028
SFFV	0.4885	>0.9999	>0.9999	>0.9999

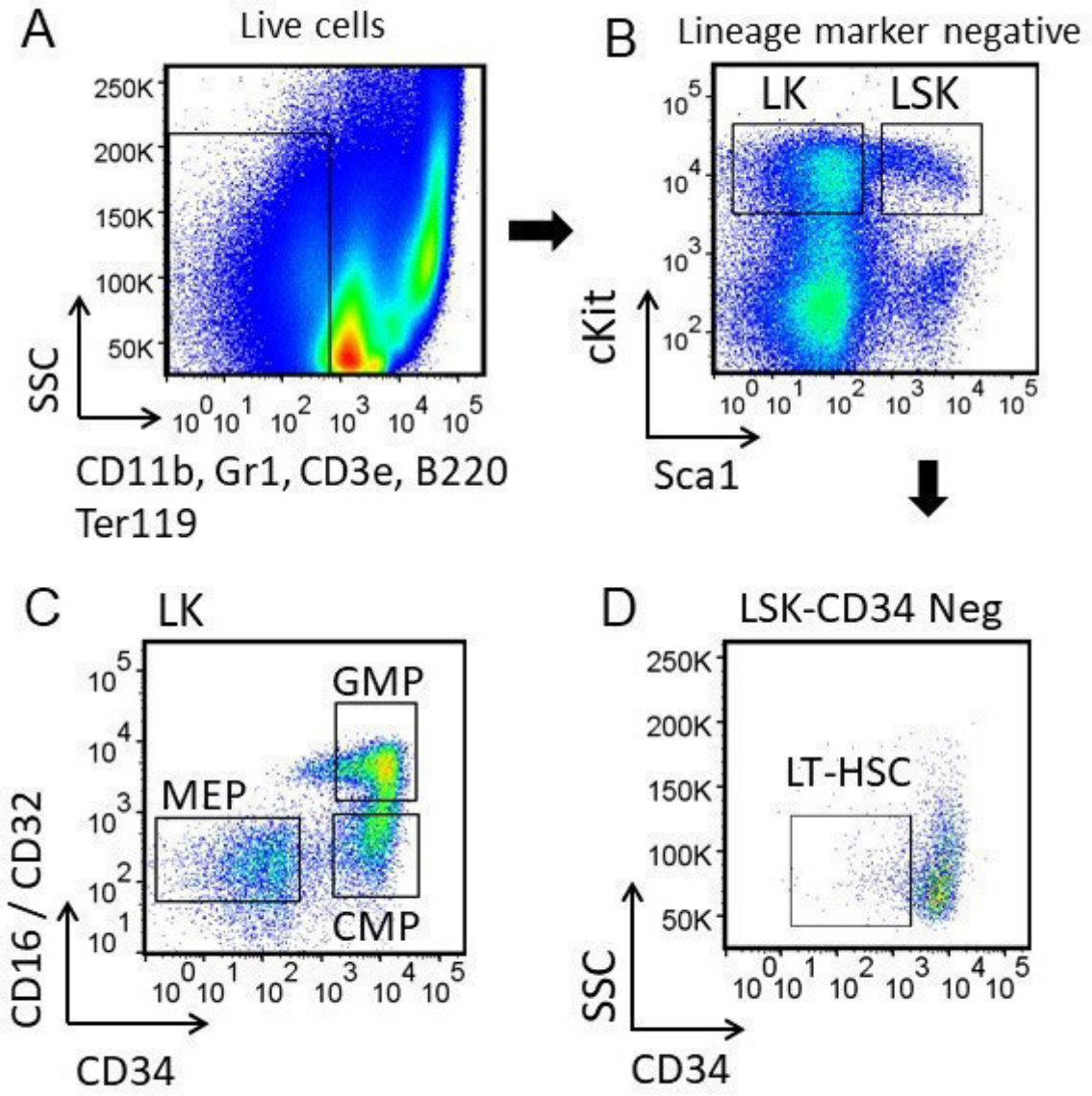


Figure 3.7.1 Cell surface marker staining for the identification of the hematopoietic stem cells and progenitors (HSPC) in the bone marrow (LT-LSK, LSK, LK, GMP, CMP, MEP, lineage marker-negative cells). (B) LK and LSK cells were identified as the cKit+/Sca1- and ckit+/Sca1+ populations in the lineage marker-negative (lin-) cells from the whole bone marrow (A), respectively. (C) Common myeloid progenitors CMP, granulocyte - macrophage progenitor GMP, megakaryocyte- erythroid progenitor MEP, were identified by their differential staining with the CD16/CD32 and CD34 marker as indicated. (D) The long-term HSC (LT-HSC) was characterized as the CD34-negative cells in the LSK cell population.

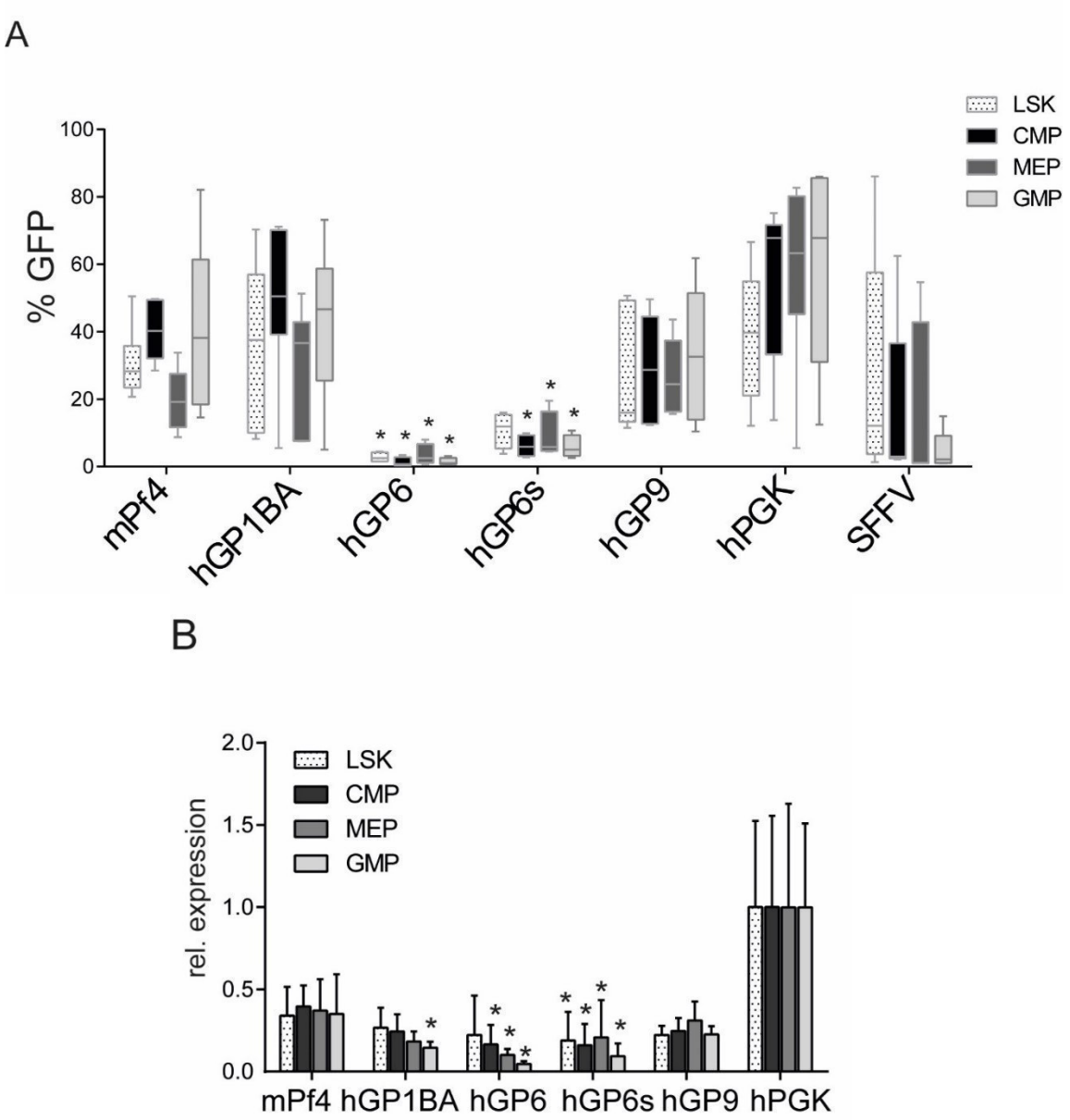


Figure 3.7.2. Expression from MK-specific lentiviral vectors in the hematopoietic stem and progenitor cells of transplanted mice. BM cells were isolated 23 weeks after transplantation. (A) Percentages of GFP positive cells and (B) the relative expression in the GFP positive cells in the respective populations. The MFI of the hPGK promoter was set to 1 and the relative expression calculated. Populations were defined as follows: LSK: lin-, Sca1+, ckit+; CMP: lin-, Sca1-, ckit+, CD34+, CD16/32-; GMP: lin-, Sca1-, ckit+, CD34+, CD16/32+; MEP: lin-, Sca1-, ckit+, CD34-, CD16/32-. Shown is the mean +/- SD. Statistical analysis was performed by Kruskal Wallis test, *statistical significance by Dunn´s multiples comparison test, MK-vectors compared to hPGK control vector. The hGP6 and hGP6s vectors were also significantly less active in HSPC compared to the other MK-specific vectors, p-values supplied in the table 3.1

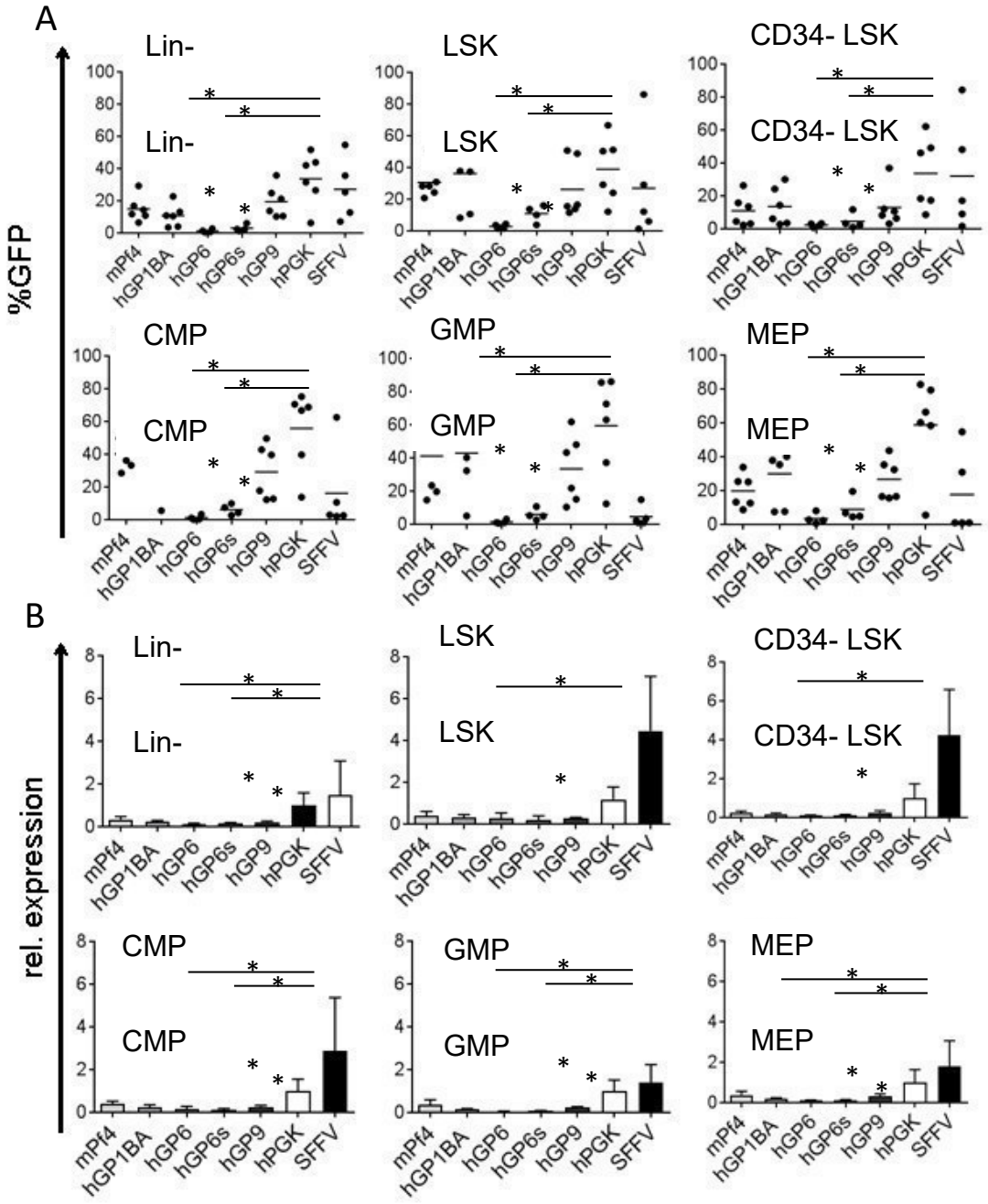


Figure 3.7.3. GFP expression from the MK-specific lentiviral vectors in the different HSPC populations in the BM. Every dot represents one mouse. GFP measurements were performed in the lineage marker-negative (lin-) cells, LSK cells, long-term LSK cells (CD34-LSK), common myeloid progenitors (CMP), granulocyte-macrophage progenitor (GMP) and megakaryocyte-erythroid progenitor (MEP)**. (A) percentage of GFP positive cells in the respective cell population (B) Relative expression of GFP in the HSPC.** MFI of GFP of PGK was set to one. Groups are constituted by 4-6 mice each. Bar represents the mean +/-SD. Statistical analysis was performed with the nonparametric Kruskal Wallis-One-way ANOVA and Dunn´s multiple comparison test.

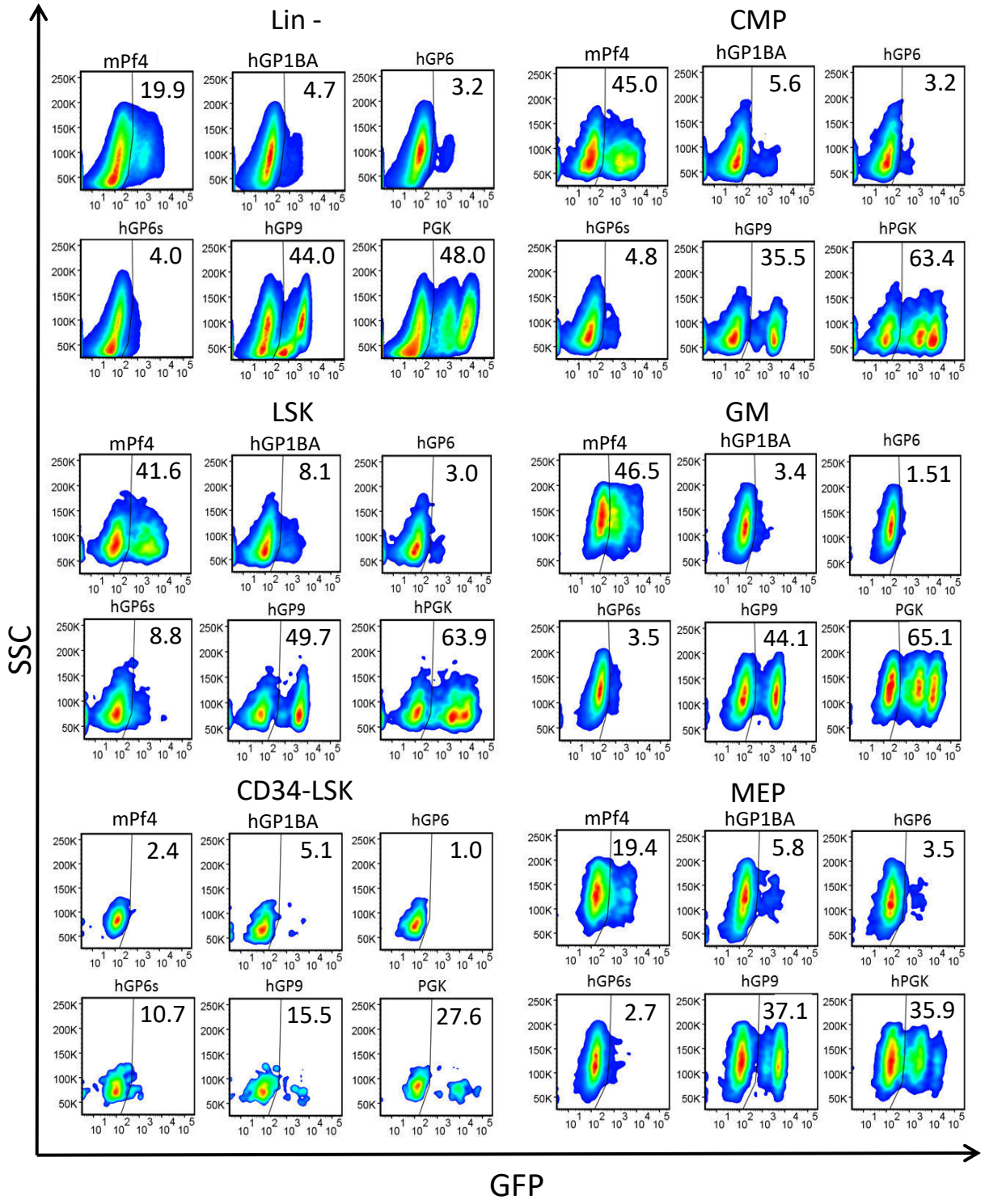


Figure 3.7.4. Examples for GFP expression in HSPC from the MK-specific lentiviral vectors. Gating strategy was performed as shown in Suppl. Fig 8. The percentage of GFP expressing cells was measured by flow cytometry. This data represents an example of one transplanted mouse per group.

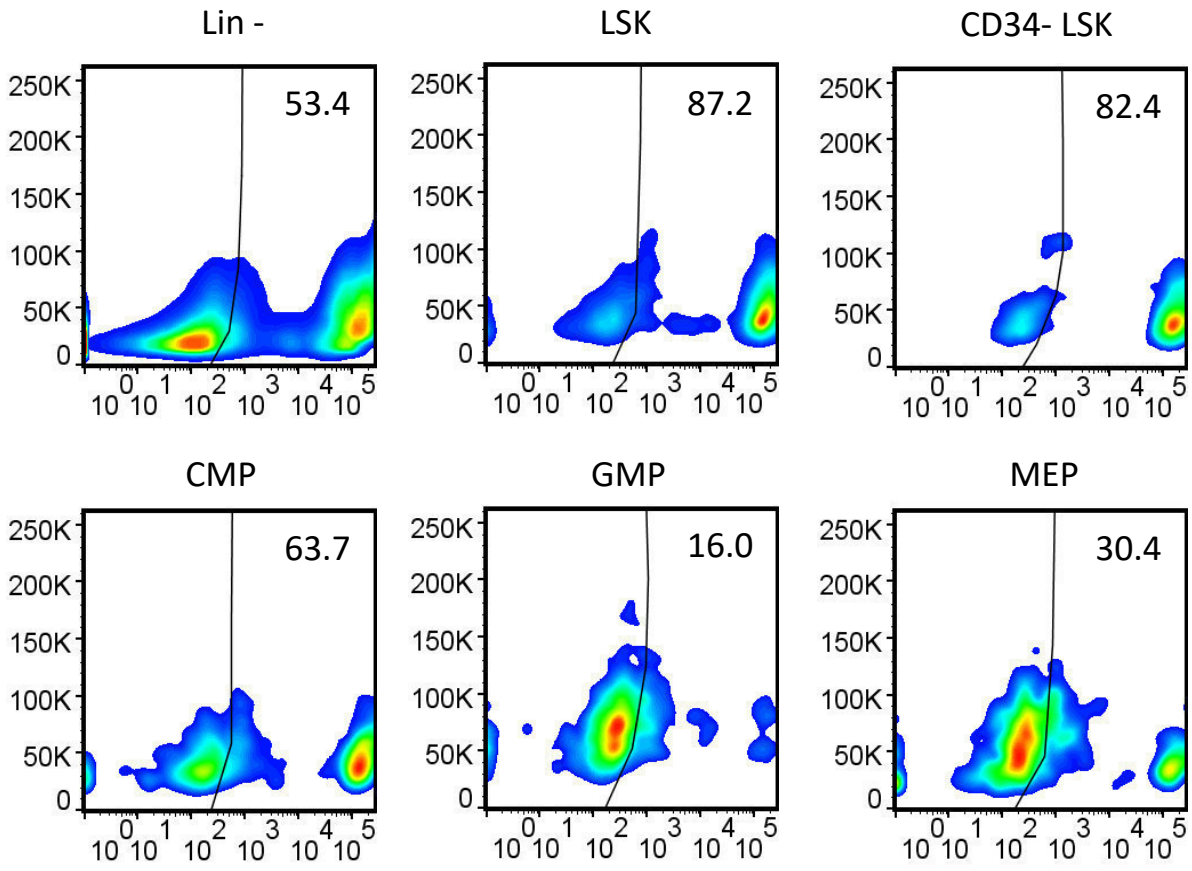


Figure 3.7.5. Example of the SFFV promoter activity in the HSPC. Percentage of GFP expressing cells is shown in the lineage marker-negative (lin-) cells, LSK, CD34- LSK, common myeloid progenitor, granulocyte- macrophage progenitor and megakaryocyte-erythroid progenitor.

To test whether the expression from the promoter fragments recapitulate the normal expression of the respective genes in the HSPC, the RNA levels of murine Gp1ba, Gp6, Gp9 and Pf4 in sorted LSK, CMP, GMP and MEP populations of wildtype C57Bl/6 mice were evaluated (Figure 3.7.6). As observed in figure 3.7.6, a substantial mRNA expression of murine Gp1ba and Gp9 in LSK, CMP and MEP cells could be confirmed. (Figure 3.7.6) which dropped with differentiation to the granulocytic/monocytic lineage (GMP). mRNA levels of Gp1ba and Gp9 were very similar, as would be expected by their function as subunits of the same receptor complex. mPf4 expression was 2.5 fold higher than Gp1ba expression in the LSK cells and increased substantially in the CMP (about 10-fold). In contrast, expression of murine Gp6 in HSPC was almost null which agrees with the low activity of the promoter fragment when introduced in the lentiviral vector in these cells in our experiments.

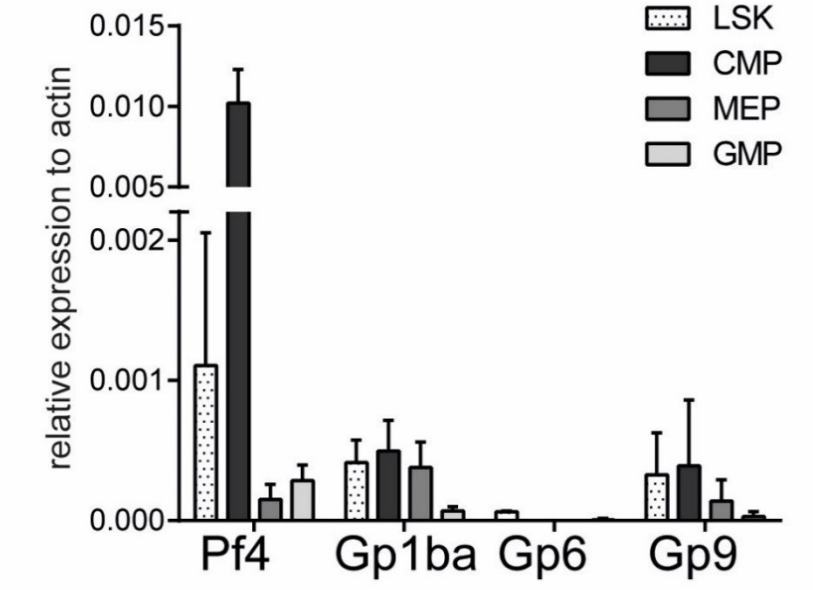


Figure 3.7.6. mRNA levels relative to actin of the murine genes Pf4, Gp6, Gp1ba and Gp9 in the indicated populations of the HSPC measured by RT-PCR. (n=3 independent cell sorts). Experiment performed by Saskia Kohlscheen at the Sorting facility provided by the Prof. Michael Rieger, at the department of Medicine, Hematology and Oncology at the Goethe University Hospital Frankfurt, in collaboration with Susanne Wingert.

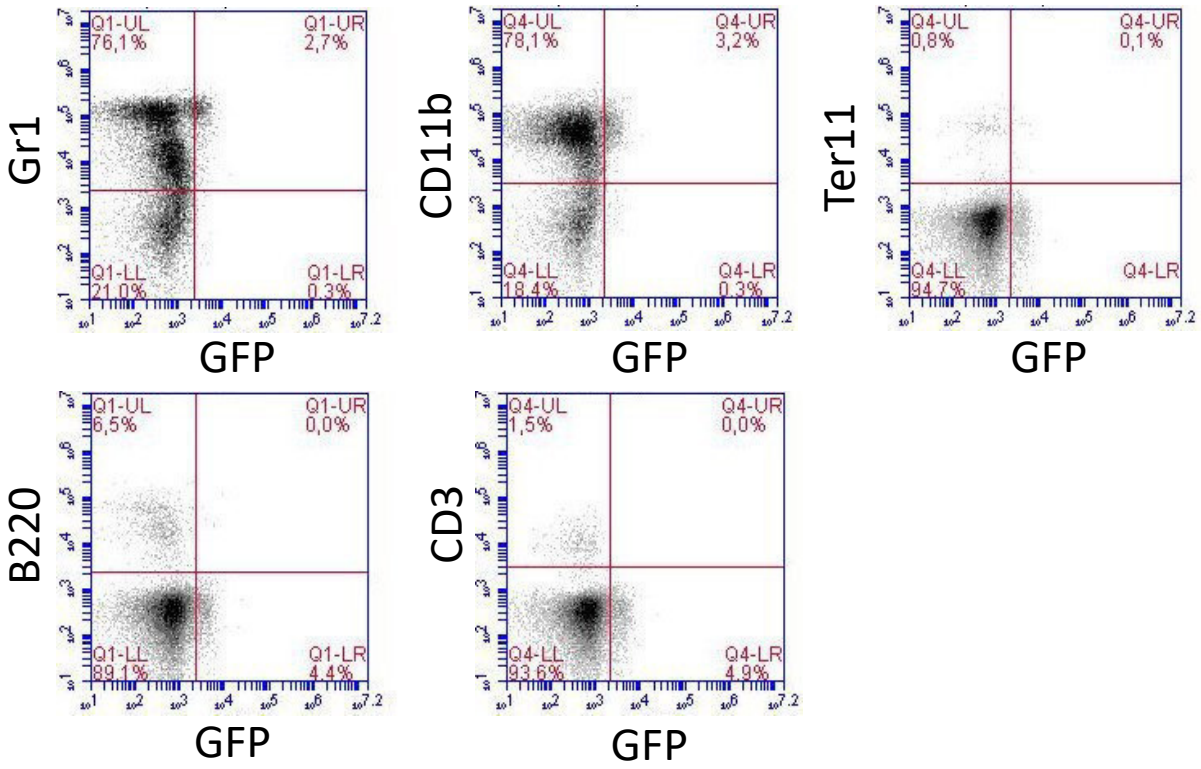


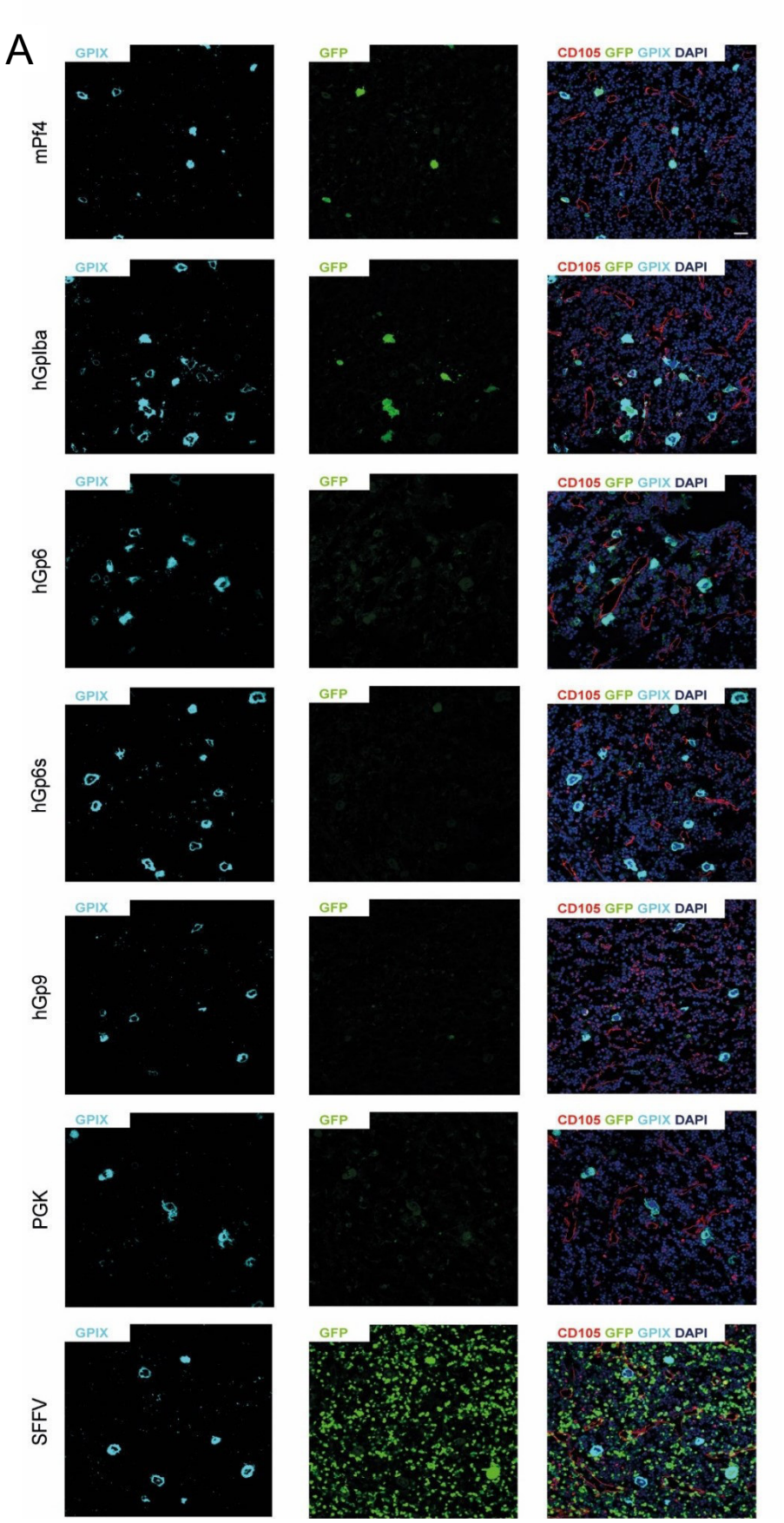
Figure 3.7.7. Example of a flow cytometric analysis of the total BM of a mouse transplanted with the hGP6s vector.

As shown in the previous figure 3.7.7, the GFP expression, driven by the hGP6 promoter detected by flow cytometer outside of megakaryocytes, resides mainly in the myeloid cells (CD11b and Gr1 positive cells) and to some extend in the erythroid progenitors of the BM detected by Ter119, note that mature erythrocytes were lysed before analysis), but not in lymphoid cells (B220 and CD3 positive).

3.8. Expression in MKs in the BM in situ

To analyze specificity and expression strength from the MK-specific lentiviral vectors in the intact BM environment, we determined GFP expression in native cryosections of whole femora of mice transplanted with transduced BM cells. To visualize the BM structure, we performed immunostainings for MK (GPIX) and blood vessels (endoglin, CD105). It has to be taken into account that not all cells in the transplant were gene marked, and therefore GFP can only be detected in a subset of megakaryocytes.

As shown in the figure 3.8.1 A, in megakaryocytes, the GFP was strongly expressed from the mPf4-vector (10 +/- 4.5-fold) and also from the hGP1BA-vector (7.4 +/- 2.6-fold; Figure 3.8.1 A, B), followed by the hGP6s-vector (4 +/- 2-fold) and the hGP6-vector (1.5 +/- 0.7-fold, all values compared to the hPGK promoter). The GFP expression from the hGP9 promoter was similar to hPGK vector (Figure 3.8.1 A, B). Importantly, with the hGP6 promoter, there was almost no fluorescent signal detected in non-MK cells when using the MK-specific vectors with minor background activity in myeloid cells, figure 3.7.7. In this sense, we have to consider that the HSPC subset in which vector activity was demonstrated in our flow cytometric analysis only contribute <1% of the BM cells and will not easily be visualized by microscopic analysis. Of note, expression from the viral SFFV promoter resulted in very strong GFP expression (15.4 +/- 0.3-fold), which was not restricted to MKs.


A
GPIX
GFP
CD105 GFP GPIX DAPI
mPf4
hGplba
hGp6
hGp6s
hGp9
PGK
SFFV

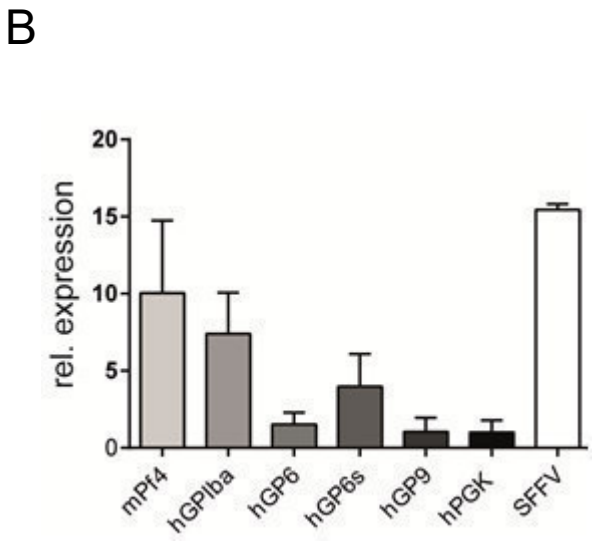


Figure 3.8.1. Expression from MK-specific lentiviral vectors in megakaryocytes in the BM in situ. (A) Confocal images of BM sections of mice transplanted with modified BM cells with lentiviral vectors as indicated. For detection of all MK, a staining for GPIX was performed, left pictures. Blood vessels were visualized by staining for CD105 (endoglin). Middle pictures: GFP expression; right pictures: overlay of the GPIX, GFP, endoglin and DAPI signals. (B) Quantification of relative GFP expression in MK cells. Kruskal-Wallis test with Dunn's multiple comparison test *$p<0.5$, **$p<0.01$. Immunostaining from bone marrow in situ and pictures taken by Sebastian Dütting, from the Department of Experimental Biomedicine-Vascular Medicine, University Hospital and Rudolf Virchow Center, University of Würzburg, Germany.

3.9. Mpl expression from MK-specific lentiviral vectors improves Mpl-/- megakaryopoiesis.

To address whether the new MK-specific lentiviral vectors allow the modification of megakaryopoiesis and platelet phenotype, we investigated the expression of the thrombopoietin receptor Mpl, by transducing Mpl-/- lineage marker-negative cells with lentiviral vectors carrying the Mpl cDNA, whose expression was driven from the lineage-specific promoters, figure 3.9.1 A. Lin-cells from Mpl-/- mice were differentiated into MK for in vitro analysis or performed BM transplantations. Mpl-deficient mice have a known defect in megakaryocyte maturation and are thrombocytopenic (Alexander et al., 1996). In *in vitro* analysis, after gene transfer, MK maturation and polyploidization were recovered by the re-expression of Mpl indicated by significantly more MKs with >8n compared to Mpl-/- MK, this effect is evidenced in cytospins (Fig. 3.9.1 B), as well in flow cytometer analysis, were more megakaryocyte with high polyploidy were observed at later stages of maturation (MKs >16n,32n and 64n), figure 3.9.2 and figure 3.9.3.

For the in vivo analysis, Mpl-/- mice transplanted with lin-BM cells transduced by the mPf4.Mpl and hGP6.Mpl vectors showed significantly elevated platelet counts compared to control mice transplanted with a GFP-encoding control vector (PGK.GFP, Fig.3.9.4. A, B). In the BM, megakaryocyte maturation was improved with a higher number of polylobulated MKs (Figure 3.9.5). One of the mPf4.Mpl mice had a transient thrombocytosis six week after transplantation (Figure 3.9.4 C). Two control mice transplanted with lin-BM cells transduced with SFFV.Mpl and PGK.Mpl vectors developed severe erythrocytosis, leukocytosis (3/6 mice) (Figure 3.9.4 C), splenomegaly (0.6–2.5g) and erythroid infiltrations in the liver (Figure 3.9.6) and had to be euthanized three to five weeks after transplantation, demonstrating the need to restrict transgene expression to MKs.

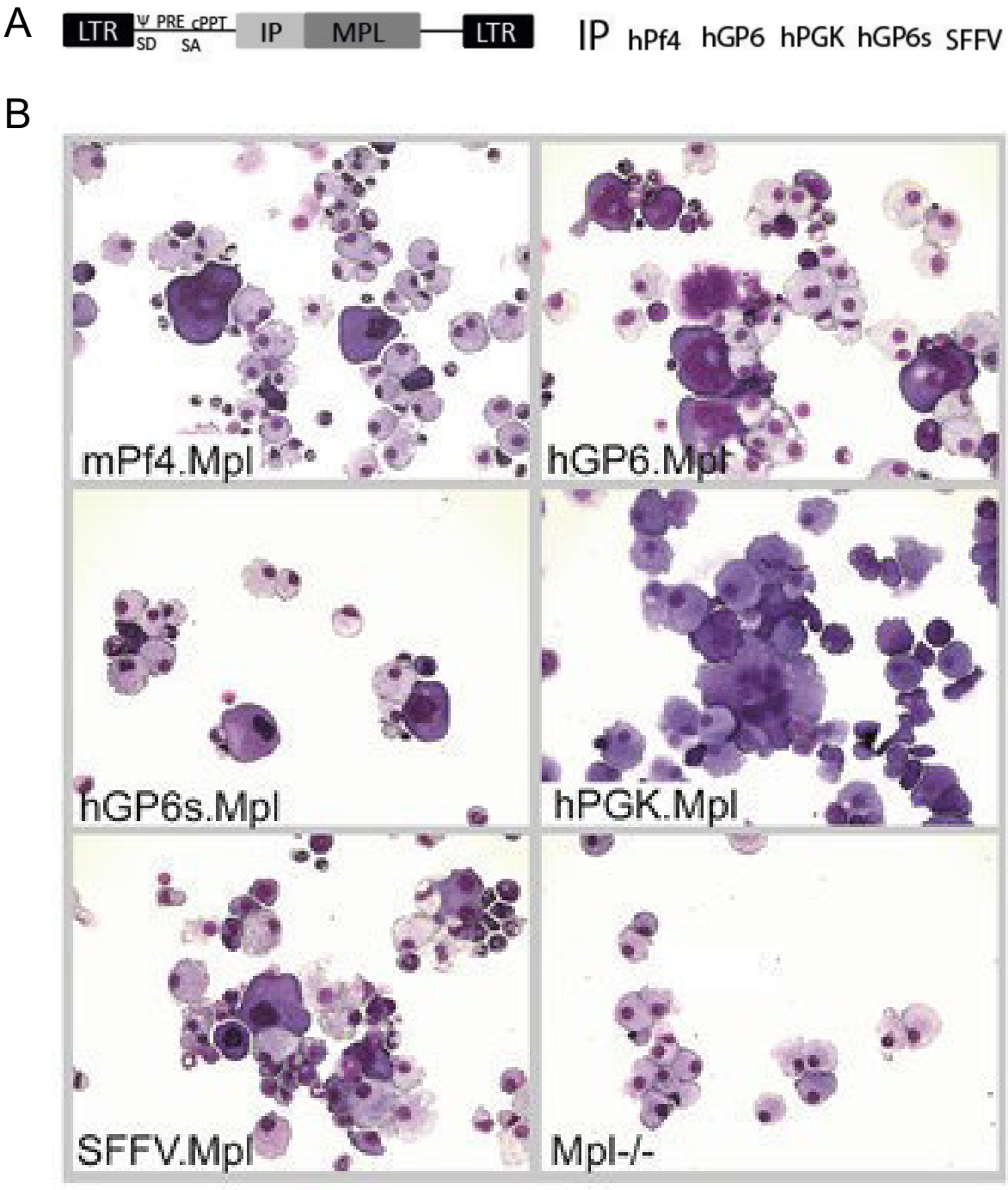


Figure 3.9.1. Mpl expression from MK-specific lentiviral vectors corrects Mpl-/- megakaryopoiesis. Lentiviral vector with the lineage specific promoter for the Mpl gene transfer (A). Cytology of in vitro differentiated Mpl-/- MK after gene transfer with the respective MK-specific vectors expressing Mpl, as indicated (B).

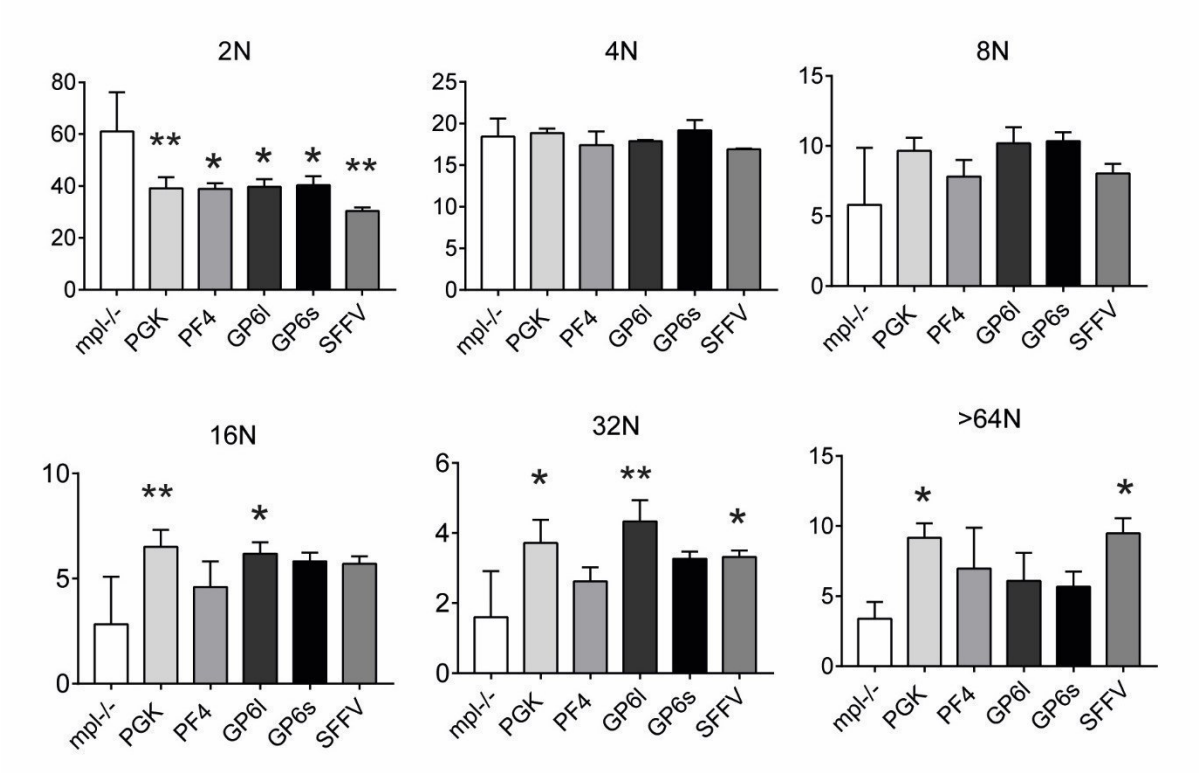


Figure 3.9.2. Polyploidization in in vitro MKs from Mpl deficient mice after Mpl gene transfer. Lineage marker-negative cells were isolated from Mpl-/- mice and transduced with lentiviral vectors expressing the Mpl receptor under the control of mPf4, hGP6 (long and short) hPGK and SFFV promoters. Cells were cultured for 2 weeks in StemSpan medium with 50ng/ml of THPO for driving differentiation into megakaryocytes. Cells were stained with propidium iodide and analyzed by flow cytometer for studies of polyploidization during megakaryopoiesis. Expression of Mpl by MK-specific lentiviral vectors improved MK maturation of Mpl-/- cells as they reached higher ploidy stages than the uncorrected Mpl-/- MK. Kruskal-Wallis test with Dunn's multiple comparison *$p<0.5$, **$p<0.01$; compared to the non-corrected Mpl-/- cells.

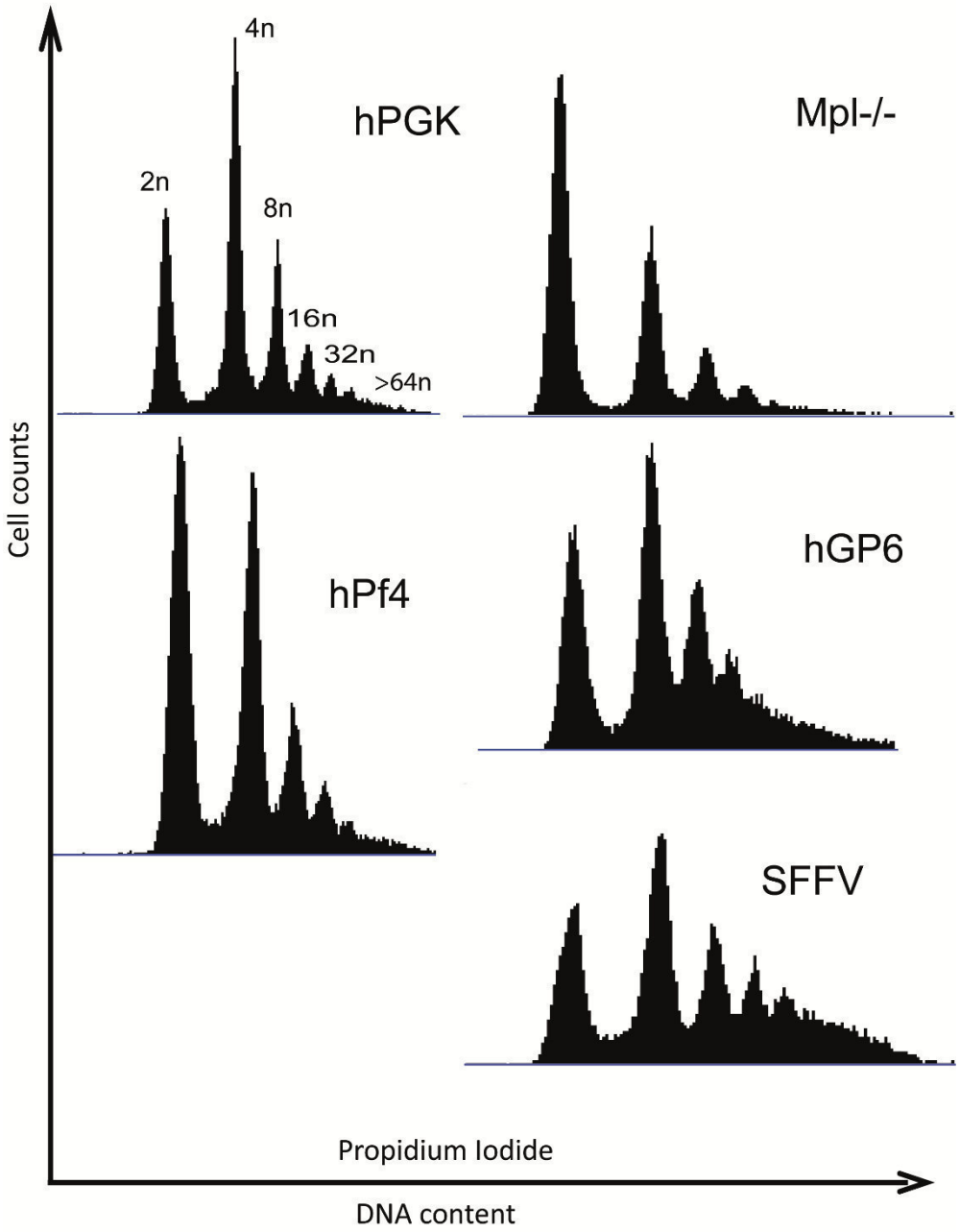


Figure 3.9.3. Histograms from transduced and differentiated Mpl-/- megakaryocytes after Mpl gene transfer. Mpl-/- cell were transduced with the lineage-specific vectors driving the expression of Mpl. Transduced cells were cultured for 2 weeks after transduction with 50ng/ml murine Thpo for megakaryocyte differentiation. After Mpl gene transfer higher stages of megakaryocyte maturation (>8N) were achieved.

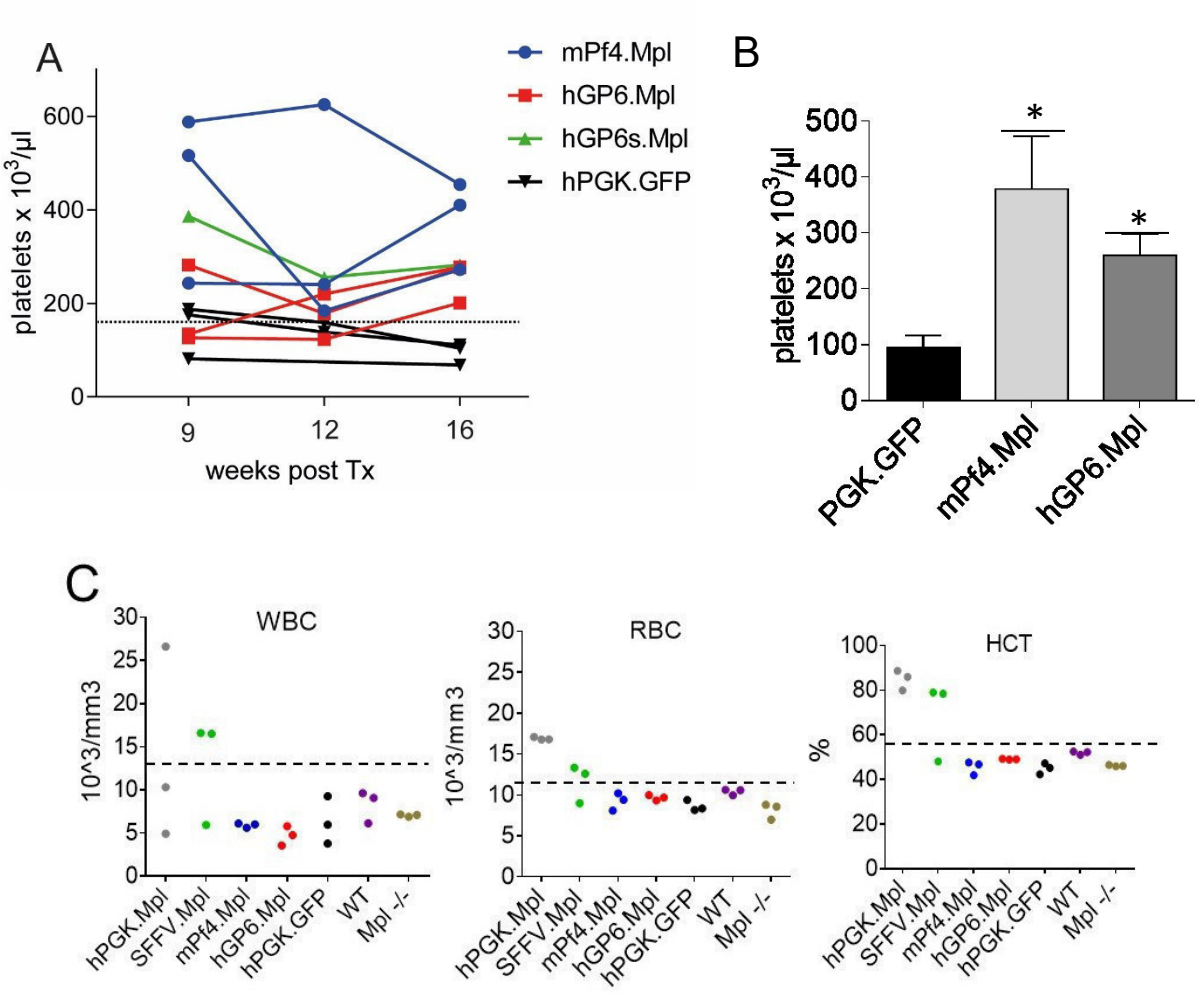


Figure 3.9.4. In vivo MPL gene transfer. (A) Platelet counts of transplanted mice over time after transplantation. Vector used are indicated. Only one mouse was transplanted with the hGP6s.Mpl vector. Gray dotted line indicates the average platelet levels in Mpl-/- (B) Summarized platelet counts at 16 weeks after transplantation. (mPf4 n=3; hGP6 n=4, including the hGP6s mouse). *p<0.05, Mann-Whitney test. (C) Blood counts of the mice transplanted with the PGK.Mpl and SFFV.Mpl lentiviral vectors at the day they were killed due to disease symptoms. As control, the blood counts of mPf4.Mpl and hGP6.Mpl mice and PGK.GFP control mice are displayed (taken 5-6 six weeks after transplantation. Gray dotted line indicates the maximum physiological level in Wild types.

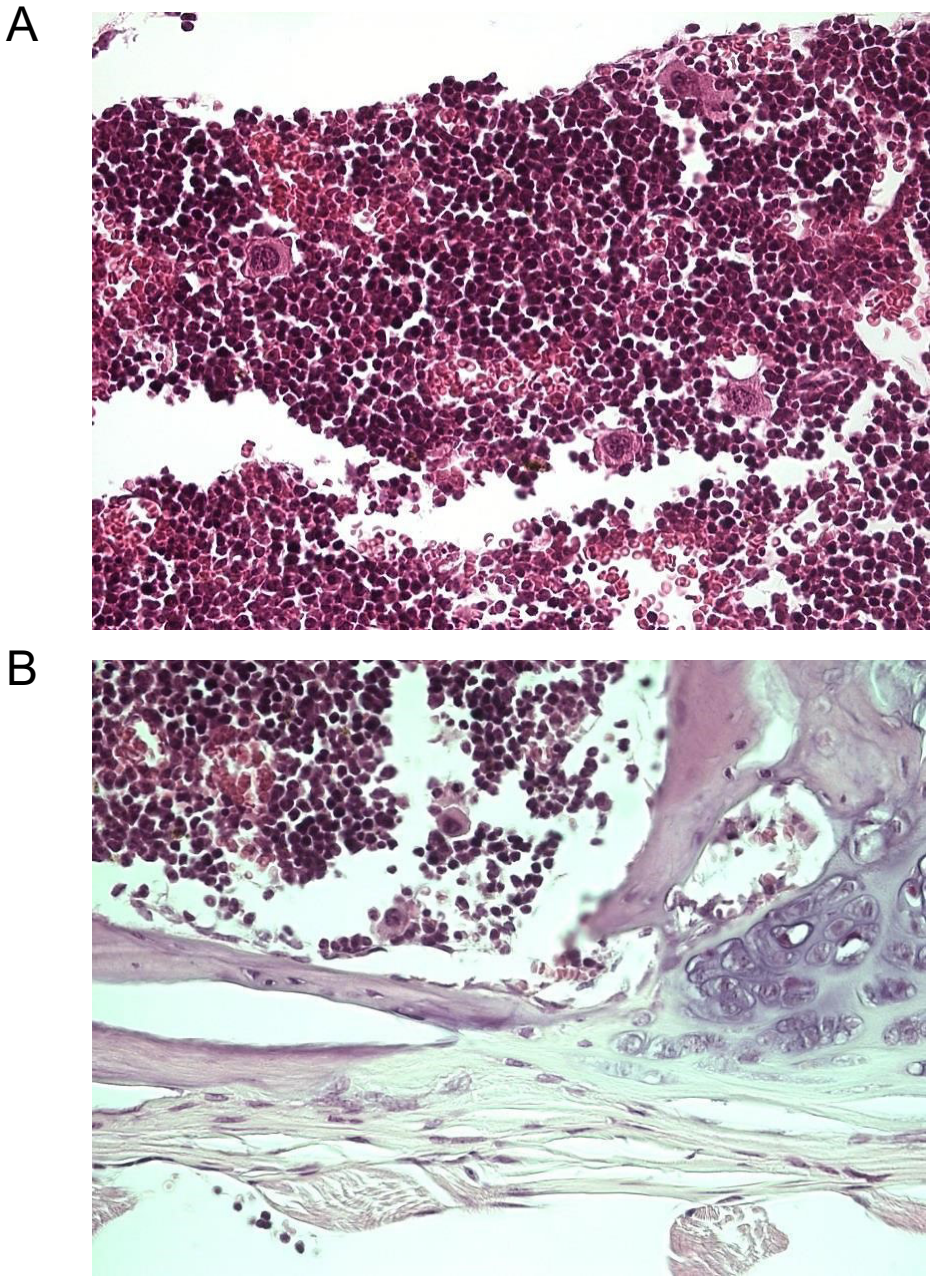


Figure 3.9.5. Histology of the bone marrow (A) Example of a correction in megakaryocyte maturation by Mpl gene transfer. Enlarged and matured megakaryocytes are pointed out by arrows. **(B)** Bone marrow section of an Mpl-/- mice. Note the few megakaryocytes, small in size with low polyploidy. (Hematoxylin/Eosin staining, 400-fold magnification).

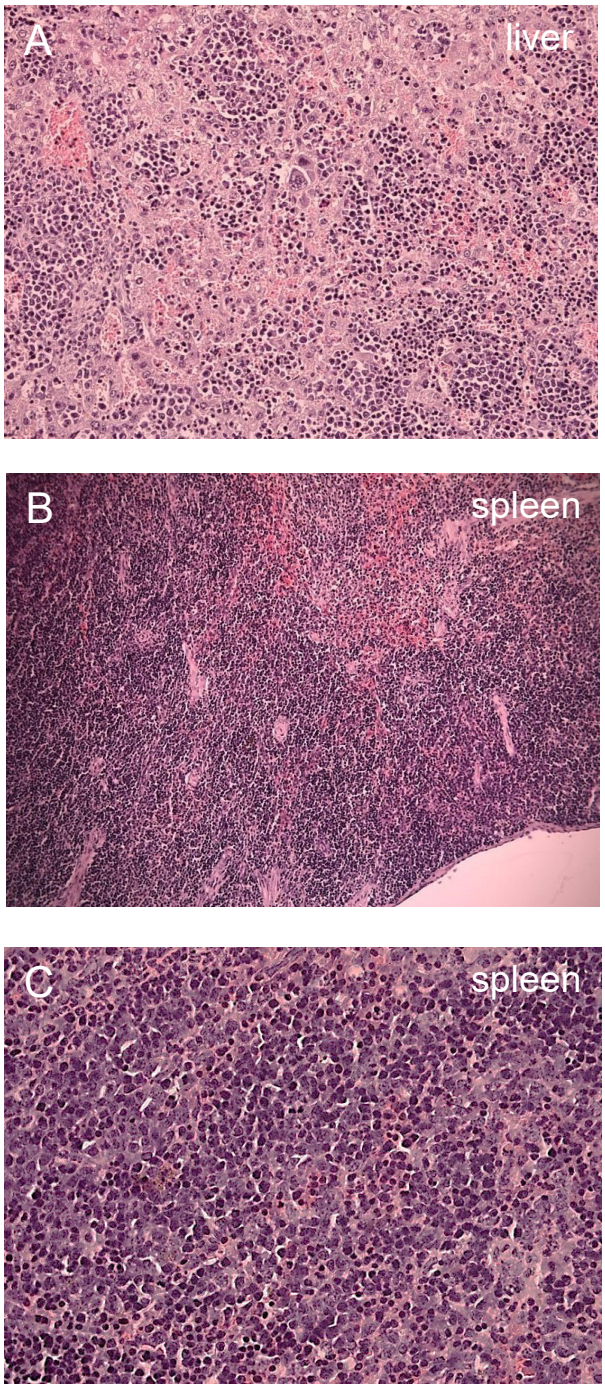


Figure 3.9.6. Expression of Mpl by the SFFV or PGK promoter in lentiviral vectors induced an erythroid hyperproliferation with infiltrations of erythroid progenitor cells in the liver (picture A, 200-fold magnification) and hyperproliferation of the red pulp in the spleen (picture B. 100-fold magnification). The prominent cell type in the red pulp of the spleen also consists of erythroid progenitors (picture C, 400-fold magnification). Paraffin-embedded sections, Hematoxylin-Eosin staining.

Chapter 4

- "Targeting protein expression to the alpha-granules of megakaryocytes and platelets by using sorting signals"

„Wenn du denkst, es geht nicht weiter - geh einfach weiter"

Georg Aescht

4.1. Targeting proteins to the alpha granule of platelets by sorting signals.

The aim of this part of the project was the targeting of proteins to the secretory alpha granules of megakaryocytes and platelets. To achieve this purpose, we followed two strategies:

- The sorting signal of RANTES (*Regulated upon Activation Normal T cell Expressed and Secreted,* member of the and CC chemokine family (Rollins, 1997)) was fused N-terminal to the destabilized GFP (d2eGFP). This sorting domains is a small peptide sequence (VIATLKDGRK). It will deliver the protein into the granules as soluble cargo.

- The transmembrane granular targeting sequence of P-selectin (the transmembrane domain and cytoplasmic tail (referred as TDCT) Section 4.2.2), was fused to d2eGFP or the B domain deleted codon optimized human coagulation Factor VIII cDNA (BDcohFVIII_TDCT). The TDCT will deliver the protein into the membrane of alpha granules.

For the expression of the fusion proteins in MK, SIN-lentiviral vectors were generated that express from the viral SFFV promoter or the platelet factor 4 promoter (Pf4p, Section 4.2.2).

4.1.1 Primary megakaryocytes contain alpha granules expressing P-selectin and von Willebrand factor.

Among the three types of platelet secretory granules, α–granules, dense granules, and lysosomes, the α–granules are the largest and most abundant. There are approximately 50–80 α–granules per platelet, ranging in size from 200–500 nm, representing roughly about 10 % of platelet volume (Frojmovic and Milton, 1982).

For the identification of alpha granules in differentiated megakaryocytes, we performed immunostainings detecting P-selectin (as transmembrane granular protein) and von Willebrand Factor as intragranular protein (Maynard et al., 2007; Blair and Flaumenhaft, 2009; Senzel et al., 2009).

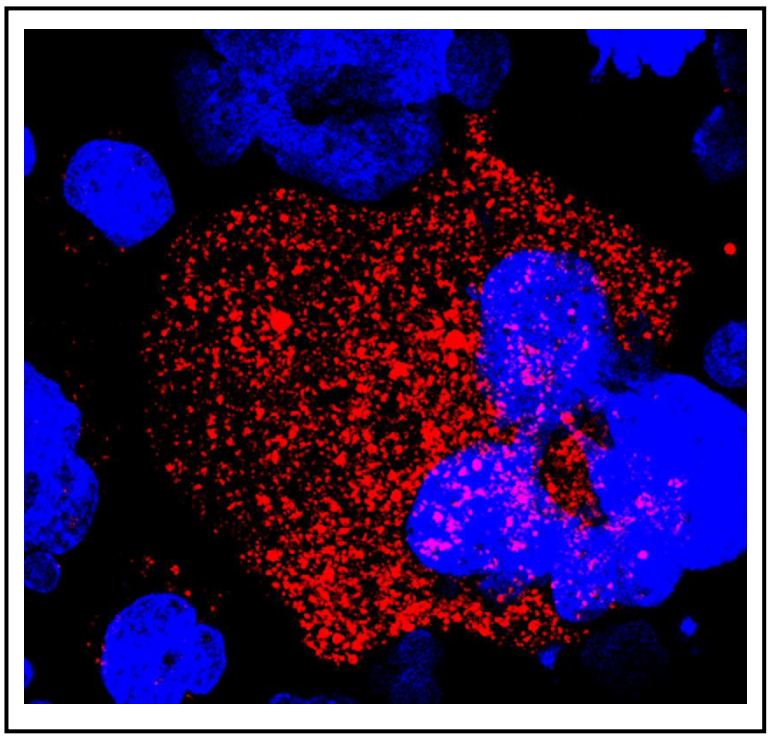


Figure 4.1.1. Primary non-transduced MK. Lin- cells were isolated from whole BM bone marrow from wild type C57BL/6J mice and differentiated into MK with the stimulation of THPO (50ug/ml). Cellular nuclei were visualized by labelling with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) antibody and visualized with a Goat anti mouse Cy3 secondary antibody (Red). Confocal microscopy 100X.

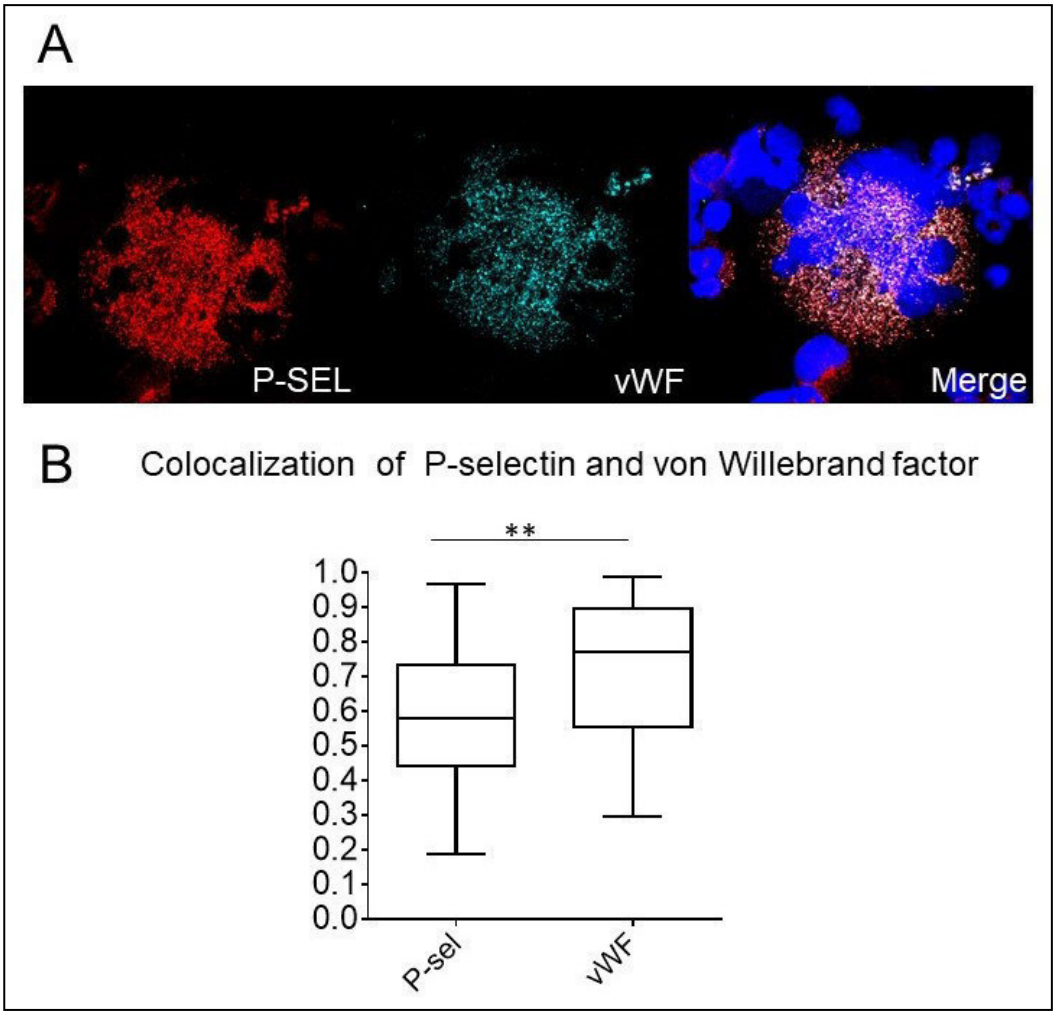


Figure 4.1.2. Confocal microscopy from non-transduced MK. (A) Inmuno- staining of primary non-transduced MK. Lin-cells were isolated from whole BM from wild type C57BL/6J mice and differentiated into MK with the stimulation of THPO (50ug/ml). Cell nuclei was visualized with DAPI (Blue). Alpha granules were marked simultaneously with a primary anti human/mouse CD62P (P-selectin) antibody, and a Rabbit anti von Willebrand Factor. Marking was visualized by using a Goat anti mouse Cy3 secondary antibody (Red) and a Goat anti rabbit Alexa Fluor 647 (Turquoise). Merge of P-selectin and von Willebrand Factor is shown in white. Confocal microscopy 100X. **(B)** Summarized analysis of the **colocalization of P-selectin (P-sel) and von Willebrand Factor (vWF) in alpha granules,** Immuno-stained MK were analyzed by laser scanning confocal microscopy (LSM). Figure shows a box and whiskers chart, based on the analysis of single cells. Statistical analysis was performed with the Mann Whitney Test, p-value =0.0056.

Taking in consideration that cargo may be distributed differentially in the alpha granules, we analyzed the grade of colocalization between the two alpha granule markers P-selectin (P-sel) and von Willebrand factor, (vWF).,. As shown in figure 4.1.2 (A), we stained secretory alpha granules of in vitro differentiated MK simultaneously with the P-selectin and von Willebrand Factor antibodies. Laser scanning Confocal Microscopy (LSM) was performed, and digital images were analyzed in the ZEN- ZEISS 2 software, blue edition. Digitalized images, as represented in figure 4.1.2 (A), showed a grade of colocalization, with overlapping spotted patterns for P-selectin (Red) and von Willebrand Factor (Turquoise) represented as white dots in the picture. For more accuracy in the interpretation, we performed single cell analysis using the ZEN- ZEISS 2 blue edition software. In this case, colocalization analysis is performed on a pixel-based basis. The image of one cell lead to the emission of thousands of pixels, where every pixel is plotted in a scatter plot diagram, based on the intensity from each selected channel. The software can simultaneously analyze two different measurements or channels from the scatterplot. In this study, we evaluated the grade of colocalization based on the weight colocalization coefficient (WCC). The WCC is based on the comparison of two channels (or colors) and is calculated by summing the values obtained in the pixels from the colocalized region for the two channels and dividing this value by the sum of the values detected in all pixels. The value for each pixel is equal to its intensity, with a maximum value of 1. The WCC was derived from Manders and collaborators in 1993 in the paper “Measurement of co-localization of objects in dual-color confocal images”. The colocalization coefficient values will range from 0 (no colocalization or 0 %) to 1 (or 100% of colocalization). In the interpretation, the emitted value of WCC from 0-1 reflects the average of frequency, in which every time the signal from one channel is measured, the signal from the other channel is also simultaneously detected in the same pixel, (both channels colocalized). For the biological interpretation in this particular case, the WCC values for the P-sel (Figure 4.1.2 B) reflects the average in the frequency or percentage in which every time that the signal for P-selecting was measured, the signal for the von Willebrand Factor (vWF) was also detected (and vice versa). Based on the weight colocalization analysis we could observe a

significant difference in the patterns of colocalization (shown with the ** and based on the statistical analysis of Mann Whitney test, P-value: 0.0056), regardless the similar pattern of colocalization observed as white dots in the figure 4.1.2(A). Having a closer look (Figure 4.1.2 B), there is more colocalization from the vWF signal to the P-selectin than from the P-selectin to the vWF. Based on the studies supporting differential protein content within the granules and considering that P-selectin is present in all alpha granules, the significant difference observed in the WCC suggests that there is a subpopulation of alpha granules that do not contain von Willebrand Factor. This significant difference however, could also be in part accentuated by inherent differences between the analyzed cells, due to possible limitations of the staining technique (differences in staining on each cell, sensitivity etc.), or due to possible differences in the maturation stage. Therefore, in the further experiments performed in this study, we continued using both markers for the evaluation of protein targeting, since both are important and abundant proteins within alpha granules and represent good examples of transmembrane and intragranular proteins. In addition, as we were using targeting signals that mediate differential targeting, the use of both proteins as alpha granular markers, facilitate de detection of differences in the targeting.

4.2.1. Intra-alpha granule targeting

As a first approach for intragranular targeting, we used the signal peptide and the sorting signal of the platelet-cytokine RANTES, previously suggested by (El Golli et al., 2005), fused it with the d2eGFP and incorporate it into a SIN-lentiviral vector, using the platelet factor 4 promoter fragment (Pf4p). This sorting signal corresponds to a small peptide sequence (10 amino acids), structurally homologous with two other sorting signals characterized as well by El Golli and collaborators. In this study, the authors describe the structure of these sorting sequences based on 3D alignments, indicating the formation of a β turn between 2 anti-parallel β strands within the protein, regardless the difference in the nucleotide sequence, see figure 4.2.1. In the original protein, these sorting signals are localized at an internal position within the protein sequence, after the ATG and the signal peptide and before the stop codon, described as follow (PF4: L^{72}-K^{81}, NAP-2: V^{94}-K^{103} and

RANTES: V^{62}-Q^{71}). Therefore, to preserve the original localization and avoid possible modifications in the folding or in the transport of the d2eGFP recombinant protein, RANTES signal peptide and RANTES sorting signal were the kept at a 5' end of the nucleotide sequence (N-terminus from the targeted protein). In the other hand, by keeping the sorting signal at this position, d2eGFP could be easily exchangeable by any other proteins of interest.

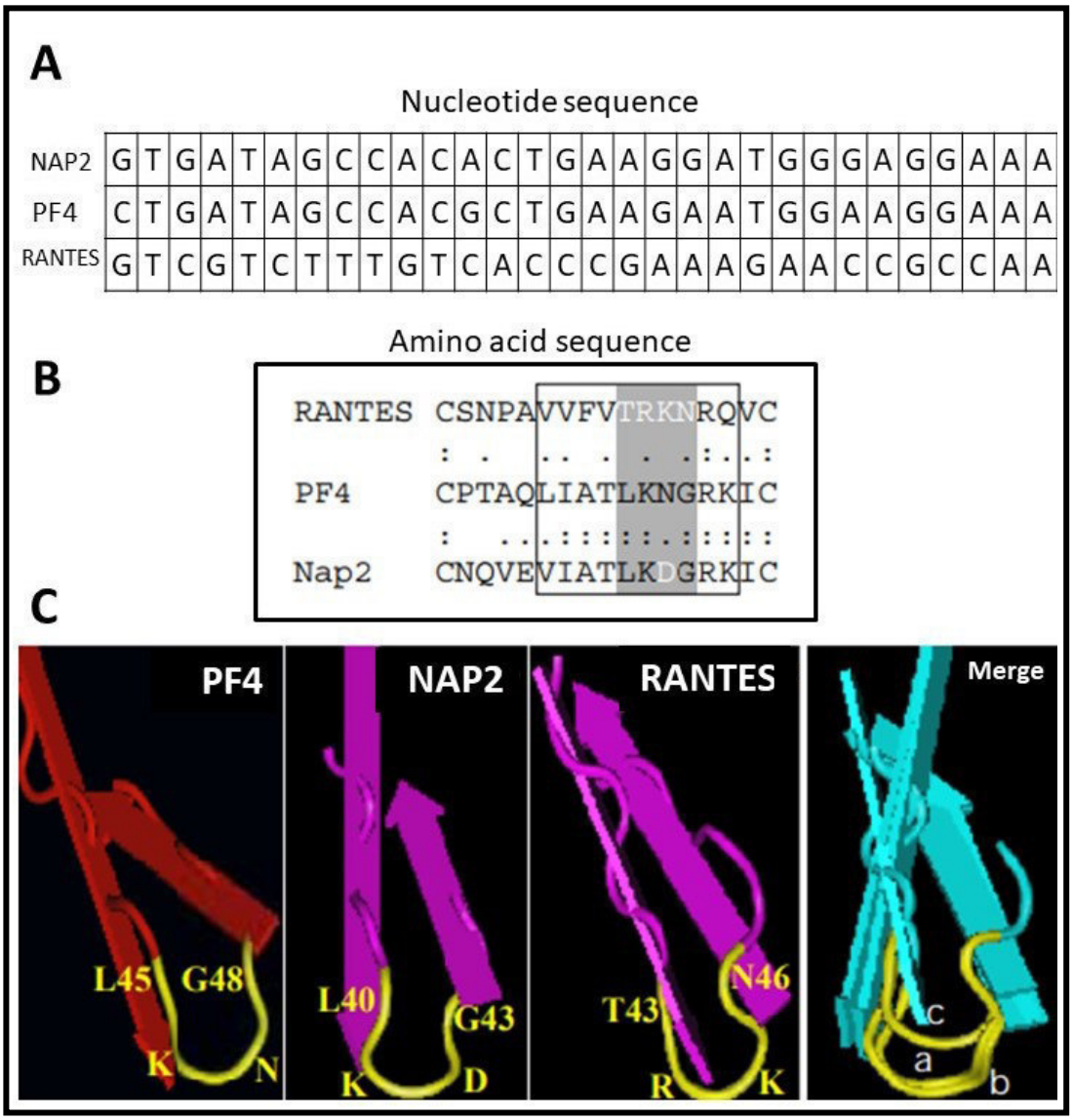


Figure 4.2.1. Analysis of the sorting signals. Nucleotide sequence (A). Amino acid sequence (B). Amino acids are showed in single letter code. The 10 amino acids are enclosed within a black square, and the targeting cores are included in a grey shadow. Sequence homologies are indicated with double points. **Comparison of the PF4, NAP2 and RANTES sorting signals in a 3D structure analysis (C)**. Merge of 3 structure is also shown and sequences are identified as (a,b,c). Large arrows correspond to β strands. Amino acid sequence (B) and 3D structure analysis (C) were taken and modified from (El Golli et al. 2005).

For the evaluation of the RANTES.d2eGFP targeting vector, we generate vector particles in a concentration range of 10^7-10^8 vp/ml after concentration by ultracentrifugation, showing that this vector was well packaged. Lin-cells from wildtype C57BL/6J mice were isolated from whole BM and transduced with the targeted and non-targeted vectors. After transduction, cells were cultivated and differentiated into MK with 50ul/ml THPO for a maximum period of 3 weeks. Before the analysis, cells were centrifugated onto microscope slides for the immunostaining. As granular markers, we used antibodies detecting P-selectin and von Willebrand factor. LSM was used for the collection of the digital images, and the images were analyzed in the ZEN- ZEISS 2 blue edition. Transduced cells with the RANTES.d2eGFP targeting vector were compared with cells transduced the non-targeting vector.colocalization analysis was performed at the single cell level, based on the digital images.

4.2.2 Intragranular targeting. Lineage specific GFP targeting vector.

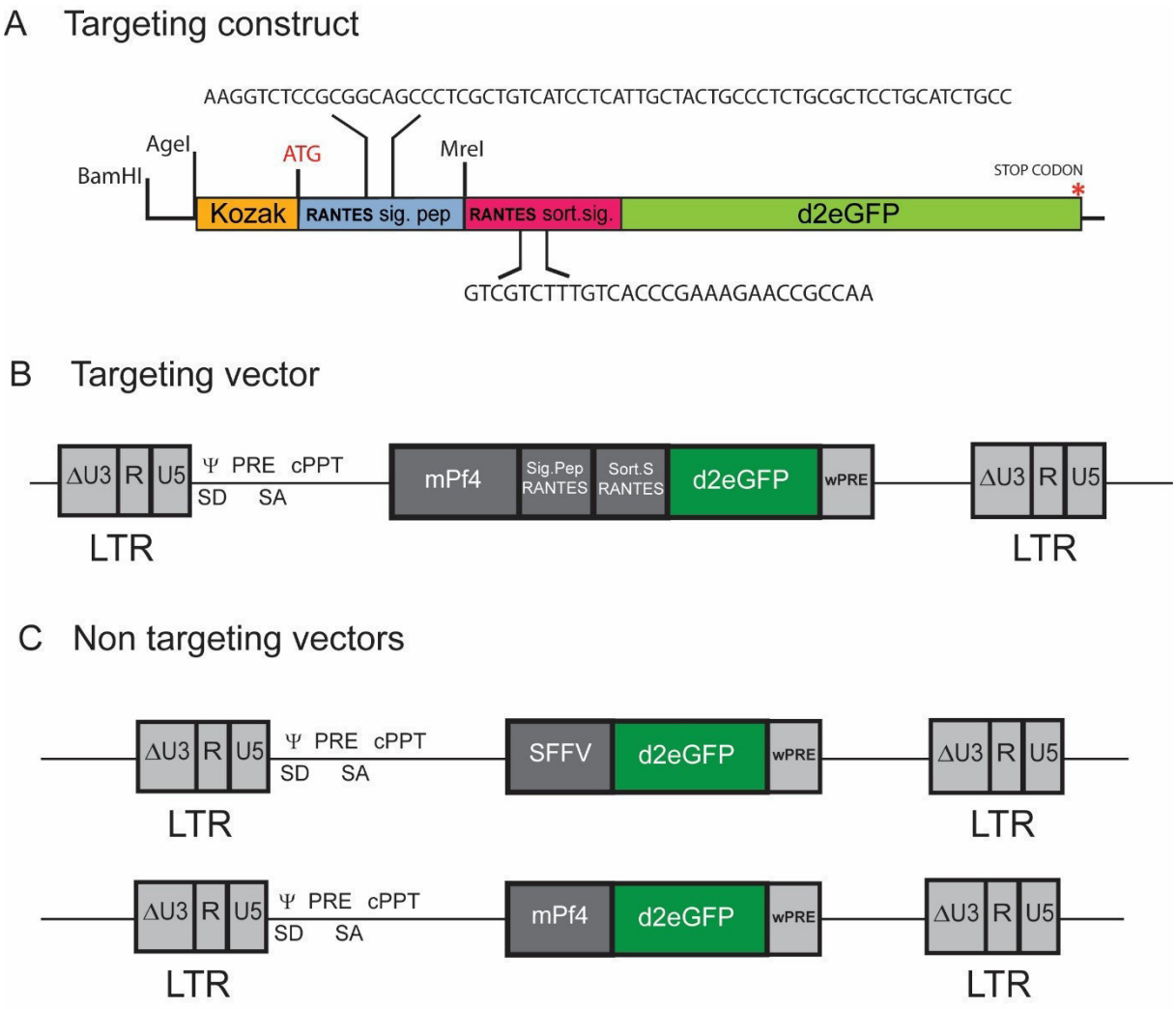


Figure 4.2.2 Vector architecture for GFP targeting. GFP fusion (A). d2eGFP was fused at the N-terminus with the signal peptide and the sorting signal of the cytokine RANTES. The targeting signal was incorporated by polymerase chains reaction, (PCR), digestion in cloning sites and ligation into the SIN-lentiviral vector. **Architecture of the targeting vector RRL.PPT.mPF4.Sig.pep.SortSigRANTES.d2eGFP.pre,** in short RANTES.d2eGFP **(B). Non-targeting vectors RRL.PPT.SFFV.d2eGFP.pre and RRL.PPT.mPf4p.d2eGFP.pre,** in short referred as SFFV.d2eGFP and mPf4p.d2eGFP **(C).**

In the figure 4.2.3 are shown the weight colocalization coefficient (WCC) from transduced MK, represented in a box and whisker bar-chart. Statistical analysis from these values reveal a significant difference in the GFP colocalization efficiency, when cells transduced with the targeting vector RANTES.d2eGFP are compared with the cells transduced with the non-targeting vector SFFV.d2eGFP (p-value <0.0001), in both P-selectin and von Willebrand Factor stained granules. Likewise, no significant difference was observed when the WCC values between the P-selectin and von Willebrand factor stained alpha-granules were compared, neither with the targeting vector (p-value 0.1593), nor with non-targeting vector (p-value 0.3007). reflecting no preference in the targeting or protein distribution within the alpha granules subpopulations in cells transduced with the targeting vector. The median of the WCC values observed from the targeting vector RANTES.d2eGFP is 0.8 (80 % of colocalization) with P-selectin stained granules and from 0.7 (70%) with von Willebrand Factor stained granules. In the case of the non-targeting vector SFFV.d2eGFP the median of the WCC observed were <0.3 (30%) both in P-selectin and von Willebrand Factor stained granules.

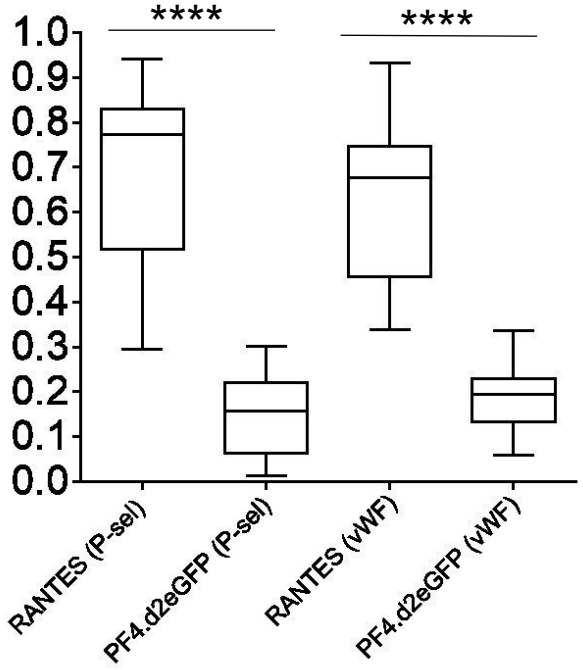


Figure 4.2.3. GFP colocalization analysis in transduced MK. Dot plot bar-charts showing the weight colocalization coefficient (WCC) of GFP either in P-selectine (P-sel) or von Willebrand Factor (vWF) stained granules. Every dot represents the value of one single cell. Statistical analysis was performed with the non-parametric Mann Whitney test, by comparing the untargeted vector (mPf4.d2eGFP) with the targeting vector RANTES.d2eGFP, (shown in the graphs as RANTES). For that, The WCC values from cells transduced with the targeting vector RANTES, were compared with the WCC of GFP expressing cells transduced with the untargeted GFP vector (PF4.d2eGFP) in both P-selectin and von Willebrand factor stained alpha granules. (****$p<0.0001$).

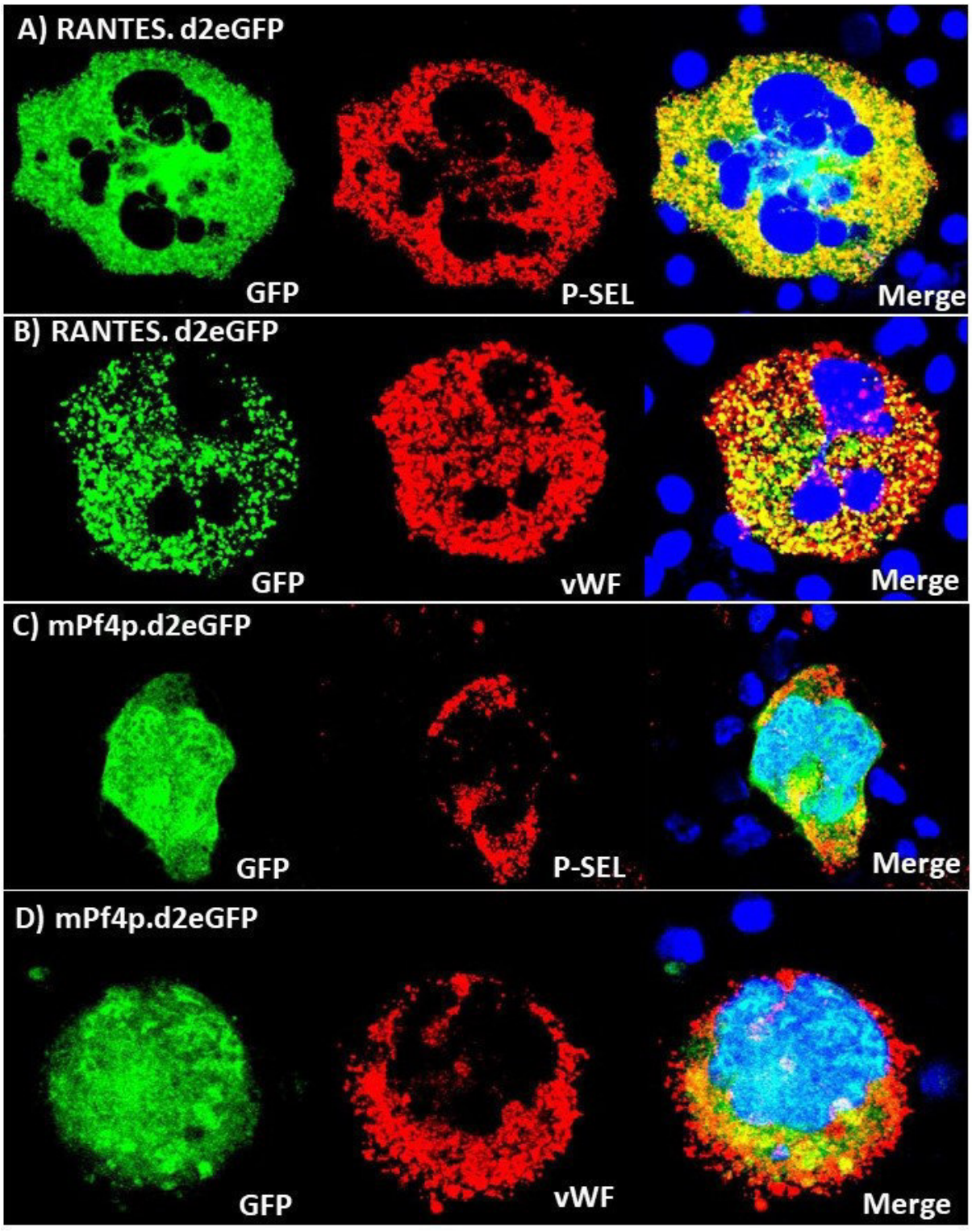

A) RANTES. d2eGFP
GFP
P-SEL
Merge
B) RANTES. d2eGFP
GFP
vWF
Merge
C) mPf4p.d2eGFP
GFP
P-SEL
Merge
D) mPf4p.d2eGFP
GFP
vWF
Merge

Figure 4.2.4. In vitro differentiated MK transduced with lentiviral targeting vectors. RANTES.d2eGFP vector, alpha granules stained with P-selectin (A), RANTES. d2eGFP vector, alpha granules stained with von Willebrand Factor (B), Non-targeting vector mPf4p.de2GFP alpha-granules stained with P-selectin (C). Non-targeting vector mPf4p.de2GFP, alpha-granules stained with von Willebrand Factor (D). Cellular Nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) or a rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti mouse Cy3 or a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in Red). Colocalization is shown by merging of P-selectin / von Willebrand Factor (Red) and the targeted GFP (green), showed in yellow. Confocal microscopy 100X.

As shown in the figure 4.2.4 (A and B), the dotted or granular GFP pattern observed in MK transduced with the RANTES.d2eGFP targeting vector, overlap the signals emitted by P-selectin and von Willebrand factor stained granules (merge in yellow), indicating an evident colocalization: The GFP evident spotty pattern (shown in green), with absence of nuclear GFP, different to what was observed with the non-targeted vector SFFV.d2eGFP. The Targeted-GFP cellular distribution clearly overlapped the cytosolic pattern from both P-selectin and von Willebrand Factor stained granules (shown in yellow, figure 4.2.4, A and B). With the non-targeting SFFV.d2eGFP and mPf4p.de2GFP vectors, a more uniform and homogeneous GFP distribution was observed; extended to the nuclear area (Figure 4.2.4, C and D), with cytosolic GFP outside from the granular areas; a clear evidence of no or lower GFP targeting to granules.

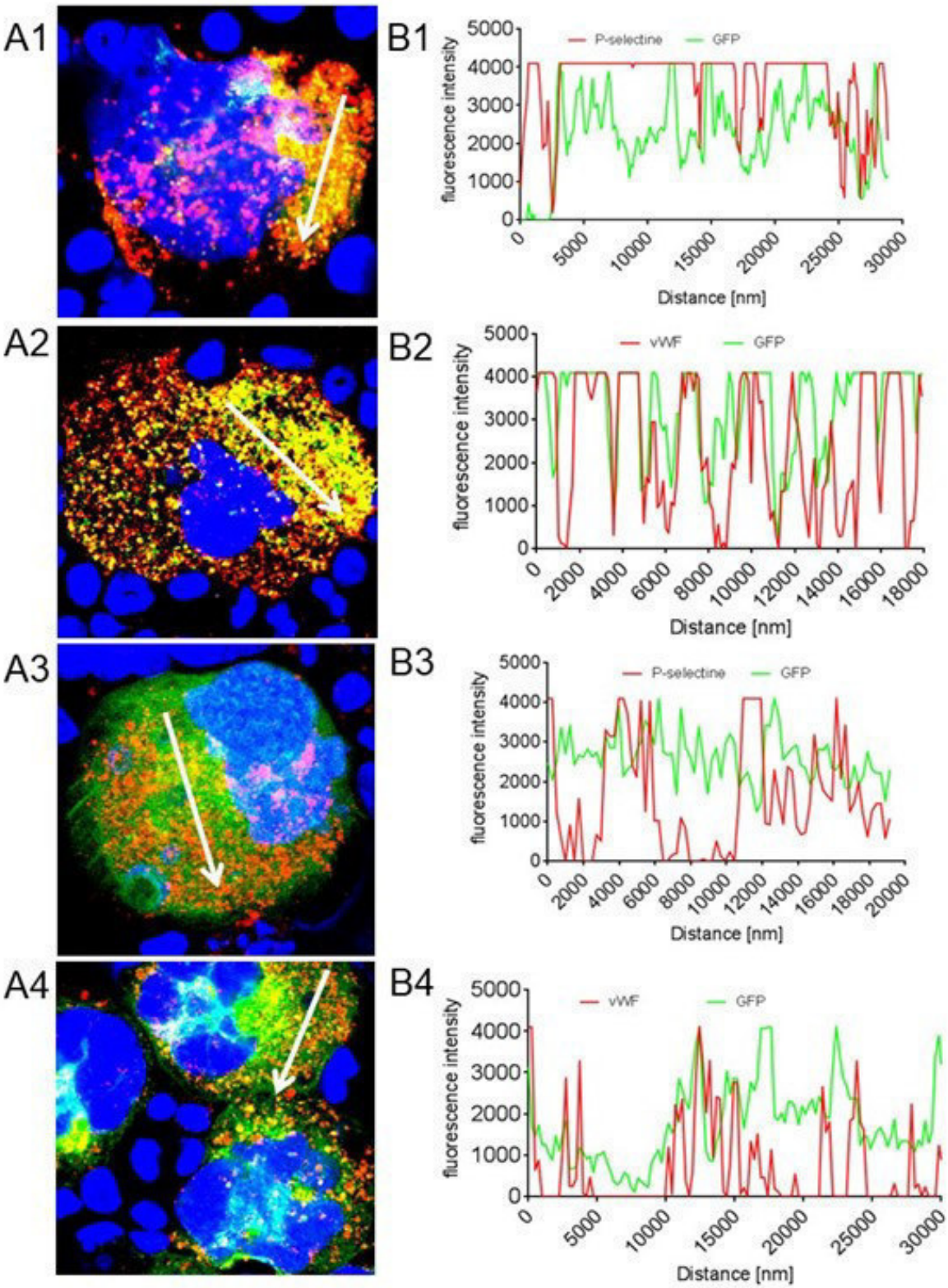

A1
B1
P-selectine
GFP
fluorescence intensity
Distance [nm]
A2
B2
vWF
GFP
fluorescence intensity
Distance [nm]
A3
B3
P-selectine
GFP
fluorescence intensity
Distance [nm]
A4
B4
vWF
GFP
fluorescence intensity
Distance [nm]

Figure 4.2.5. In vitro differentiated MK transduced with lentiviral targeting vectors (A). RANTES.d2eGFP, alpha granules stained with P-selectin (**A1**), RANTES.d2eGFP, alpha granules stained with von Willebrand Factor (**A2**). Non-targeting vector mPf4p.de2GFP alpha-granules stained with P-selectin (**A3**). Non-targeting vector mPf4p.d2eGFP, alpha-granules stained with von Willebrand Factor (**A4**). Cellular Nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) or a rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti mouse Cy3 or a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in Red). Colocalization is shown by merging of P-selectin / von Willebrand Factor (Red) and the targeted GFP (green), showed in yellow. Confocal microscopy 100X. **Fluorescence intensity analysis (B1-B4).** The profiles (B1-B4) shows the overlapping of the fluorescence intensities from the P-selectin and von Willebrand Factor with the GFP signal, measured per nanometer, either in the targeting vector RANTES.d2eGFP (B1-B2) or in non-targeting vector mPf4p.de2GFP (B3-B4) along the marked line with the white arrow.

These results were corroborated in the fluorescent intensity graphs (figure 4.2.5 B1 and B2 respectively), where for a more accurate analysis, we measured the fluorescent intensity from both red and green channels (P-sel/GFP and vWF/GFP) per nanometer (nm) in a specific area of transduced cells (shown as a white arrow in figures 4.2.5 A1-4and 4.2.6, C and D). These values were measured by the software ZEISS ZEN blue edition and plotted afterwards in a graph, to compare the fluorescence intensity from P-selectin or von Willebrand factor with the fluorescent intensity of GFP, per the distance in nanometers (Figures 4.2.5 B1-B4 and 4.2.6, E and F). These graphs allowed us to compare more specifically the overlapping and superposition of the signals, giving us a better and a clearer vision of the GFP-colocalization into the alpha-granules. As observed in the figure 4.2.5 (B1-2), there is an evident and clear overlapping of the GFP signal expressed from the targeting vector RANTES.d2eGFP with both P-selecting and von Willebrand Factor stained granules; reflecting with this a specific GFP targeting along the studied area (white arrow figure 4.2.5 A1-A2). This was not observed in cells transduced with the SFFV.d2eGFP and mPf4p.de2GFP vectors, evidenced in the fluorescent intensity graphs (figure 4.2.5 B3 and B4, figure 4.2.6 E and F) where the GFP signal (shown in green line) was detected in areas of no superposition with the P-selectin and von Willebrand Factor signals (shown in red), indicating the presence of GFP in cytosolic regions outside of the alpha granules.

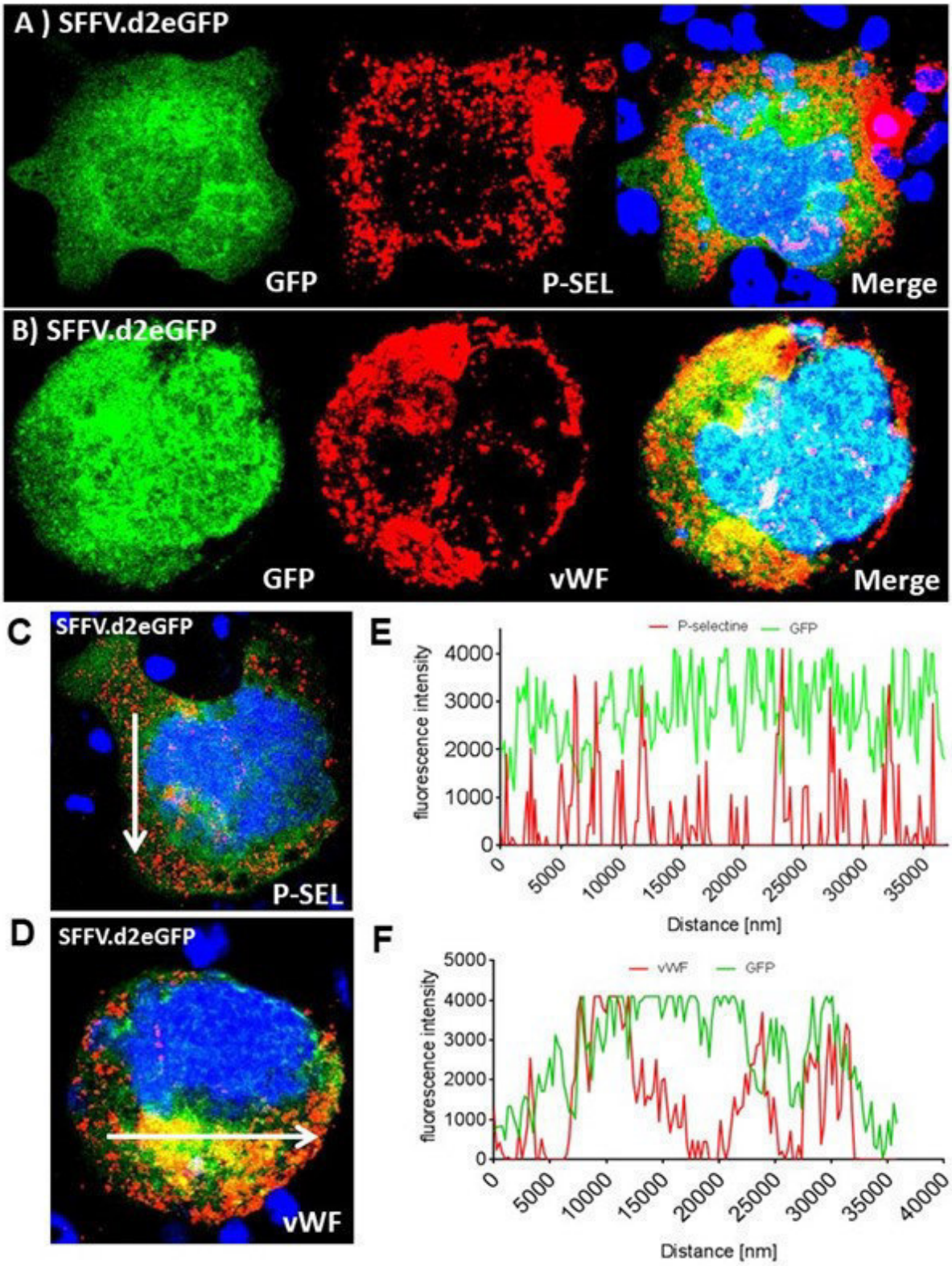

A) SFFV.d2eGFP
GFP
P-SEL
Merge
B) SFFV.d2eGFP
GFP
vWF
Merge
C
SFFV.d2eGFP
P-SEL
D
SFFV.d2eGFP
vWF
E
P-selectine
GFP
fluorescence intensity
4000
3000
2000
1000
0
0
5000
10000
15000
20000
25000
30000
35000
Distance [nm]
F
vWF
GFP
fluorescence intensity
5000
4000
3000
2000
1000
0
0
5000
10000
15000
20000
25000
30000
35000
40000
Distance [nm]

Figure 4.2.6. In vitro differentiated MK transduced with lentiviral non-targeting vectors SFFV.de2GFP. Cellular nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) antibody and visualized by using a Goat anti mouse Cy3 secondary antibody (Red). Colocalization is shown by merging of P-selectin (Red) and targeted GFP (green), showed in yellow. Alpha granules were stained with a primary rabbit anti von Willebrand Factor (vWF) antibody and visualized by using a Goat anti rabbit Alexa fluor 467 secondary antibody (Red). Colocalization is shown by merging of von Willebrand Factor (Red) and targeted GFP (green), showed in yellow. Confocal microscopy 100X. **Fluorescence intensity analysis (E-F).** The profiles (B1-B4) shows the overlapping from the fluorescence intensities of P-selectin or von Willebrand Factor (Red) and GFP (green) per nanometer along the line marked by the white arrow.

These results show that the inclusion of a targeting signal have indeed an effect in the protein trafficking, modifying thereby the transport and protein trafficking, allowing a controlled storage in specific cellular compartments, that, in other conditions, would have been ubiquitously expressed and distributed in the cytosol of the cell. The use of a lineage-specific promoter as the platelet factor 4 promoter (PF4p), has an effect in targeting the protein expression to a cellular lineage as shown before (chapter 3), but not to an any subcellular compartment, since no difference in the GFP expression patterns was observed between the cells transduced with the PF4.d2eGFP and SFFV.d2eGFP non-targeting vectors as expected.

4.3 Transmembrane alpha granule targeting.

As a second targeting approach, we aimed to target proteins to the membrane of the alpha granules of MK and platelets. To achieve this, we used the sorting signal or targeting domain of P-selectin that also translocates to the plasma membrane after platelet activation (Disdier et al., 1992; Koedam et al., 1992; Hartwell et al., 1998; Modderman et al., 1998; Furie et al., 2001).

4.3.1 Targeting d2eGFP to the membrane of alpha granules by using the P selectin sorting signal

As first construct, the signal peptide of P-selectin was fused tod2eGFP as reporter, followed by the transmembrane domain and cytoplasmic tail of P-selectin, see figure 4.3.1. The coding sequence of this new fusion protein, named P-sel.sig.pep.d2eGFP.TDCT (in short P-sel.d2eGFP.TDCT), was inserted into a SIN-lentiviral vector and used for transduction of Lin- cells for MK differentiation. Targeting of protein was evaluated in colocalization analysis by laser scanning confocal microscopy (LSM).

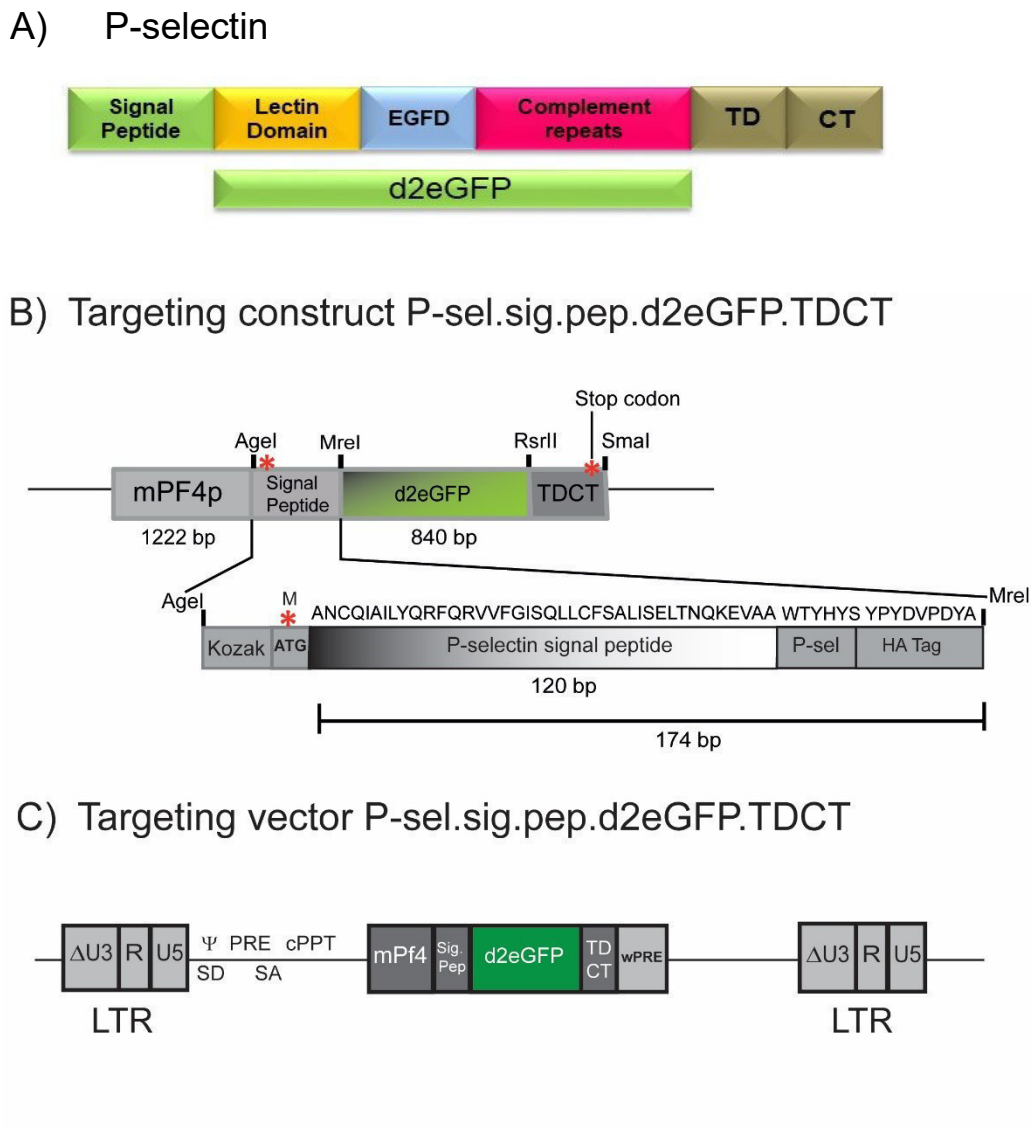


Figure 4.3.1. Fusion of P-selectin sequences to d2eGFP for the generation of the smembrane targeting vector. **P-selectin protein structure (A).** For the generation of the targeting construct, the lectin domain, the epidermal grow factor domain and the complement repeats (sushi domains) were substituted by a destabilized GFP variant (d2eGFP). Just the P-selectin signal peptide (N-terminus), the transmembrane domain (CT) and the cytoplasmic tail (CT) (C-terminus) remained in the recombinant protein. **P-selectin-d2eGFP targeting construct (B).** By PCR, we fused the d2eGFP to the N-terminus with the signal peptide (41 amino acids) and the HA-Tag for extracellular detection and differentiation of the recombinant form the wild type P selectin. At the C terminus, (d2eGFP) was fused with the transmembrane domain and cytoplasmic tail (TDCT) of P-selectin protein, conformed by 59 amino acids as follow:

(LTYFGGAVASTIGLIMGGTLLALLRKRFRQKDDGKCPLNPHSHLGTYGVFTNAAFDPSP)
(Disdier et al., 1992), used as a sorting signal for the protein targeting. In the final constructs, by using the restriction sites (MreI / RsrII), d2eGFP can be replaced for any other coding sequence of choice. **Targeting lentiviral vector RRL.PPT.mPf4.Psel.sig.pep.d2GFP.TDCT (C).** For transduction of Lin-cells, the coding sequence of the fusion protein was inserted into a SIN-lentiviral vector by the AgeI and SmaI restriction sites. The expression of the recombinant protein was driven by the lineage-specific promoter murine platelet factor 4 (mPF4p).

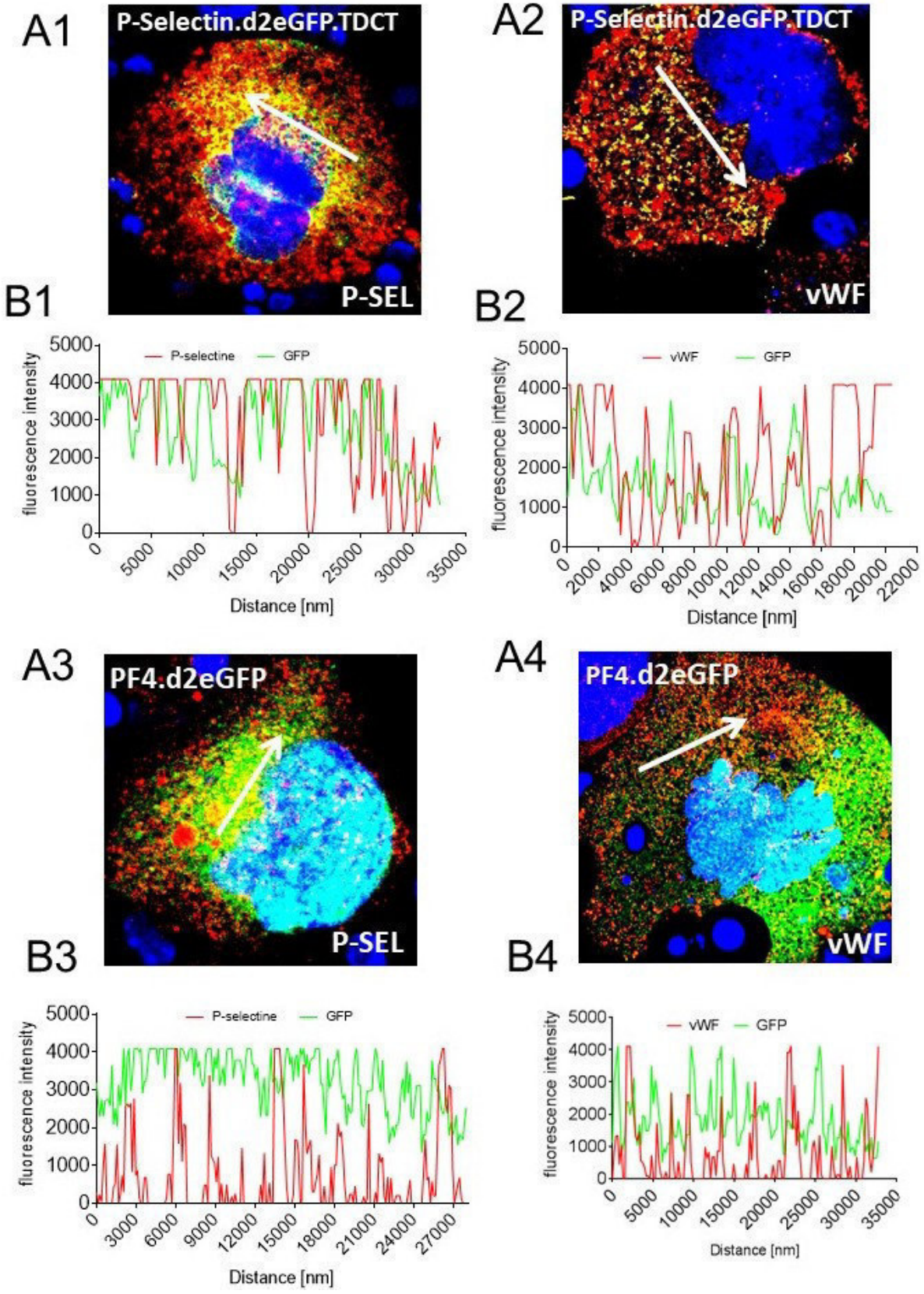

A1
P-Selectin.d2eGFP.TDCT
P-SEL
A2
P-Selectin.d2eGFP.TDCT
vWF
B1
P-selectine
GFP
fluorescence intensity
Distance [nm]
B2
vWF
GFP
fluorescence intensity
Distance [nm]
A3
PF4.d2eGFP
P-SEL
A4
PF4.d2eGFP
vWF
B3
P-selectine
GFP
fluorescence intensity
Distance [nm]
B4
vWF
GFP
fluorescence intensity
Distance [nm]

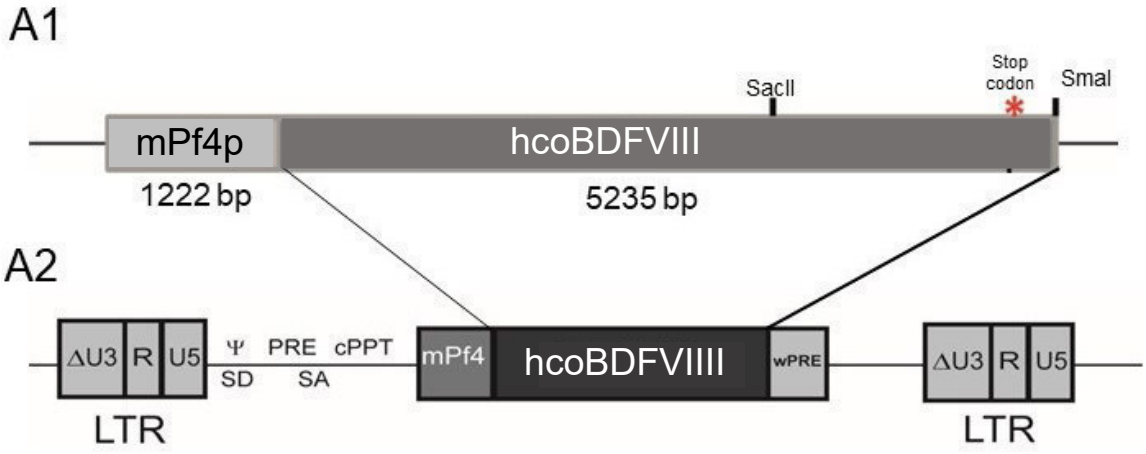
Figure 4.3.2. Colocalization analysis of transduced MK. In vitro differentiated MK transduced with lentiviral targeting vectors (A). Vector P-sel.sig.pep.d2eGFP.TDCT, alpha granules stained with P-selectin (P-SEL) (A1), alpha granules stained with von Willebrand Factor (vWF) (A2). Non-targeting vector Pf4p.de2GFP alpha-granules stained with P-selectin (P-SEL) (A3). Non-targeting vector Pf4p.de2GFP, alpha-granules stained with von Willebrand Factor (vWF) (A4). Cell nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) or a rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti mouse Cy3 or a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in Red). Colocalization is shown by merging of P-selectin / von Willebrand Factor (Red) and the targeted GFP (green), shown in yellow. LSM 100X. Fluorescence intensity analysis (B1-B4). The profiles (B1-B4) show the overlapping of the fluorescence intensities from the P-selectin and von Willebrand Factor with the GFP signal, measured per nanometer, either in the targeting vector P-sel. SigPep.d2eGFP.TDCT (B1-B2) or in non-targeting vector PF4p.de2GFP (B3-B4) along the marked line by the white arrow.

As shown in the figure 4.3.2, the dotted GFP pattern observed in MK transduced with the P-sel.sig.pep.d2eGFP.TDCT targeting vector, overlap the signals emitted by P-selectin (A1) and von Willebrand factor (A2) stained granules (merge shown in yellow), indicating an evident colocalization, corroborated in the fluorescent intensity graphs (figure 4.3.2, B1 and B2 respectively), by a clear signal overlapping in the studied area (white arrow in figure 4.3.2 A1 and A2). The untargeted GFP expressed by the non-targeting vector Pf4p.d2eGFP, is clearly evident in the fluorescent intensity graphs (figure 4.2.2 B3 and B4) where the GFP signal (green line) is observed in areas of not superposition with the signals of P-selectin (B3) and von Willebrand Factor (B4), indicating the presence of GFP in cytosolic regions outside of the alpha granules. These results indicate that the inclusion of the transmembrane domain and cytoplasmic tail of P-selectin drive the storage of target proteins to the alpha granules of MK. This targeting did not show any preference of colocalization between P-selectin and von Willebrand Factor.

4.3.2. Targeting of human coagulation Factor VIII to the membrane of alpha granules.

Aiming to study targeting of proteins in a more physiological context, and willing to take this model up in the future for a potential therapeutic approach, we tried to target the human B domain-deleted coagulation factor VIII (FVIII) to the membrane of the secretory alpha granules of platelets. This recombinant coagulation factor would be stored (like P-selectin does) in the alpha granules, but this time rather as a transmembrane protein of the alpha granules, and not intragranular. This targeting configuration would allow to expose the coagulation factor VIII in the membrane of the platelets as a cell surface protein, once the alpha granules have been released after platelet activation. This targeting model is based on the cellular trafficking of P-selectin during granular secretion, as well as in the natural cell based coagulation process, where the Factor VIII together with Factor IX are bound to the phospholipidic cell surface membrane of platelets, to activate Factor X via the intrinsic tenase complex, figure 1.11 (Blostein et al., 2003; Hoffman, 2003; Soeda et al., 2009). For the performance of these experiments, the B domain deleted Factor VIII used in this study, named as hcoBDFVIII (in short FVIII), was kindly provided by Dr. Joachim Schwäble, Institute of Transfusion medicine, Blutspendedienst Frankfurt am Main. In the following figure, (figure 4.3.3) we show the structure and the cloning strategy of the targeted and non-targeted Factor VIII used in this study.

pRRL.PPT.mPf4p.hcoBDFVIII



pRRL.PPT.mPf4p.hcoBDFVIII_TDCT

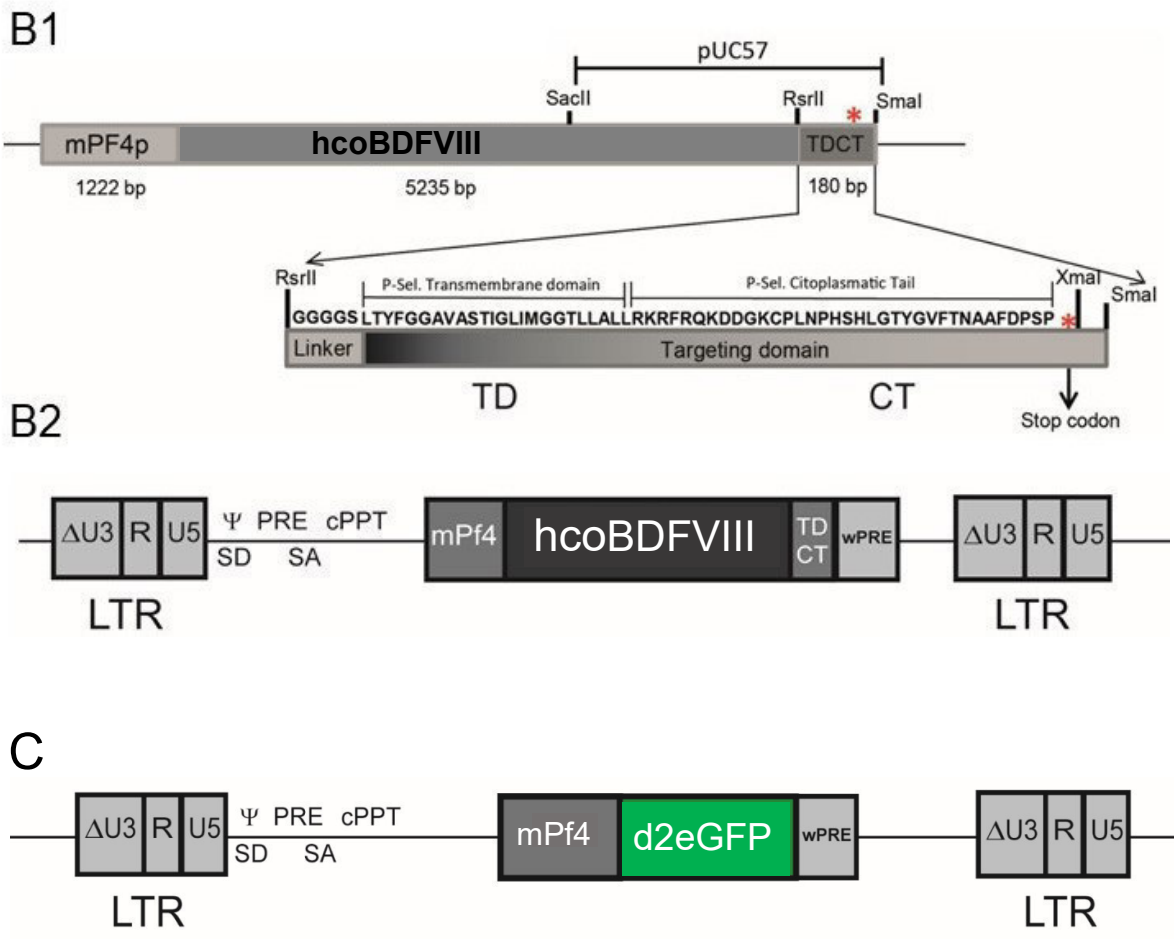


Figure 4.3.3. Targeting vectors of the human coagulation factor 8 protein. **Untargeted human B domain deleted codon optimized coagulation factor VIII (A)**. **Construct (A1) Vector (A2).** The B domain deleted codon optimized human Factor VIII without any targeting signal (named in short FVIII), was inserted into a SIN-lentiviral vector, using the murine platelet factor 4 promoter for driving transgene expression. **Targeted B domain deleted codon optimized human FVIII (in short FVIII_TDCT) (B). Targeting construct (B1). Targeting Vector (B2)**. The transmembrane and cytoplasmic domain (TDCT) of P-selectin was fused at the C-terminus of the B domain deleted codon optimized human FVIII, and cloned into a pUC57 expression plasmid vector, using the SacII and SmaI cloning sites at the N and C- terminus respectively (plasmid from GenScript, USA,Inc). The RsrII restriction cloning site was additionally include, between the recombinant fragment of the C-terminus of FVIII and the TDCT (A1), for possible further cloning purposes. For the generation of the targeted recombinant FVIII, the untargeted human codon optimized coagulation factor VIII (A2) was digested with SacII/SmaI, purified and ligated with the SacII/SmaI digested recombinant fragment from the pUC57 cloning vector. The new targeting vector (B2) would include the original FVIII from (A1) fused with the P-selecting sorting signal TDCT. **Untargeted control vector (C).** As negative control, we used a SIN-lentiviral vector including the murine platelet factor four promoter (mPf4p) driving the expression of the destabilized enhanced GFP (d2eGFP).

For the evaluation of the targeting efficiency of the FVIII_TDCT vector and compare it to the non-targeting FVIII and mPf4.d2eGFP vectors, we transduced Lin- cells with the targeting (FVIII_TDCT) and non-targeting (FVIII and mPf4.d2eGFP) vectors. Cells were then cultured and differentiated into MK, followed by immunostainings for the detection of the transgenic proteins (FVIII and d2eGFP) using confocal laser scanning microscopy (LSM). As alpha granule markers we use P-selectin (P-SEL) and von Willebrand Factor (vWF). As shown in figures 4.3.4 and 4.3.5, there is an evident signal overlap (shown in both cases in yellow) between the d2eGFP or targeted FVIII (FVIII_TDCT) (both proteins shown in green) with the P-selectin / von Willebrand marker (shown in red), as expected. This colocalization of signals is evidenced by the strong dotted pattern in both FVIII/ FVIII.TDCT with the P-selectin / von Willebrand alpha granule markers, exposing the same cellular disposition. On the other hand, the subcellular localization of the d2eGFP observed from the non-targeting vector mPF4p.d2eGFP, covers regions where no specific signals for the alpha granules markers were detected, showing a clear difference in comparison with the targeting vectors.

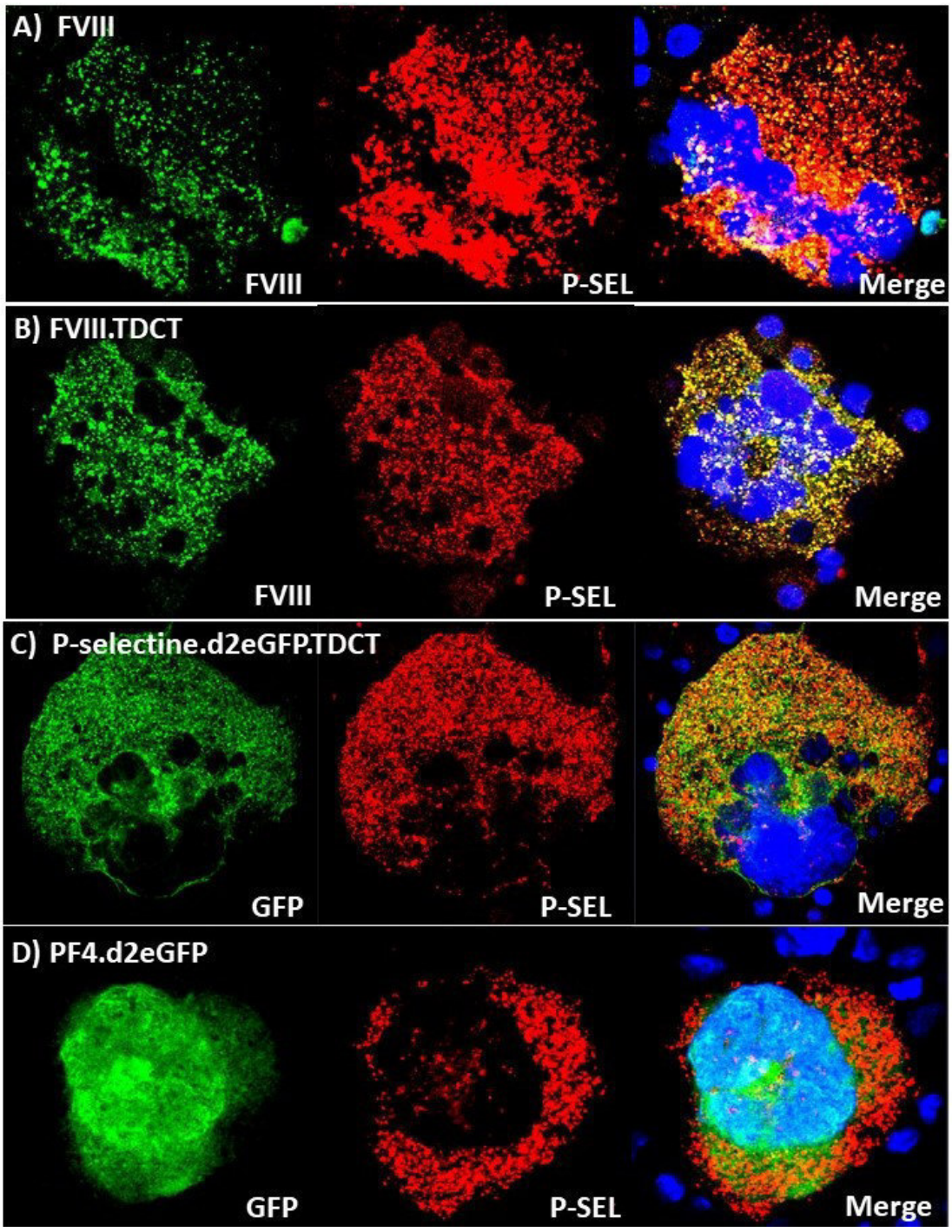


Figure 4.3.4. Transmembrane alpha granule targeting. **Non-Targeting FVIII vector (FVIII) (A). Targeting FVIII vector (FVIII.TDCT) (B). P-selectin Targeting vector (P-selectin.d2eGFP.TDCT) (C). Non-targeting GFP vector, mPF4.d2eGFP (D).** Cell nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin, shown as P-SEL) and visualized by using a Goat anti mouse Cy3 secondary antibody (shown in Red). Colocalization is shown by merging of P-selectin (Red) with the targeted and non-targeted FVIII / GFP (green), shown in yellow. LSM 100X.

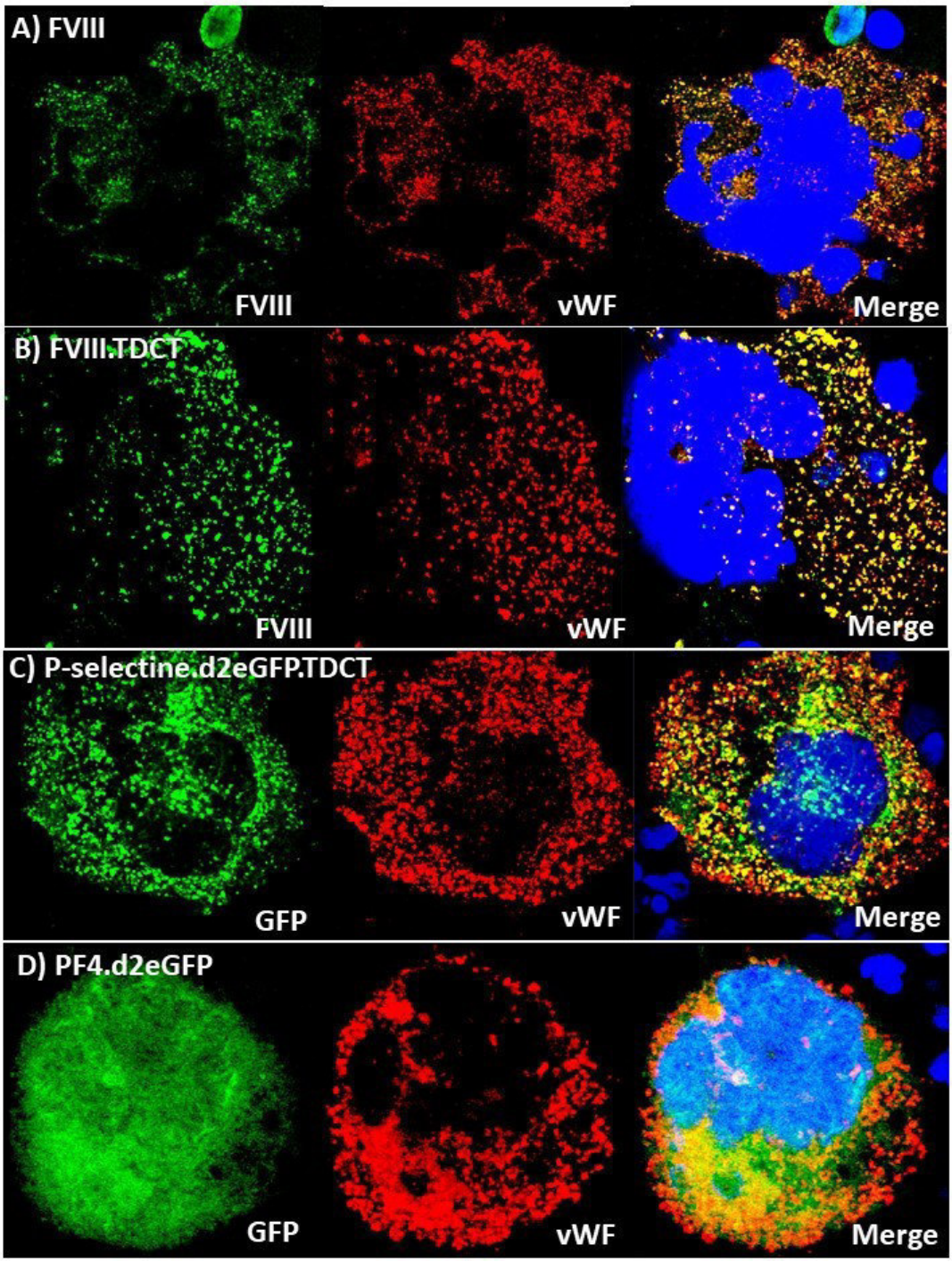


Figure 4.3.5. Transmembrane alpha granule targeting. **Non-Targeting FVIII vector (FVIII) (A). Targeting FVIII vector (FVIII.TDCT) (B). P-selectin Targeting vector (P-selectin.d2eGFP.TDCT) (C). Non-targeting GFP vector, mPF4.d2eGFP (D).** Cellular nuclei content was visualized with DAPI (blue). Alpha granules were stained with a primary rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in red as vWF). Colocalization is shown by merging of von Willebrand Factor (red) with the targeted and non-targeted FVIII / GFP (green), shown in yellow. LSM 100X.

The specific targeting of FVIII (expressed from the non-targeting vector, mPF4p.hBDcoFVIII) was surprising since no targeting signal was included. However, it was already described in previous studies that overexpression of Factor VIII in different cells lines, in the presence of the von Willebrand factor, leads to a modification of the FVIII trafficking within the cell and the storage of the coagulation factor in the secretory granules. (Rosenberg et al., 1998; van den Biggelaar et al., 2011). This effect was shown to be due to an intracellular interaction and binding of FVIII to the von Willebrand factor. The interaction leads to a modification from a constitutive to a regulated secretory pathway and thereby producing an intracellular storage pool of both proteins. This storage, however, has been described to be "intragranular", and it is mediated by a sorting signal contained within vWF protein (propeptide-D2 domain of vWf). However, since our targeting approach is different, we also expected to observe some differences in the FVIII targeting detected with our lentiviral vector constructs. For this purpose, we performed bidirectional colocalization analysis in the transduced megakaryocytes, as shown in figure 4.3.6. This bidirectional analysis takes into consideration not just the colocalization or distribution of the GFP compared to the alpha granule markers, as a reference for intracellular FVIII and GFP distribution, but also the colocalization of the alpha granule markers P-selectine and von Willebrand factor with the respective transgenes FVIII /FVIII_TDCT / d2eGFP. This analysis was important to demonstrate that as expected the FVIII targeting was mediated just by the interaction with von Willebrand Factor.

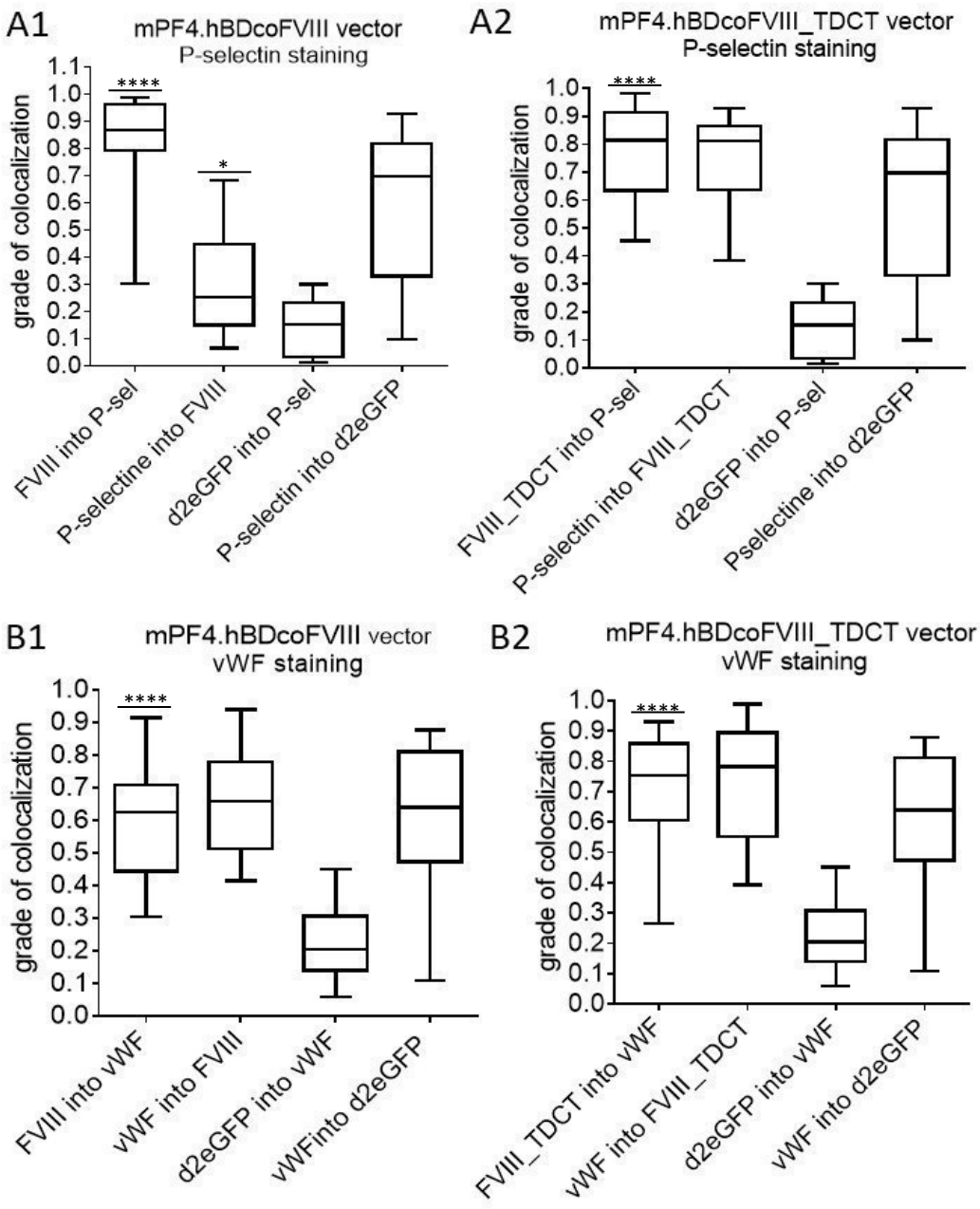


Figure 4.3.6. FVIII colocalization analysis in transduced megakaryocytes. Box and whiskers-charts showing the weight colocalization coefficient (WCC) of the targeting FVIII vector (mPf4.hcoBDFVIII.TDCT), shown in the charts as FVIII_TDCT and the non-targeting FVIII vector (mPf4.hcoBDFVIII), shown as FVIII for the FVIII expression, in both P-selectin (charts A1, A2) or von Willebrand Factor stained granules (charts B1, B2). As negative control for protein targeting, we used the GFP non-targeting vector (mPf4.d2eGFP), shown as d2eGFP, compared in P-selectin- and von Willebrand-positive granules as well. Statistical analysis was performed with the Mann Whitney test, by comparing the non-targeting vector (mPf4.hcoBDFVIII) with the targeting vector (mPf4.hcoBDFVIII.TDCT).

For the performance of these experiments, lin- cells isolated from whole BM were transduced with the vectors using a MOI of 5 and cultivated for a maximum period of 3 weeks and differentiated into MK for the analysis by immunostainings and LSM. As observed in the figure 4.3.6, for the evaluation of the FVIII targeting efficiency, the WCC values from the FVIII and FVIII_TDCT vectors in P-selectin and von Willebrand Factor stained granules were compared to the WCC values observed in the cells transduced with non-targeting GFP vector (mPf4.d2GFP, shown as d2GFP) in P-selectin and von Willebrand Factor stained granules, in a bidirectional colocalization analysis. That means to compare not just the colocalization of the signal from the expression of the FVIII and FVIII_TDCT protein with the P-selectin and von Willebrand alpha granules markers was evaluated (shown in the graphs as FVIII into P-sel, FVIII_TDCT into P-sel and FVIII into vWF / FVIII_TDCT into vWF), but also the colocalization of the signal emitted by the P-selectin and the von Willebrand Factor alpha granules marker with the FVIII and FVIII_TDCT, referred in the graphs as P-selectin into FVIII / P-selectin into FVIII_TDCT (shown in A1 and A2) and vWF into FVIII / vWF into FVIII_TDCT (shown in B1 and B2). These measurements allowed us to evaluated not just the cellular localization and distribution of the FVIII and FVIII_TDCT within the cells, but also to evaluate the alpha granule content. To have the reference from a non-targeting GFP vector, the WCC values from cells transduced with the mPf4p.d2eGFP vector (named in the graphs as d2eGFP) were also analyzed and consider as negative controls. These measurements are shown in the graphs as d2eGFP into P-sel / d2eGFP into vWF (in A1 and B1), as well as P-selectin into d2eGFP and vWF into d2eGFP (in A2 and B2). The WCC values from d2eGFP into P-sel and d2eGFP into vWF reflects the localization of the d2eGFP within the cells when it is compared to what is observed for the alpha granule markers P-selectin and von Willebrand Factor. The other two measurements, P-selectin into d2eGFP and vWF into d2eGFP, reflects the frequency in which the P-selectine and von Willebrand Factor stained granules contains GFP.

When comparing in figure 4.3.6 the non-targeting FVIII vector (mPF4.hcoBDFVIII) with the targeting mPf4.hcoBDFVIII.TDCT vector, we observed that there was no significant difference in the amount of FVIII which is targeted to the P-selecting containing alpha granules, p-value 0.1548 (FVIII into P-sel / FVIII_TDCT into P-sel, figure A1 and A2), meaning that indeed there is a targeting of the both FVIII and FVIII_TDCT into the P-selectin stained alpha granules. However, there is a remarkable significant difference ($p<0.0001$) when comparing the grade of colocalization of the P-selectin marker with the FVIII_TDCT to the FVIII (observed in P-selectin into FVIII/ FVIII_TDCT, in A1 and A2). These results indicate that in the case of the cells transduced with the targeting FVIII_TDCT vector, there was a major percentage of secretory alpha granules containing Factor VIII protein than with the non-targeting FVIII vector. When analyzing the same vectors in the context of the von Willebrand factor stained granules (FVIII into vWF and FVIII_TDCT into vWF, see in figure B1 and B2), a significant difference in the colocalization of the FVIII and FVIII_TDCT into the von Willebrand Factor containing granules was observed (**$p=0.0020$); this means that despite of the colocalization of FVIII in the alpha granules, the grade of FVIII targeting in the FVIII_TDCT expressing cells is higher as with the non-targeted FVIII expressing cells, however, no significant difference in the colocalization of the Willebrand factor containing granules with FVIII and FVIII_TDCT could be demonstrated ($p= 0.0745$), shown as vWF into FVIII/FVIII_TDCT in (B1 and B2). All these results translate, that the FVIII_TDCT is efficiently targeted to the alpha granules of MK with no evidence of significant difference within the alpha granules subpopulation. On the other hand, the FVIII it is also targeted to the secretory granules but with a preferential targeting to the von Willebrand factor containing granules. The targeting observed with the FVIII, is probably mediated by an intracellular binding and interaction of the von Willebrand Factor with the FVIII, as previously described in (Rosenberg et al., 1998), reflected in this case by the preferential colocalization and cellular distribution to the secretory alpha granules containing von Willebrand Factor, clearly observed in vWF into FVIII (B1) in comparison to P-selectin into FVIII (A1). In this case, vWf can direct the

intracellular trafficking of FVIII to a regulated secretory pathway, thereby producing an intracellular storage pool of both proteins.

On the other hand, when comparing both FVIII and FVIII_TDCT vectors with the non-targeting GFP vector, mPf4.d2eGFP (shown as d2eGFP), we see that despite of the amount of GFP that could be found within the alpha granules (observed in P-selectin and vWF into GFP), the GFP was not uniquely localized in the secretory compartment but rather probably as a cytosolic protein This is different to what was observed with the FVIII vectors, where no differences in the bidirectional colocalization analysis was observed (when comparing WCC from d2eGFP into P-sel and d2eGFP into vWF vs FVIII /FVIII_TDCT into P-sel and into vWF), with a significant difference of (****), in both cases for P-sel and vWF markers, $p < 0.0001$. In light of the above findings, we can conclude that with the mPf4.d2eGFP vector, as expected, we did not achieve any specific GFP targeting, reflected by the very low grade of colocalization observed (0.1-0.4 in d2eGFP into P-sel /vWF), with no preferential distribution or association with any alpha granule markers (shown in P-sel / vWF into d2eGFP, with no statistic significant differences, p-value of 0.0640 /0.093 respectively).

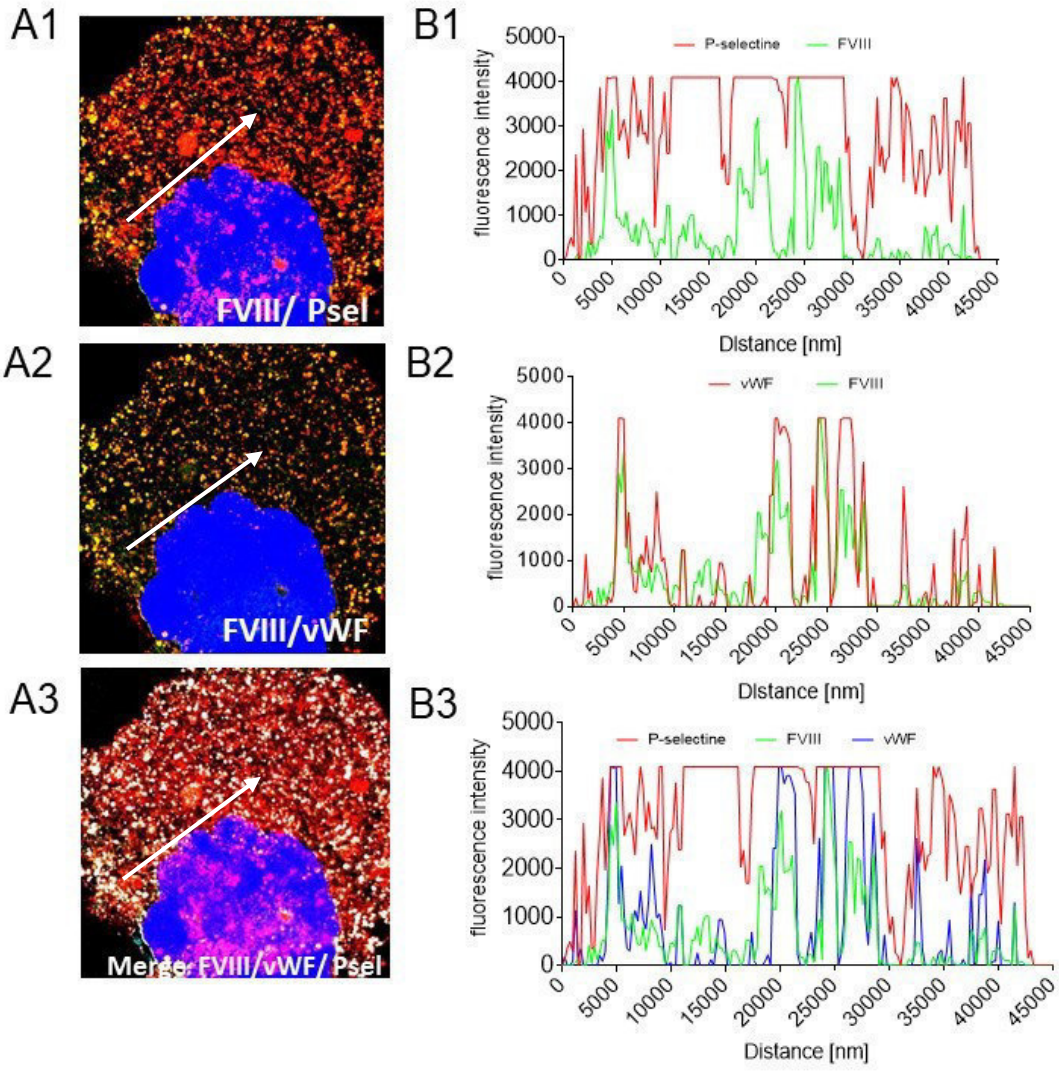


Figure 4.3.7. Colocalization analysis of transduced megakaryocytes. In vitro differentiated MK transduced with the human B domain deleted codon optimized coagulation factor VIII (mPf4.hcoBDFVIII, shown here as FVIII) Lentiviral non-targeting vector (A). **Alpha granules stained for P-selectin (Psel)**, merge of FVIII and P-selectin shown in yellow (A1). **Alpha granules stained for von Willebrand Factor (vWF),** merge of FVIII and vWF shown in yellow (A2), Merge of the 3 signals: **FVIII, P-selectin and von Willebrand Factor (A3),** colocalization shown in white. Cell Nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) or a rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti mouse Cy3 or a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in red). LSM 100X. **Fluorescence intensity analysis (B1-B3).** The profiles (B1-B2) shows the overlapping of the fluorescence intensities from the P-selectin (in Red, B1) and von Willebrand Factor (in Red B2) with the FVIII signal (shown in green), measured per nanometer, along the marked line by the white arrow. **B3** merging of P-selectin signal (Red), von Willebrand Factor (in blue) and FVIII (in green). This analysis is based on transduced cells stained for both alpha granular markers.

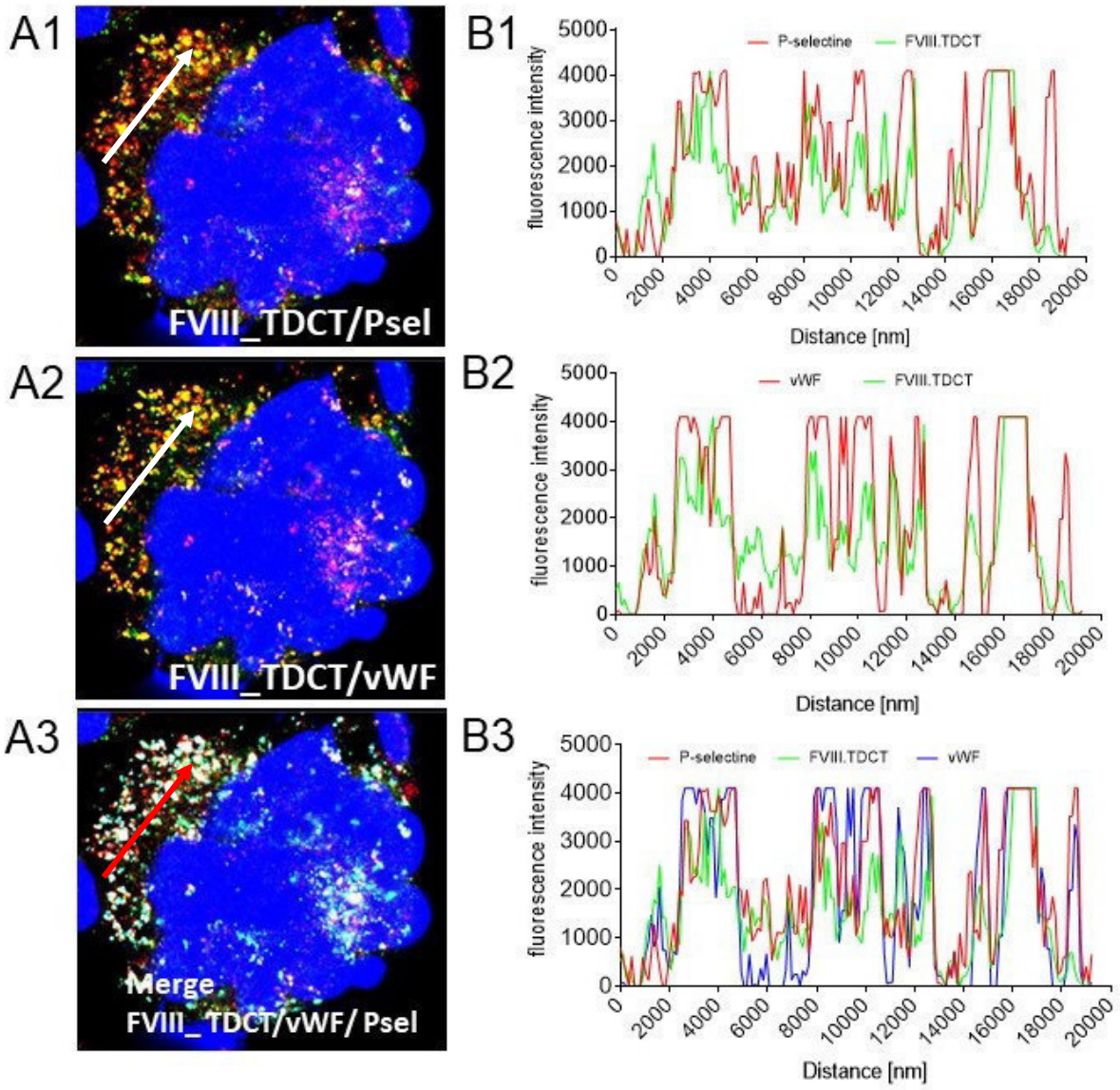


Figure 4.3.8. Colocalization analysis of transduced megakaryocytes. In vitro differentiated megakaryocytes transduced with the human B domain deleted codon optimized coagulation factor VIII (mPF4.hBDcoFVIII_TDCT, in short FVIII_TDCT) Lentiviral targeting vector (A). **Alpha granules stained for P-selectin (Psel),** merge of FVIII_TDCT and P-sel shown in yellow **(A1). Alpha granules stained for von Willebrand Factor (vWF) (A2)**, merge of FVIII_TDCT and vWF shown in yellow. Colocalization of the 3 signals: FVIII_TDCT, P-selectin and von Willebrand Factor**,** shown in white **(A3).** Cell Nuclei content was visualized with DAPI (Blue). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) or a rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti mouse Cy3 or a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in Red). LSM 100X. **Fluorescence intensity analysis (B1-B3).** The profiles (B1-B2) shows the overlapping of the fluorescence intensities from the P-selectin (in Red, B1) and von Willebrand Factor (in Red B2) with the FVIII signal (shown in

green), measured per nanometer, along the marked line by the white arrow. **B3** merging of P-selectin signal (Red), von Willebrand Factor (in blue) and FVIII (in green). This analysis is based on transduced cells stained for both alpha granular markers.

To corroborate the results observed in the colocalization analysis (Figure 4.3.6), we performed fluorescence intensities analysis, as shown in figures 4.3.7 and 4.3.8. The dotted FVIII_TDCT pattern obtained from MK transduced with the mPF4.cohBDFVIII_TDCT targeting vector (figure 4.3.8), overlaps and coincide with the signals emitted by P-selectin (A1) and von Willebrand factor (A2) stained granules (merge shown in yellow), indicating a strong and evident colocalization. This was corroborated in the fluorescent intensity graphs (figure 4.3.8, B1 and B2 respectively) by the clear overlapping signals in the studied area (white arrow in figure 4.3.8 A1 and A2). A clear superposition was observed between the red line (P-sel/ vWF) and the green line (FVIII) in B1 and B2. The same effect was observed when we overlap the 3 signals: FVIII, P-sel and vWF, as shown in A3 (merge in white) and B3. These results indicate no differential or preferential targeting of FVIII_TDCT between the P-selectin and von Willebrand Factor containing granules, as previously shown in the colocalization analysis, figure 4.3.6. Therefore, the inclusion of the transmembrane domain and cytoplasmic tail of P-selectin drive the storage of target proteins to the alpha granules of megakaryocytes without any preference of colocalization between P-selectin and von Willebrand Factor markers.

On the other hand, the “untargeted” FVIII expressed from the mPF4.hBDcoFVIII non-targeting vector (figure 4.3.6, B1), presented a clear evidence of differential and preferential FVIII targeting into alpha granules that contain von Willebrand factor, demonstrated by the fluorescent intensity graphs (figure 4.3.7, B1, and B2), giving the evidence that the targeting in the FVIII is mediated by its interaction with the von Willebrand factor.

4.4. Genetically modified platelets.

To evaluate the effect of the targeting vectors in the platelets, we performed bone marrow transplantations into wild type C57BL/6J recipient mice. Lineage marker-negative cells isolated from whole bone marrow of wild type C57BL/6 mice, were transduced in vitro with the targeting vectors: mPf4.RANTES.d2eGFP (RANTES.d2eGFP), P-sel.Sig.pep.d2eGFP.TDCT (P-sel.d2eGFP), mPf4.hBDcoFVIII_TDCT (FVIII_TDCT) and the non-targeting control vectors mPf4.hBDcoFVIII (FVIII) / PF4.d2eGFP, followed by transplantation into recipient wild type C57BL/6 mice (4 mice per vector). One of the SFFV.GFP surviving transplanted mice from the lineage specific vectors study (see chapter 3) was also used for the analysis as a negative control. 12-16 weeks after engraftment, platelets were isolated from whole blood and stained for immunofluorescent analysis using the Laser Scanning Confocal Microscopy (LSM). Flow cytometry was utilized to detect the donor cell chimerism and engraftment. The analysis of the genetically modified platelets was based on a single cell basis by means of immunostaining of F-actin (for cellular structure visualization), P-selectin or von Willebrand Factor (for alpha granules detection), and GFP / FVIII / FVIII_TDCT as the transgene expression. To appreciate better the differences in the targeting and in the colocalization, analysis of genetically modified platelets was performed on activated platelets. For this, platelets were isolated from whole blood of transplanted mice as washed platelets and activated with 0.2 μM of thrombin before staining.

12 weeks after transplantation a leukocyte chimerism in transplanted mice between 2 and 20 percent was observed, and transgene expression in platelets varied in the range of 1 to 3 percent. Although the overall engraftment was low, in some of the transplanted mice transgene expression was enough to perform single cell analysis by LSM and obtain preliminary data about the targeting in platelets in vivo. This limitation however, made itnot possible to pursue functional analysis or to quantify the extend of targeting in platelets similar to what was performed in MK in vitro.

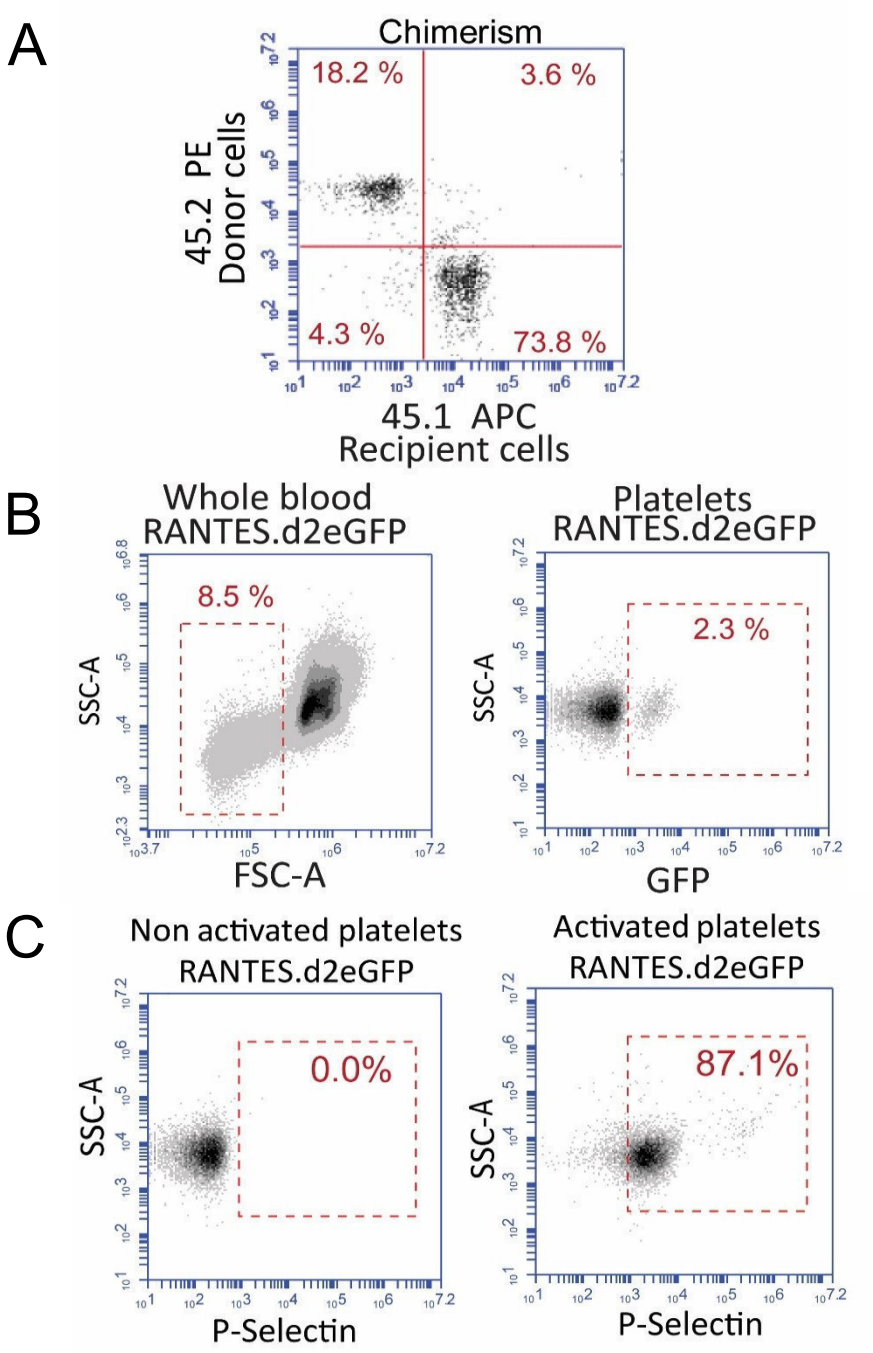


Figure 4.4.1 Example of analysis of the genetically modified platelets from transplanted mice with the RRL.PPT.mPf4.RANTES.sig.pep.sort.sig.d2eGFP targeting vector. Analysis of chimerism (A). Leukocytes from whole blood of transplanted mice were isolated and stained for the CD45.1 (cells from the recipient mice) and the CD45.2 (cells from donor mice). **GFP expression in genetically modified platelets (B)**. First dot blot (left side) represents the gate for the platelets in whole blood. Second dot blot (Right side) represent the percentage of GFP expressing platelets. **Platelet activation test in genetically modified platelets (C).** Washed platelets were isolated from whole blood and activated with thrombin (0.2μM). P-selectin (PE) was detected as activation marker.

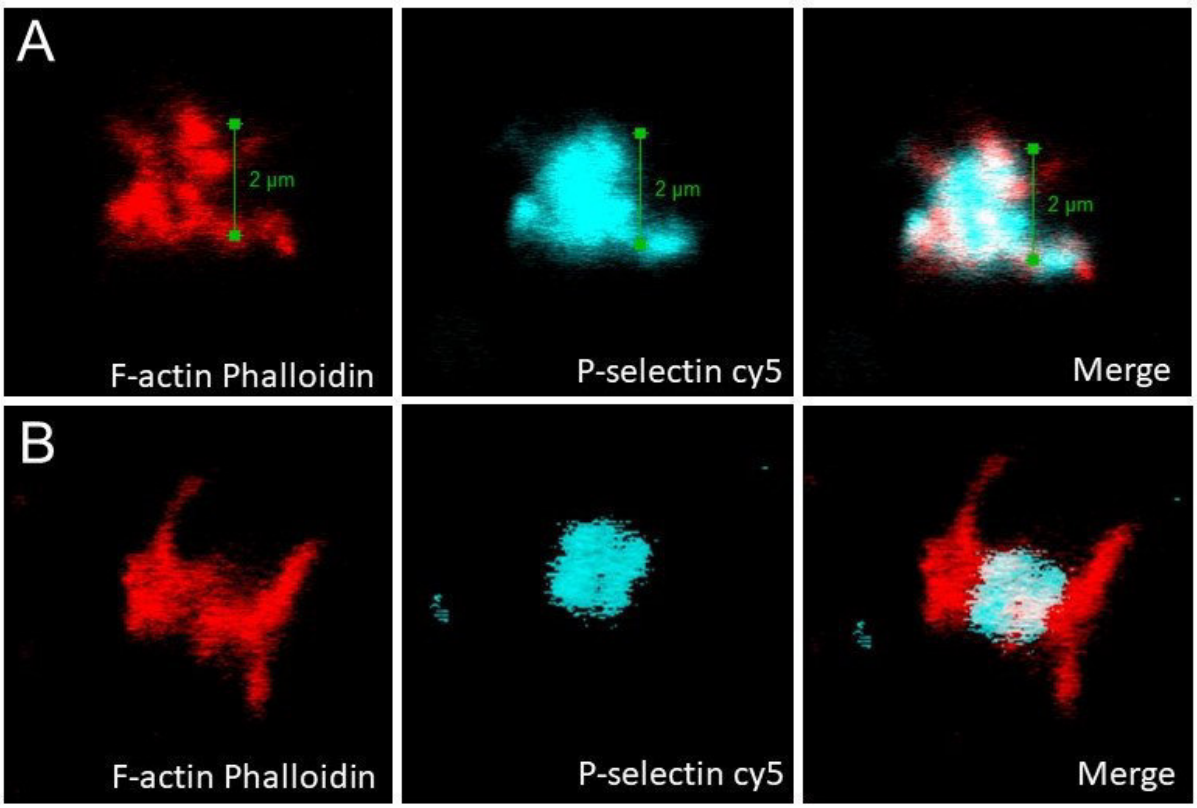


Figure 4.4.2. LSM in washed activated platelets. Platelets were isolated from whole blood of control mice (non-transplanted) and stained for F-actin with phalloidin. As alpha granule markers, the platelets were stained for **P-selectin (A)** and **von Willebrand Factor (B)**. LSM 100x. Washed platelets were activated with thrombin (0.2µM).

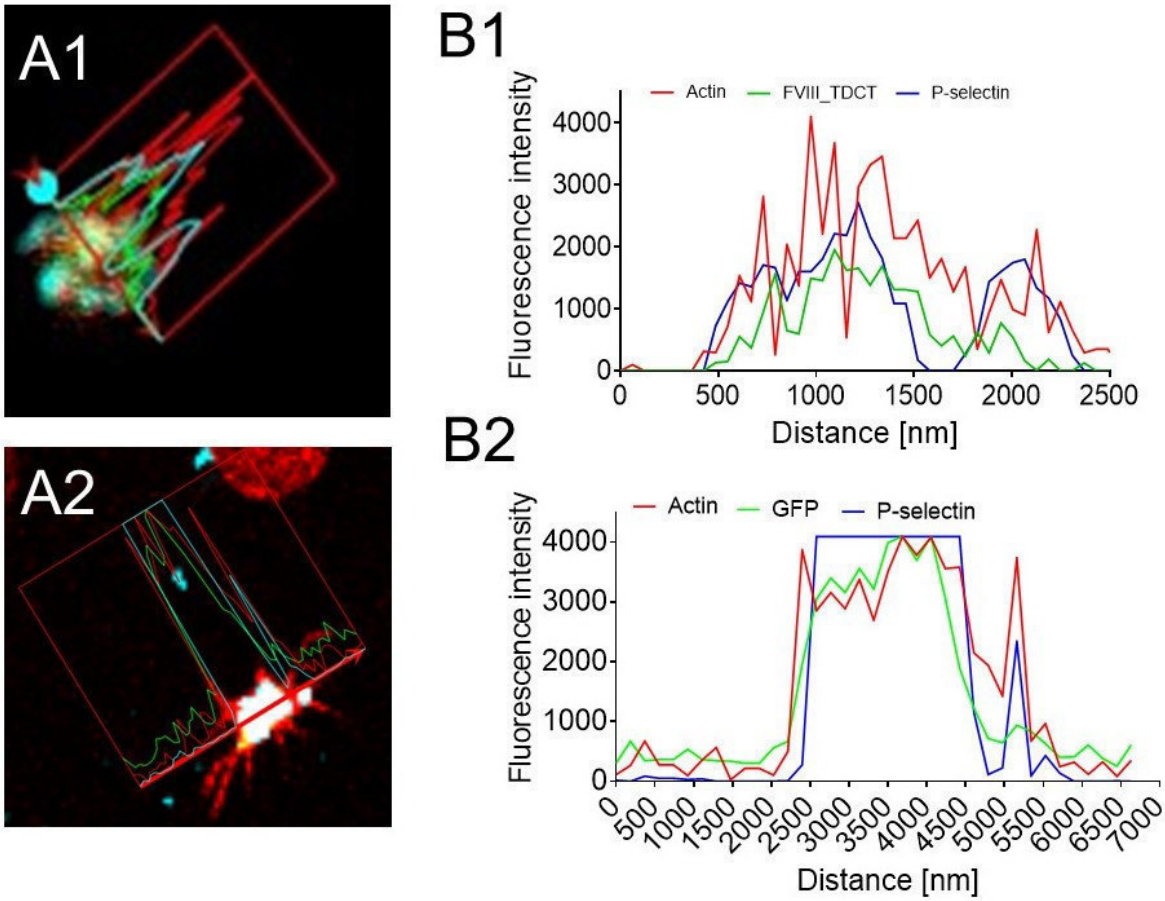


Figure 4.4.3. LSM in washed activated platelets. The picture shows the image of platelets isolated from whole blood of transplanted mice with the mPF4.hBDcoFVIII_TDCT targeting vector, referred as FVIII_TDCT (A1-B1) and the SFFV.GFP (referred as GFP) vector (A2-B2). Platelets were stained for F-actin with phalloidin (shown in red). Alpha granules were stained with a primary anti human/mouse CD62P (P-selectin) and visualized by using a goat anti mouse Cy5 (shown in turquoise). FVIII_TDCT was stained with a primary sheep anti human FVIII antibody labeled with FITC (shown in Green). The pictures represent an amplification of a selected region for a better observation of distribution and localization of the FVIII_TDCT and the d2eGFP signal. **Fluorescence intensity analysis (B1-B2).** Analysis of colocalization from the amplified region. The graphs show the overlapping of the fluorescence intensities from the alpha granule marker P-selectin (shown in blue) with the FVIII_TDCT or d2eGFP signal (shown in green). F-actin (in Red). Fluorescence intensities are measured per nanometer, along the marked line. LSM 100X.

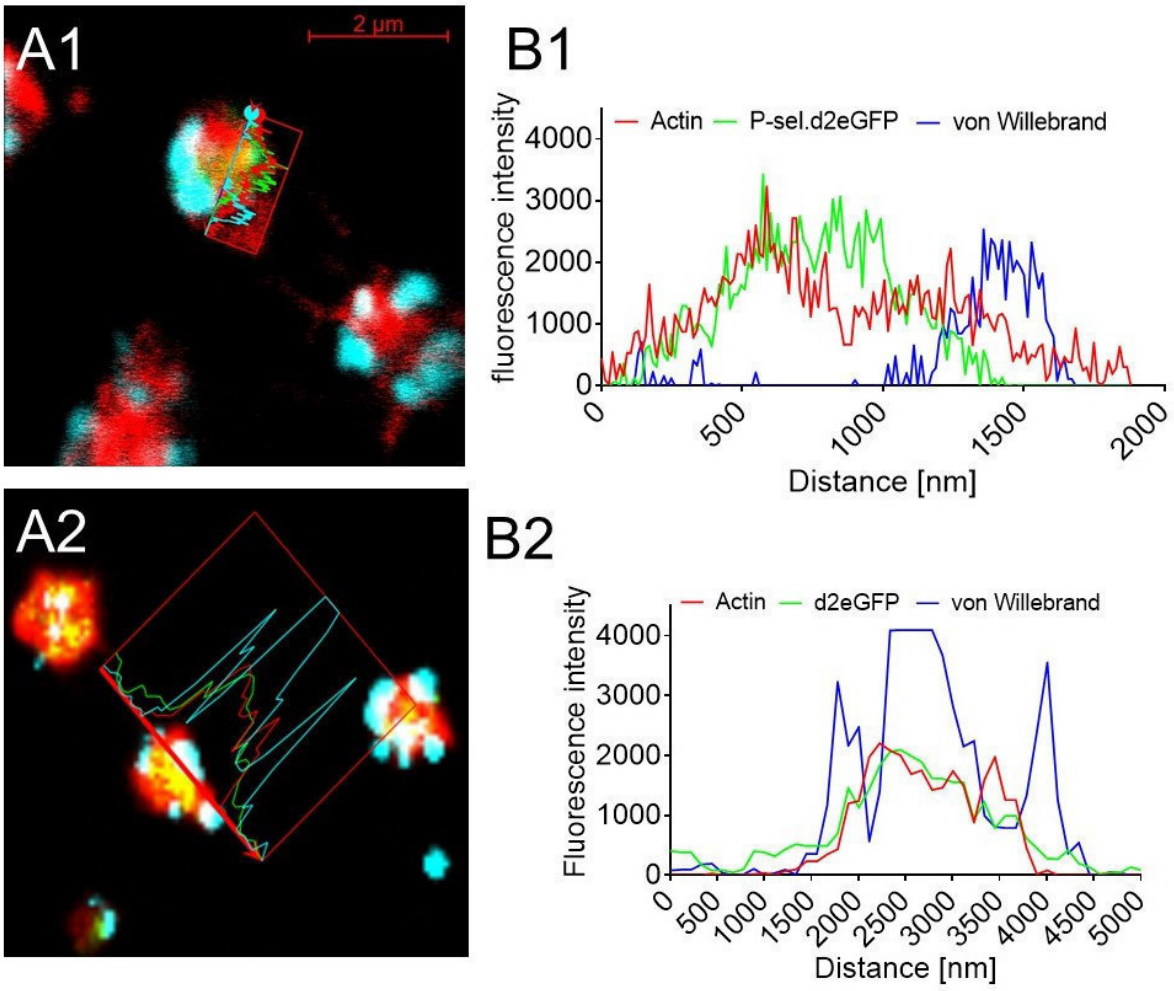


Figure 4.4.4. LSM of washed activated platelets. Example of platelets isolated from whole blood of transplanted mice with the P-Sel.sig.pep.d2eGFP.TDCT (referred as P-sel.d2eGFP) targeting vector and the non-targeting vector Pf4.d2eGFP (d2eGFP) **(A1-A2)**. Platelets were stained for F-actin with phalloidin (shown in red). Alpha granules were stained with a rabbit anti von Willebrand Factor antibody and visualized by using a Goat anti rabbit Alexa Fluor 647 secondary antibody (shown in turquoise). The pictures represent an amplification of a region selected for a better observation of distribution and localization of the d2eGFP signal. **(B1-B2) Fluorescence intensity analysis.** Analysis of colocalization from the amplified region. The graphs show the overlapping of the fluorescence intensities from the F-Actin (in red), d2eGFP/ P-sel.d2eGFP (shown in green) and von Willebrand Factor (shown in blue). Fluorescence intensities are measured per nanometer, along the marked line. LSM 100x

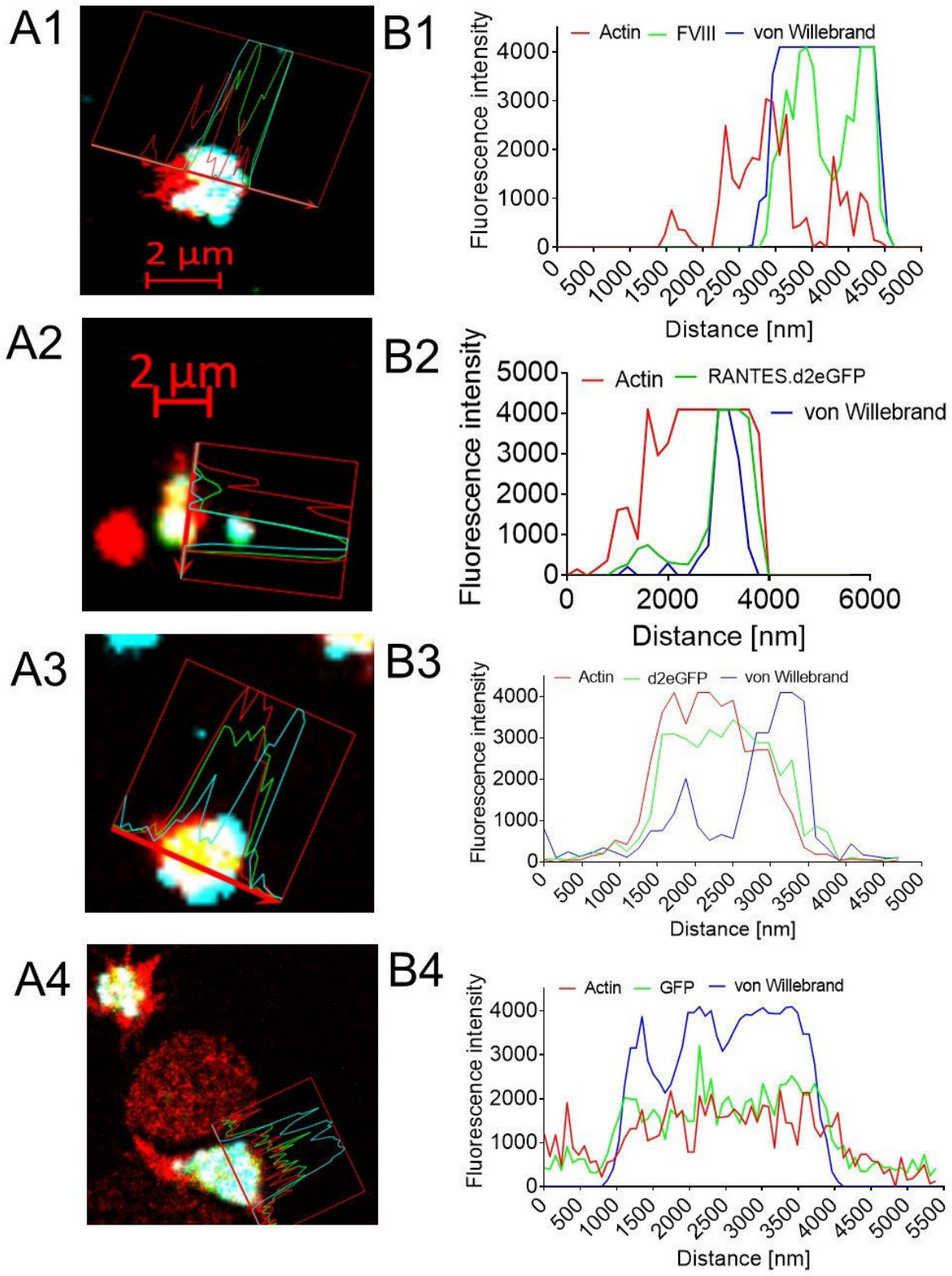

A1
B1
2 µm
Actin
FVIII
von Willebrand
Fluorescence intensity
Distance [nm]
A2
B2
2 µm
Actin
RANTES.d2eGFP
von Willebrand
Fluorescence intensity
Distance [nm]
A3
B3
Actin
d2eGFP
von Willebrand
Fluorescence intensity
Distance [nm]
A4
B4
Actin
GFP
von Willebrand
Fluorescence intensity
Distance [nm]

Figure 4.4.5. LSM of washed activated platelets. (A1-A2). Example of platelets isolated from whole blood of transplanted mice with the RANTES.d2eGFP (referred as RANTES.d2eGFP) targeting vector and the non-targeting vectors mPf4.hBDcoFVIII (shown as FVIII), Pf4.d2eGFP (shown as d2eGFP) and SFFV.GFP (named as GFP). Platelets were stained for F-actin with phalloidin (shown in red). Alpha granules were stained with a rabbit anti von Willebrand Factor antibody and visualized by using a goat anti rabbit Alexa Fluor 647 secondary antibody (shown in turquoise). The pictures represent an amplification of a region selected for a better observation of distribution and localization of the d2eGFP signal. Colocalization of the transgene with F-Actin is shown in yellow, colocalization of the 3 signals is shown in white. **(B1-B4) Fluorescence intensity analysis.** Analysis of colocalization from the amplified region. The graphs show the overlapping of the fluorescence intensities from the F-Actin (in red), d2eGFP/ FVIII (shown in green) and von Willebrand Factor (shown in blue). Fluorescence intensities are measured per nanometer, along the marked line. LSM 100x.

Due to the low chimerism in transplanted mice (between 2 to 20%, example in figure 4.4.1), also the percentage of transgene expression in most of transplanted mice was low (0.5-3 %). The low chimerism may be due to an insufficient conditioning before transplantation, leading to a poor donor cells engraftment; however, blood cells reconstitution was not impaired, as observed by the normal blood counts in transplanted mice (data not shown). Furthermore, 12 weeks after transplantation we could detect already transgene expression in genetically modified platelets, figure 4.4.1 (B). We also were able to activate genetically modified platelets with thrombin, as shown in figure 4.4.1 (C) by the detection of P-selectin. Therefore, we can conclude that the genetic modification did not impair hematopoiesis and that genetically modified platelets were able to express the transgene and can be normally activated.

The transmembrane granular targeting by the TDCT domain in the vectorsGFP_TDCT and FVIII_TDCT, should colocalize GFP or FVIII to the alpha granule membrane in platelets, as previously shown in MK. After activation, the signal should be translocated to the cell surface of platelets.

We found that even when the evaluated 3 markers (FVIII_TDCT, P-selectin and F-actin) remains within the cellular compartment after platelet activation (because there are not secreted), targeted FVIII colocalized more with the transmembrane granular marker P-

selectin than with the intracellular marker F-actin (Figure 4.4.3 B1). The observed results coincide with what was observed from our studies in MK and would preliminary indicate, that targeted FVIII is translocated to the cell surface after platelet activation in the same way as the normal P-selectin protein would do. This is different to what was observed in platelets from mice transduced with the non-targeting Pf4.d2eGFP vector, where the d2eGFP was localized more with the intracellular marker F-actin (figure 4.43 A2-B2). To compare these results to what would be observed by using an intragranular marker, genetically modified platelets with the P-Sel.sig.pep.d2eGFP.TDCT were activated and stained for colocalization analysis using von Willebrand Factor as alpha granular marker. In this case, LSM analysis of the GFP_TDCT, referred as P-sel.d2eGFP in figure 4.4.4 A1-B1 (because besides containing the TDCT sorting signal, it also contains the P-selectin signal peptide), it showed no colocalization with the alpha granule marker von Willebrand factor in activated platelets. This is contrary to what was observed in MK where a clear colocalization with von Willebrand Factor was detected; nevertheless, this was expected since although in MK both proteins are located in the alpha granules, in the platelets, after cell activation GFP_TDCT it is translocated to the cell surface, same as the normal P-selectin protein, and therefore will not colocalize with the intragranular protein von Willebrand factor, because it is not secreted, in contrast to von Willebrand factor, which is released to the extracellular space from the cell after platelet activation. On the other hand, similar to what was observed in MK, we observed that non-targeted FVIII (figure 4.4.4 in A1-B1) colocalize in activated platelets with the intragranular protein von Willebrand Factor, due to their mutual binding, which modifies its cellular trafficking and therefore, its granular targeting and subsequent secretion is expected after platelet activation.

Likewise, digital images of LSM from activated platelets in mice transplanted with the RANTES.d2eGFP vector (figure 4.4.5 A2-B2) showed that targeted GFP (RANTES.d2eGFP), as previously observed in the MK, colocalize with the intragranular marker von Willebrand factor (vWF). This could be an indirect proof of GFP cargo release, since the von Willebrand factor is normally released after platelet activation. Analysis of GFP colocalization in activated platelets from non-targeting vectors Pf4.d2eGFP and

SFFV.GFP, indicate that after platelet activation, the localization and distribution of GFP from non-targeting vectors remain in areas where von Willebrand Factor is not detected, as shown in yellow in figures 4.4.5 A3-A4 and B3, B4, indicating with this, the GFP cytosolic localization. This is also corroborated by the colocalization of non-targeted GFP with the cytosolic marker F-actin (figure 4.4.5 B3-B4). It is also possible to observe less GFP colocalization with the von Willebrand Factor alpha granule marker, in comparison with the cells transduced with the RANTES.sig.pep.sort.sig.d2eGFP targeting vector, where a more clear and evident overlap and colocalization of the GFP with the von Willebrand Factor is observed (figures 4.4.5).

However, LSM analysis has some technical limitations and therefore assumptions from these experiments are limited Instead, the corroboration in functional tests like ELISAs and coagulation tests must be further performed. However, due to the small number of transgenic cells in my experiement the required tests could not be performed. Nevertheless, the initial results from colocalization analysis in genetically modified platelets corroborated what we observed earlier in the MKs . Therefore, we can conclude that we were able to generate genetically modified platelets that carried transgenic cargo within their granules without affecting their normal function.

Chapter 5

- Discussion
- Outlook
- Summary

„Man merkt nie, was schon getan wurde, man sieht immer nur, was noch zu tun bleibt. “

Marie Curie

5.1. Generating genetically modified platelets, safety considerations for gene therapy

In this study, we developed lentiviral vectors aiming for specific expression in megakaryocytes and platelets, by using previously described promoter fragments of the MK-specific genes hGP1BA, hGP6, hGP9, mPf4. We showed an unprecedented accurate, detailed and extensive characterization of these vectors in in-vitro and in-vivo models. These findings improved our understanding of the tissue specificity of the promoter fragments within the context of lentiviral vectors, as well as on their possible further use in future experimental therapies. These considerations bring us closer to prevent and avoiding off target effects when using specific promoter fragments. In addition, it also highlights and move forwards our understanding of a powerful approach of manipulating HSCs for the purposes of studying megakaryopoiesis and platelet production in vitro and in vivo. The importance of this study lies in the possibility of using lineage specific vectors, as an accurate and faster option to modulate gene function in vivo and in vitro; by using these vectors, it will be possible to overexpress or knockdown gene expression and with this, modulate platelet function. This offers a faster mechanism to develop disease mouse models for hematological disorders. Classical knockout or transgenic mouse models rely on the development of a new mouse strain which can be tedious and time consuming. An alternative approach is the genetic modification of hematopoietic stem cells (HSC) by retro or lentiviral vector transduction and subsequent transplantation into recipient mice. As these vectors stably integrate into the genome, they guarantee the long-term expression of a transgene in the hematopoietic system. The concept of retro/lentiviral gene transfer has also proven to be successful in clinical gene therapy trials for the treatment of monogenetic diseases with hematological phenotype such as primary immunodeficiencies (Aiuti et al., 2013; Cicalese and Aiuti, 2015).

On the other hand, nowadays the use of lineage-specific vectors has become more important in terms of the safety issues because this will reduce off-target effects by transgene expression. Without transcriptional control, any genetic modification at the hematopoietic stem cell level will express directly in the hematopoietic stem cells and all the blood cells derived from them. Unwanted expression in HSC can be therefore

considered a major problem, since this could have an effect on hematopoietic differentiation or even lead to transformation (leukemia). This is well recognized and in the field of gene therapy this phenomenon of transgene toxicity is termed "phenotoxicity" (Baum et al., 2003). In other studies, toxicity was observed after expression of therapeutic proteins in HSC; for example the subunits of the NADPH oxidase complex for the treatment of chronic granulomatous disease (Grez et al., 2011) and expression of the galactocerebrosidase (GALC) for the treatment of Globoid cell leukodystrophy (Gentner et al., 2010). Lymphocyte toxicity was also shown after expression of WASP for the treatment of Wiskott Aldrich Syndrome (Toscano et al., 2008) or expression of cytidine deaminase, an enzyme supplying drug resistance to cells (Lachmann et al., 2012). To address this question in our study, we expressed the thrombopoietin receptor Mpl by the mPf4 and hGP6 vectors and transduced Mpl-/- lin- cells and transplanted into Mpl-/- mice; we further compared the performance of these two vectors with vectors expressing Mpl from the PGK or the SFFV promoters. Mice expressing Mpl by these two ubiquitous promoters developed erythrocytosis and splenomegaly within the first five weeks after transplantation and had to be killed due to clinical symptoms. Mpl expression from the mPf4 and hGP6 vectors, however, increased platelet counts compared to uncorrected and transplanted Mpl-/- mice with no signs of adverse reaction due to Mpl expression. This experiment demonstrates that the MK-specific vectors, including the hGP6 vector, could induce phenotypical changes in platelets in vivo, could modulate megakaryopoiesis and more importantly, we could also show that unrestricted expression can cause severe problems in vivo. Therefore, in our study we supply further evidence supporting the concept that the ectopic expression can disturb normal hematopoiesis. To give an example of a potential application in the research field of platelet biology research, one could imagine studying secretory functions in platelets, by overexpression of proteins that can interact with the secretory pathway; in these cases, it would be of great importance not to disturb secretory processes in leukocytes as this would have immunological consequences in vivo. Therefore, a deep knowledge and characterization of potential off target expression from the lineage-specific vectors is indispensable for the establishment of new experimental therapies.

5.2. Targeting transgene expression to megakaryocytes and platelets by lineage-specific lentiviral vectors.

In vitro experiments

Already in the in vitro assays we could initially observed the significant advantages of using lineage specific vectors, when compared it with the non-targeting vectors, not just by showing differences in the folds increase of GFP expression after differentiation, but also in the higher promoter activity observed in later stages of megakaryopoiesis, demonstrating an indeed lineage specificity. In vitro assays in non-megakaryocytic cells (primary cells and cell lineage) showed lower off target GFP expression in these cells (in percentage and intensity), when comparing with cells transduced with the non-specific PGK vector. These vectors also significantly increased megakaryocyte production and maturation from Mpl knockout lineage marker negative cells after MPL gene transfer, demonstrating that physiological Mpl expression levels could be achieved.

In vivo experiments

The advantage of using lineage-specific lentiviral vectors was also evidenced in our in vivo models, showing after bone marrow transplants the lower off-target GFP expression not just in the leucocytes, but also from B-, T- and myeloid cells from the spleens of transplanted mice. We demonstrated that the *hGP6* promoter in the lentiviral vector conferred the highest specificity with adequate expression levels similar to the hPGK-vector in platelets or even higher in MKs. The truncation of the *hGP6* promoter into a shorter fragment of 350 bp (*hGP6s*) did not change its specificity or expression in MK. This is an important fact to consider, given the size limitation of the expression cassette when using lentiviral vectors. We found that the major advantage of the hGP6 vectors is their low activity in the hematopoietic stem and progenitor cells (HSPC). Although the overall expression strength by the hGP6 vector was relatively lower than for example the mPf4 vector, it showed to expresses at the same level as the hPGK promoter, which is often applied in gene therapy studies and performs with sufficient strength to induce phenotypical changes (González-Murillo et al., 2010; Huston et al., 2011). The hGP6 vector also had a similar strength than the hGP1BA vector, a vector that was used in a previous study in our

group and it was able to correct the thrombocytopenia in Mpl-/- mice. We also found, that the mPf4-vector had the strongest activity in platelets and MKs followed by the hGP1BA-vector; however, both showed also considerable off target expression in leukocytes and HSPC. The hGP9-vector showed the weakest activity and no specificity although the promoter was described as MK-specific.

Furthermore, we applied in situ confocal microscopy to visualize expression in MK in the BM. To our knowledge this is the first use of this technique to compare expression strength and specificity from different lentiviral vectors in MKs in the BM. This allowed the direct quantification of expression in the MKs which is difficult to analyze by flow cytometry due to their low number and susceptibility to damage during isolation from the BM. The analysis of MK in situ demonstrated the expression from the mPf4 vector to be ~65% of that from the viral SFFV promoter which makes it a powerful tool to modify MK. However, our accompanying analysis by flow cytometry demonstrated strong activity of the mPf4 and hGP1BA vectors in HSC which would have been missed by microscopic analysis only.

Recent evidence has demonstrated a CD41+ population in the murine LSK cell pool. These cells were shown to be myeloid- or MK-biased. Expression analysis of endogenous genes in these cells clearly demonstrate the expression of platelet factor 4 in HSCs (Calaminus et al., 2012; Gekas and Graf, 2013; Miyawaki et al., 2015). Also the members of the GPIb-V-IX complex can be found in transcriptional profiles of HSC (Gekas and Graf, 2013; Cabezas-Wallscheid et al., 2014; Miyawaki et al., 2015). It has been described that after inflammatory stimulation, the expression in HSC is further upregulated (Haas et al., 2015). In combination with our own analysis, which also confirmed the endogenous cellular mRNA expression of GP1ba, Gp9 and Pf4 in the HSPC in wildtype mice; the activity of the respective promoter fragments of these genes in this compartment was therefore not completely unexpected. We detected unexpected high expression of Pf4 cellular mRNA in CMP cells which was also not recapitulated from the *mPf4* promoter fragment. However, Gp6 was found not to be expressed in HSPC and the GP6 promoter fragment used in our study showed only very low activity in these cells.

Finally, we demonstrate that expression of a functional protein, the Thpo receptor Mpl, by MK-specific vectors can alter MKs and platelets phenotypes in vivo. We have to consider that MPL deficiency is a complex hematopoietic disorder, with a major negative effect in the hematopoietic stem cells, affecting no just platelet counts but also the process of hematopoiesis in general, a phenotype well recapitulated in the Mpl knock out mouse. (Kimura et al., 1998). In our experiments we could show the effects of using lineage specific vectors with different stem cell activity. In these experiments the mPf4-vector exhibited superior effects compared to hGP6 promoter fragments, due to its higher activity in MK and stem cells. However, this promoter also showed transient adverse reactions in one of the transplanted mice. The effect of the hGP6 promoter fragment was evidenced by the increased in the platelet counts after gene transfer, but in a relative lower range compared to mPf4 due to its lower activity in the stem cells, as expected. Additionally, we further demonstrate the detrimental effects of ectopic Mpl expression, from the ubiquitous hPGK promoter which has a similar strength than the hGP6, showing that uncontrolled expression of Mpl in hematopoiesis induces pathological erythroid hyperproliferation, as previously observed in one earlier study (Heckl et al., 2011). This agrees with the concept that ectopic expression of transgenes in hematopoietic cells, may cause adverse reaction also in the context of other hematopoietic lineages

This means that the use of the promoter fragments restricted to megakaryocyte and platelet specific expression should be adapted according to the differentiation stages where the effect is required. For effects at more undifferentiated stages or even at the HSCs level, promoter fragments like mPf4 and hGP1BA should be take into consideration, but in cases where a more restricted and specific platelet expression is desired, the hGP6 promoter fragments should be instead used. In this sense, the human GP6 promoter fragment (hGP6, -697 to +29) was already efficiently used to study gene function in vivo in one further study. In this case, hyperactive variants of Cdc42 and RhoA were overexpressed under the control of hGP6 using lentiviral vectors, in order to show that Cdc42 and RhoA act as a regulatory circuit in coordination with the GP1Bα signaling pathway to coordinate MK polarization and transendothelial platelet biogenesis in vivo (Dütting et al., 2017).

The new MK-specific lentiviral vectors will allow the modification of the MKs and platelets in a safer way, by transplantation of transduced HSC and can very flexibly be used to develop in-vivo mouse models by exchanging the transgenes or the genetic background of the target HSCs. In contrast to knockout mouse models, lentiviral gene transfer is especially well suited for the overexpression of genes but may also be employed for MK-specific knockdown, the delivery of Cre or other modifying enzymes.

5.3 Difference of expression regardless the similarities about transcription factor binding sites

Megakaryopoiesis is modulated by multiple extrinsic and intrinsic signals, as well as by complex networks pathways that regulate the differentiation of MK both positively and negatively. All these cellular changes are mostly controlled by transcription factors. Some of the reported transcription factors that influence the process of megakaryopoiesis include GATA-1, Friend of GATA-1 (FOG-1), GATA-2, FLI-1, PU.1, NF-E2, and RUNX1. Most of these important transcriptions binding sites are present within the selected sequence of the studied promoters, therefore, since MK-specific promoters share transcription factor binding sites (ETS, FLI1, GATA-1, SP1, PU.1 (Hashimoto and Ware, 1995; Bastian et al., 1996; Minami et al., 1998; Holmes et al., 2002; Wang et al., 2002; Szalai et al., 2006), it was surprising to see the differences in promoter performance when introduced into the lentiviral vectors. Especially when analyzing the GP9 promoter fragment used in our study, which did not confer any specificity. However, it is important to consider that in the context of lentiviral gene transfer, only fragments of the cellular promoters can be used due to the size limitation of the vectors, and therefore they will reflect only to a certain extend the activity of the original cellular promoters, as regulatory elements may not be included within the studied fragments. Therefore, this is a limitation that should be always considered when using cellular promoter fragments, and this fact highlights the importance of a previous characterization in terms of strength, target specificity and off target expression.

5.4. Other studies restricting protein expression

Previous studies have also used cellular promoter fragments and included them in viral vectors to restrict protein expression to specific cell types. In the megakaryocyte and platelet field however, before our study, the Glycoprotein alpha II beta (GPIIb murine (m) and human (h)) has been used for the development of megakaryocyte specific vectors, since it has been described that this promoter also contains multiple Ets and GATA binding sites directing MK-specificity (Uzan et al., 1991; Prandini et al., 1992; Prandini et al., 1996). Before our study, it was considered the promoter most well studied when aiming megakaryocyte specific expression; mostly from initial studies, coming from the work of Wilcox and collaborators, when transduced human CD34+ peripheral blood cells with a murine leukemia virus (MuLV) vector, controlled by the human integrin αIIb promoter (nucleotides −889 to +35) and analyzed the transgene expression of the platelet alloantigen PlA-2 form Pro33 (a part of the integrin β3 subunit), as well as the Escherichia coli β-galactosidase in the promegakaryocyte cell line (Dami) and CD34+ cells (Wilcox et al., 1999). Further studies aimed megakaryocyte specific expression as well by using this promoter, to express proteins like coagulation factor 8 (FVIII), achieving in some cases long-term FVIII expression and improving or inclusive correcting the hemophilic phenotype (Shi et al., 2007; Kuether et al., 2012; Du et al., 2013; Schroeder et al., 2014; Shi et al., 2014;). This promoter has also been used to express ectopic FIX protein in the platelets achieving correction of the Hemophilic B phenotype (Zhang et al., 2010; Chen et al., 2014), as well for the expression of some cell surface receptors like the human Integrin beta3 (using a gamma retroviral vectors) in CD34+ cells of Glanzmann patients, achieving an ex vivo phenotype correction (Wilcox et al., 2000). It has also been used to express the human GPIIb protein in a bone marrow transplantation model in αIIbβ3 deficient Dogs, showing an improve of the hemostatic function (Fang et al., 2011), as well as for the expression of the human GPIBA in DAMI cells and CD34+ cells (Shi et al., 2004), with detectable hGP1BA on the surface, and in bone marrow transplantations in a mouse model for Bernard-Soulier syndrome with a phenotype correction in 70% of the positive platelets (Kanaji et al., 2012). However, most of these studies have just characterized the promoter

fragments in the context of the viral vectors on the target cells or their progenitors, without showing in detail the probable off target expression in other cell types. Other promoters, like the human Glycoprotein V (GPV), a subunit of the platelet GPIb-V-IX, receptor of the von Willebrand factor and thrombin, and also specifically found in platelets and mature megakaryocytes, was also characterized. A -1413/+25 promoter fragment used in a luciferase reporter assay, showed promoter activity in Dami and HEL (a megakaryocytic and erythroid cell lines) but not in K562, HL60, or HeLa cells (non-megakaryocytic cell lines); detecting a putative enhancer region in the -1413/-903 segment containing GATA and Ets binding sites (Lepage et al., 1999). One year later, the promotor activity of a -481/+22 promoter fragment from the mouse GPV gene was also evaluated it in mouse bone marrow cells and the human DAMI cell lines, by using a luciferase reporter gene assay and green fluorescence protein (GFP), observing that the full -481/+22 fragment could drive promoter activity in human as well as mouse megakaryocytic cells. Likewise, further gene analysis revealed that the mechanisms for megakaryocyte-specific maturation-dependent regulation of the GPV gene transcription is highly conserved between mouse and human, shown by the detection of GATA and Ets motifs; and reporting with this, a functional conservation of the platelet glycoprotein V promoter between mouse and human (Sato et al., 2000). Another well described promoter fragment, is the one from the Thrombopoietin receptor, (MPL) (study from Deveaux et al., 1996) for human, and the (c-Mpl) for the mouse promoter (Sunohara et al., 2008), Identifying GATA1 and Ets binding sites for both promoter fragments; furthermore the c-Mpl promoter fragment was already used in 2 studies (Heckl et al., 2011) and (Wicke et al., 2010), to over express the murine receptor Mpl in Mpl-/- mice using Gammaretroviral and lentiviral vectors, showing the importance of using the lineage specific promoter to prevents toxicity of ectopic expression (Wicke et al., 2010), and to correct the thrombocytopenic phenotype in a Mpl-/- mouse model (Heckl et al., 2011).

5.5 Use of murine promoter fragments in human cells. Is it relevant for therapeutic purposes?

In our study we showed in the in vivo and in vitro experiments that human promoter fragments were able to express in murine cells and that murine promoter fragments also express in human cells. It is well known that gene expression is mediated initially by the initiation of transcription. For this to occur, DNA binding proteins, also known as transcription factors (TFs) or trans regulatory elements, must recognize short DNA sequences which are protein binding sites (referred as well as transcription binding sites or cis regulatory elements), mostly localized at the promoter region of genes and thus linked to transcriptional regulation. The specific recognition of this cis-regulatory regions is essential for correct gene regulation (Todeschini et al., 2014). The recognition of such DNA sequences is necessary for the binding of the TFs that recruit the transcriptional machinery during initiation of transcription. In most cases, the DNA binding sites required for the transcription and expression from these promoters are conserved between in the mouse and humans, and many TFs recognize similar consensus DNA-binding sites (Eeckhoute et al., 2009). The tissue specificity of transcription regulation in all organisms will depend on the ability of the TFs (or trans elements) to interact with these cis-regulatory regions (or CRRs) of DNA. The specific sequences within the CRRs which is recognized by the transcription factors are called response elements (REs). The ability of a TF to bind to a specific RE relies on biophysical interactions between the protein structure and the DNA. In this sense, an effective transcription reaction can only take place if all the required (cis and trans) factors cooperate and interact to recruit the whole transcriptional machinery (Levine, 2010). In contrast to what is observed in prokaryotes, eukaryotic TFs typically recognize shorter sequences and therefore clustering of sites is important to achieve specific recognition (Wunderlich and Mirny, 2009). This clustering often involves interactions with other TFs which also bind neighboring sites, as well the and the interaction with cofactors and proteins that bind to neighboring DNA/chromatin sites.

In this particular case, the binding sites presents in the majority of the studied promoters are specific for GATA-1, FLI-1, PU-1, and NF-E2, which in previous studies it

has been determined that the binding sites for these transcriptions factors are highly conserved between mouse and human (Fisher and Scott, 1998; Suske, 1999; Ferreira et al., 2005; Burda et al., 2010; Svenson et al., 2010; Lentjes et al., 2016), allowing by this transgene expression in an allogenic context. In this sense, MK-specific gene promoters have in common the presence of transcriptions factor binding sites for GATA-1, ETS-factors (Fli-1), SP1 and in some cases PU-1. So far there is no evidence that the regulation of MK-specific genes may be fundamentally different in mouse and human cells. In the GP6 promoter GATA-1 and ETS1 bind at position -176 and -45, respectively, both in the murine and human promoter. The region of 257 bp (-182 to +75) is 77% homologue between mouse and human (Furihata and Kunicki, 2002). Further elements upstream the core promoter contribute to transcription, including SP1 sites, and longer fragments have been shown to express at higher levels (Furihata and Kunicki, 2002; Holmes et al., 2002). Later, a hGP6 shorter promoter was tested in the study by (Ohmori et al., 2006) who selected the fragment based on the study by Holmes et al. However, the deletion of the region between -315 and -118 was shown to significantly attenuate promoter activity (Holmes et al), and therefore, we did not further reduce promoter size.

The human GP1BA promoter contains binding sites for GATA-1 and ETS1 at positions -93 and 150, respectively (Hashimoto and Ware, 1995a). These binding sites were found to be conserved between human and mouse (Kitaguchi et al., 1997). In the study of Hashimoto and Ware it was also demonstrated that the region between -253 to +330 had the strongest promoter activity and therefore we chose this region for our studies (present and earlier studies, (Heckl et al., 2011)). Furthermore, the functionality of the human GP1BA promoter in the mouse was demonstrated by (Ware et al., 1993), by expressing the human GP1BA by the human GP1BA promoter in a transgenic approach. They demonstrated MK restricted expression and faithful regulation of the human GP1BA promoter by the murine transcription factors in the murine environment. In the case of GP9 promoter, we tested murine and human fragments with similar length but decided to use the human promoter in the in vivo experiments as the expression levels were a bit higher than from the murine one, although both promoter fragments did not perform promising. The platelet factor 4

promoter contains binding sites for GATA-1 at position -30 and ETS1 at -51 and -73 which are, similar to the GP6 promoter, homologue between mouse and men (Okada et al., 2011). An additional conserved GATA-1 site at -132 was also described by (Hamlett et al., 2008). In our study, we investigated the murine Pf4 promoter, the sequence is therefore identical to the one in the Pf4-Cre mouse, although much shorter but covering the core promoter. Another but often employed Pf4 promoter for studies in mice, in fact, is derived of the rat genome (Ravid et al., 1991b; Nguyen et al., 2005). In our study, we utilized promoter fragments that were previously described by others, since We did not aim for the characterization of new promoter sequences. In most cases the human gene is better described than the murine. However, in the context of gene transfer to the genome, heterologous DNA sequences will be recognized by transcription factors irrespective of whether the sequence is copied from the murine or human sequence as the consensus sequences are well conserved and, consequently, murine transcription factors will also bind a human sequence in the mouse genome. Therefore, for therapy purposes, the use of allogenic promoters should not represent a risk, as long promoter activity has been corroborated in advance.

5.6. Effect of the vector copy number and vector integration site on the differential percentage and intensity of expression

The percentage of GFP positive cells depends on the transduction efficiency which is controlled by the multiplicity of infection (MOI) used during the transduction. With increasing MOI, also the number of integrated vector copies will increase. A transduction efficiency above 40% will typically be associated with >2 vector copies per genome (Kustikova et al., 2003). The percentage of GFP positive platelets in vivo is then further affected by the percentage of engrafted modified stem cells, therefore 100% genetically modified hematopoiesis after engraftment of ex vivo transduced HSC is usually not achieved. In our study, we aimed for a copy number below 5, thereby a gene marking of 20-60% is in the range of what we wanted to achieve. We assumed that a higher number of integrated vector copies will also affect the specificity of expression, as the genomic

location of the integration also impacts on transcription from the vector, especially if integrations take place close to transcriptional start sites of genes. To prevent the interference of the genome on vector expression, insulators or boundary elements may be also incorporated into the long terminal repeats of the vector. This could be explored in further studies, but it was not addressed in the study presented here. In this sense, we consider that the observed results regarding strength and percentage of expression are due to the intrinsic promoter activity and not due to the direct influence of the vector copy number. This can be corroborated when we compare for instance the activity of hGP1BA promoter, with a vector copy number in their majority of 1 or 2 (just 1 mouse with 4) and a percentage of expression up to 90 % in the platelets and up 70% in the HSCs, to the activity of hGP6 (short fragment) with a vector copy number from 1 -4 and an expression in platelets almost up to 60 % and lower than 15 % in the HSCs. Even one of the mice from the long fragment of hGP6 got 8 vector copy and nevertheless in all cases the expression in HSCs was lower as 15%, which is something very good, since ensures lineage-restricted expression. On the other hand, if we analyze mPf4 promoter with vector copies up to 6, it shows percentage of expressing cells up to 80 % in platelets and up to 50% in the HSCs. In all cases however, it is then important trying to keep low vector copies to avoid affecting the specificity of expression. The possible activation of cellular pro-oncogenes as a result of clonal transformation, is a potential limitation in a therapeutic approaches when using integrating viral vectors. Lentiviral vectors have an important advantage over the gamma-retroviral vectors because they have the tendency to integrate into gene bodies and away from promoter or CpG islands. The use of the SIN configurations in lentiviral vector approaches and the use of physiological promoters, instead of the LTR's own enhancer/promoters, reduces but not abolish completely the risk.

5.7. Sorting Signal motifs.

Through our experiments in megakaryocytes we demonstrated the effect of the N-terminal fusion of the sorting signal of RANTES (peptide sequence VFVTRKNRQVC) on protein localization. In this regard we showed how the destabilized GFP variant, d2eGFP, was selectively targeted to the alpha granules of the transduced megakaryocytes; a significant difference in the GFP colocalization efficiency was detected when cells transduced with the targeting vector, RANTES.d2eGFP, were compared with the cells transduced with the non-targeting vector Pf4.d2eGFP (p-value <0.0001), in both P-selectin and von Willebrand Factor stained granules. More importantly, no significant difference was observed when the WCC values between the P-selectin and von Willebrand factor stained alpha-granules were compared, neither with the targeting vector (p-value 0.1593), nor with non-targeting vector (p-value 0.3007), reflecting no preference in the targeting or protein distribution within the alpha granules subpopulations in cells transduced with the targeting vector. Our study complements and agrees to the study of intragranular targeting performed by (Briquet-Laugier et al., 2004). Initially they handled the hypothesis that the signal peptide of PF4 is not sufficient to promote granule storage. Their in vitro experiments in the megakaryocytic cell lineage DAMI, primary megakaryocytes and AT20 cells (which is a cell line derived from mouse pituitary gland and exhibit regulated secretory granules containing ACTH and Willebrand Factor), demonstrated that PF4-GFP is targeted to ACTH-containing granules in AtT20 cells as well to alpha granules of megakaryocytes differentiated from CD34+ cells. This was not observed with the untargeted construct SP4-GFP (PF4 signal peptide + GFP). A lack of colocalization in secretory granules was also observed by analyzing eGFP alone. They used in this study however, the complete sequence of the platelet factor 4 cytokine and not a short peptide. They suggested however, the presence of a sequence within PF4 that mediates the targeting. They also reported that from all the megakaryocytic lineage they tested CHRF-288, Meg01, K562, CMK, HEL and DAMI, only the latter exhibited significant VWF expression and granule storage. In our in vitro experiments, we also tried to differentiate with phorbol esters (as recommended in publications) the human MEG-O1 and murine L8057 cell lines into megakaryocytes, with

no encouraging results; they were not able to express neither of the megakaryocytic markers, nor the von Willebrand Factor or P-selectin alpha granule markers (data not shown). Therefore, we decided to use only primary cells for the in-vitro analysis. However, this limitation on the availability of a good megakaryocytic cell line made it impossible to use selection markers for the establishment of a permanent and homogeneous megakaryocytic cell model for the study of the targeting vectors; this led to the limitation of heterogeneous short term cultures, since the cells did not maturate at the same time and not all the cell were transduced with the same efficiency. Nevertheless, regardless its disadvantages, primary cells are by far the closest and most precise cell model.

One year later, in 2005, a similar study, trying to identify the sequence that mediate PF4 targeting, reported the generation of PF4 mutants, fused to GFP in pcDNA3 expression plasmids and their evaluation in AT20 transfected cells by confocal analysis (El Golli et al., 2005). They were able to identify the 10 amino acids peptide sequence (41–50, LIATLKNGRK) responsible for the PF4 targeting. Alanine scanning mutagenesis of LIATLKNGRK narrowed the targeting sequence to LKNG; finding as well that LKNG is poorly conserved within the chemokine family. However, three-dimensional alignments of RANTES with the PF4 cytokine, together with the same region from another cytokine, the human α-granule chemokines Nap-2 (neutrophil-activating peptide), revealed that LKNG, described as a surface-exposed hydrophilic turn/loop, matched Nap-2 (LKDG) and RANTES (TRKN) peptides with similar molecular and structural features (even though when RANTES showed no homology in the sequence), (Malkowski et al., 1995; Mayo et al., 1995; Shaw et al., 2004). In this sense, these 3 cytokines share a β turn or reverse turn between two anti-parallel β strands. Moreover Nap-2 and RANTES peptides exhibited the same α-granule targeting efficiency than LKNG when SP-(LKDG)-GFP and SP-(TRKN)-GFP were expressed in DAMI cells. However, further studies or analysis of these 3 sorting signals in primary megakaryocytes or platelets, using lentiviral vectors were not performed.

From the structural point of view, the LKNG is located within the hydrophobic peptide LIATLKNGRKISL, which in native state forms a two-stranded antiparallel /β-sheet structure and turns into a reverse turn/loop in aqueous solution (Ilyina et al., 1994;

Zhang et al., 1994). This 3 motifs previously studied by (El Golli et al., 2005) LKNG,LKDG and TRKN, exhibit high hydrophilicity (due to the presence of three hydrophilic amino acids out of four), including some ionic residues such as K for LKNG, KD for LKDG, and RK for TRKN. Due to the positively charged (Lys and Arg), the LKNG is exposed at the surface of the PF4 molecule. In the PF4 molecule, all positively charged residues (Lys and Arg) form a positively charged ring within the tetrameric unit, in same way of clusters or charged domains along the PF4 sphere (Ilyina et al., 1994; Zhang et al., 1994). Likewise, the three-dimensional solution structure of cytokine RANTES was determined by (Chung et al., 2002). It was described as ellipsoid, constituted as a dimer at high concentration of cytokine. Each monomer folds to form a three-stranded β-sheet arranged in a Greek key motif, with an N-terminal extended region, a C-terminal helix and a smaller stable hydrophilic core, a reverse turn from residue 43 to residue 47, which connects the second strand to the third, running from Gln 48 to Ala 51. (see in figure 5.1). Interesting, the topology of the RANTES monomer is also very similar to what have been previously reported for other chemokines, like for example, the human NAP-2 (Malkowski et al., 1995). Interesting is however, that regardless the relevant sequence differences which generally is observed between the CC and the CXC chemokines, the topology of the structure of these two types of cytokines is conserved. Here we see for example that PF4 and NAP-2 as CXC cytokine, have a more similar reverse turn (LKNG / LKDG, respectively), compared with the one observed in RANTES as CC cytokine (TRKN). However, the physical and biochemical properties are highly conserved.

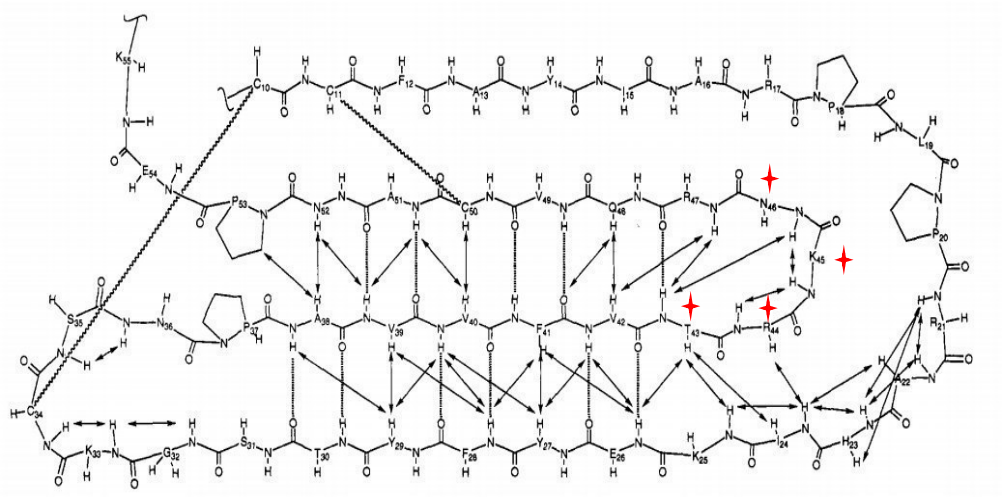


Figure 5.1. Representation of a monomer, antiparallel β-sheet structure of RANTES. Three strands of a β-sheet encompassing residues 26-30, 38-43, and 48-51 and a C-terminal helix (residues 56—65). Two disulfide bonds pair Cys 10 with Cys 34 and Cys 11 with Cys 50. At the center of the dimer, a small segment of antiparallel β-sheet is formed by residues 8-10 and 10'-8'. Intermonomer Hydrogen bonds exist between the NH of Cys 10 from each monomer to the backbone carbonyl of Thr 8 in the other monomer and between the NH of Cys 50 and the backbone carbonyl of Ser 5 (Chung et al., 2002).

Reverse turn or β-turns are thought to act likely as sites for initiation of protein folding. They are conformed by short-range interactions and bring together parts of the peptide chains that are more distant, allowing with this to direct subsequent folding events (Jaenicke, 1991; Madan et al., 2014). Since β-turns are mostly surface-exposed motifs, they are likely to participate in protein interactions, either related to ligand binding, molecular recognition, protein–protein as well as for protein–nucleic acid interactions. They are frequently sites of post-translational modifications as well, such as phosphorylation and glycosylation, which are used to enhance interactions (Schaeffer and Daggett, 2011). Given the similarity between these motifs, it is possible to take into consideration, the idea that these three motifs mediate the sorting of proteins under the same mechanism. However, the question whether these motifs bind to a specific sorting receptor for the targeting of proteins (Cool et al., 1997) or if another mechanism such as pH-dependent aggregation take place (Taupenot et al., 2005), still remains to be answered.

Comparing the sorting sequences of the alpha granule cytokines to what has been described for sorting signals of proteins in other cells, we see that neither the LKNG, nor the LKDG, TKNR, or the decapeptides LIATLKNGRK, VVFVTRKNRQ, VIATLKDGRK, resemble any of the structural sorting signals that have been described so far or before for other proteins, such as Pro-opiomelanocortin (Cool et al., 1995; Loh et al., 2002), chromogranin B (Chanat et al., 1993), prohormone convertase PC2 and furin (Creemers et al., 1996), pro-convertase PC5 (Bie, 1996), von Willebrand factor or even P-selectin. At the same time, carboxypeptidase E (CPE), which in certain cases can act as a sorting receptor (see below), it needs itself a sorting signal to mediate its own granular targeting and receptor function. This signal it has been described as a domain within the C-terminal 25 residues of CPE, mediating its functions as a signal for both lipids raft in the trans golgi network (TGN) association and the sorting of CPE to the regulated secretory pathway (Zhang et al., 2003). Additionally, VVFVTRKNRQ is also structurally and biochemically different from the 13-amino acid loop (hydrophobic acid) of the proopiomelanocortin, which is the ACTH precursor recognized by the sorting receptor CPE (Cool et al., 1995; Cool et al., 1997). Therefore, it is unlikely for CPE to be the sorting receptor for TRKN or its cytokine analogs because these motifs do not accomplish the requirements needed it for interaction with CPE. This agrees with the fact that in CPE-/- mice, the sorting of PF4 or any other alpha granule protein is not impaired (Methia et al., 1999). The true is, that the question whether this motif is engaged with a "sorting receptor" (Cool et al., 1997) or if interact through another sorting mechanism such as pH-dependent aggregation, still remains to be answered (Taupenot et al., 2005). However, glycosaminoglycans (GAGs) have been suspected to be involved in the process (Coombe, 2008). In this sense GAGs play a role as co-receptors for some cytokines like NAP-2, IL8 and PF4. In addition, GAGs are thought to be important for the localization of cytokines, both in the subcellular compartment by providing a mechanism for retaining chemokines facilitating the formation of chemokine gradients. (Handel et al., 2005; Mulloy and Rider, 2006), and extracellular compartment by means of their required interaction with GAGs for the cytokines in vivo function (Hamel et al., 2009)

The models proposed to explain the mechanisms for sorting of protein cargo and granular release by the regulated secretory pathway (RSP) and were based on observation in neuro-endocrine cells. Due to the similar RSP, and their ability of releasing secretory granules, some studies also have used non-megakaryocytic cell lines, like the AtT20 cells, which are derived from mouse pituitary gland and exhibit a regulated secretory granule containing ACTH. This cell line has been previously extensively utilized to study trafficking to intracellular storage organelles of membrane proteins, including the megakaryocyte membrane protein P-selectin, as well as secretory proteins such as VWF (Richardson, 1983).

Two models have been proposed for protein sorting: "**aggregation-mediated sorting**" and "**receptor-mediated sorting**". The aggregation-mediated model describes the mechanism that larger soluble proteins are probably packaged into α–granules by aggregation of protein monomers, such as Granin-family proteins; in this case, proteins such as chromogranin A (CgA) and B (CgB) aggregates within the acidic and high-calcium environment of the Trans Golgi Network (TGN). During this process, granin proteins start to surround and accumulate the proteins that will be sorted. The cargo is then loaded to the secretory granules (SGs) by the interaction of the disulfide loop at their N-terminal of the granin protein CgA and CgB to the budding granular membrane. The "loading" of the cargo occurs when the disulfide loop extends outside from the aggregate and binds to a supposed "loop receptors" on the secretory granule membrane (Taupenot et al., 2005; Takeuchi and Hosaka, 2008; Bartolomucci et al., 2011). Although this model still waits for further elucidation of more detailed mechanisms, large, self-assembling proteins such as von Willebrand Factor and multimerin (a Factor V binding protein) have been proposed to be sorted into immature vesicles by homoaggregation (Hayward et al., 1999; Huang et al., 2008).

During von Willebrand Factor targeting, vWF self-assembles into large homoaggregates that ultimately form tubular structures, occupying a distinct sub-compartment within **α**-granules, a process dependent on the presence of a clathrin coat and the adaptor protein AP-1 (Cramer et al., 1985; Lui-Roberts et al., 2005; Huang et al., 2008; Nightingale and Cutler, 2013). It has also been shown that heterologous expression of vWf can drive de novo formation of granules to recruit membrane proteins in cell lines that have a regulated secretory pathway (for example in AtT-20, HEK293, or RIN 5F cells), but not in those cells lines that lack such a pathway (CHO, COS, or 3T3 cells) (Wagner et al., 1991; Hop et al., 2000; Blagoveshchenskaya et al., 2002). Studies with von Willebrand factor evidencing the determinants regions governing the assembly of vWF multimers comes from the 80s, and the first evidence of existences from a possible sorting signal within the von Willebrand Factor were published in the 90s (Wagner et al., 1991). This sequence was identified in the pre-polypeptide region (VWFpp), containing the vWF signal peptide D1 and the D2 domain. This region plays a very important role in vWF biosynthesis by promoting interdimer disulfide bond formation, a process which takes place in acidic cellular compartments and its required for the multimeric conformation (Wagner et al., 1986; Wise et al., 1988). However, in 2000 it was shown that the signals for multimerization of vWF are different from the signals for trafficking of vWF to storage, evidencing that vWF storage and multimerization are 2 independent intracellular processes (Haberichter et al., 2000; Rosenberg et al., 2002). Later on 2003, two amino acids, 416 in VWFpp and 869 in the mature VWF molecule, were identified as critical for the granular storage of VWF (Haberichter et al., 2002; Haberichter et al., 2003; Haberichter et al., 2009). Wagner and colleagues suggested that this region may either contain a targeting signal or it may promote aggregation, which then enables the granule storage of the protein. This hypothesis was further supported by studies showing the targeting of ectopic proteins by their fusion with the pre-polypeptide region of von Willebrand factor such as FVIII (Du et al., 2013), α-chain of C3 (Haberichter et al., 2002), as well as the mediation of cotrafficking of ectopic proteins into secretory granules by their co expression together with the von Willebrand factor in heterologous systems IL8 (Romani de Wit et al., 2003), growth hormone (GH) (Datta et

al., 1999), P-selectin (Hop et al., 2000) and even FVIII as well, a process which has been described to be mediated by the the C1-domain of factor VIII, as essential in the assembly with von Willebrand factor and activated factor FIX (Rosenberg et al., 1998; van den Biggelaar et al., 2009; Ebberink et al., 2017). In our study we also were able that the human codon optimized B domain deleted FVIII was retargeted more efficiently in those secretary granules of MK that contained vWF.

In parallel with the aggregation process in the TGN and immature SGs, microaggregates of secretory proteins can also be tethered by transmembrane receptors to the membrane domains from the budding granules. In this model, also called "receptor mediated", other members of the granin family such as carboxypeptidase E (CPE) and/or secretogranin III (SgIII) function as sorting receptors at the membrane of the budding secretory granule in the Trans Golgi Network (Cool et al., 1997; Zhang et al., 2003; Takeuchi and Hosaka, 2008; Ji et al., 2017). These proteins have a high-affinity cholesterol binding domain. Since secretory granule membranes contain high levels of cholesterol, the protein cargo is loaded to the secretory granules by the interaction of the granin protein receptors with the cholesterol enriched domain or lipid raft from the budding vesicle (Tanguy et al., 2016). These "receptors" bind the cargo and CgA at a high-calcium and acidic PH in the TGN (Taupenot et al., 2005), bringing them to the cholesterol-rich, budding secretory granule regions. Proteins are then transferred from CPE to SgIII, and further to CgA. The CgA begins to form aggregates with peptide hormones with the increase of calcium levels in low pH. After cleavage of SgIII-binding region, CgA aggregates move from the periphery to inside the secretory granules to make a dense-core complex (Hosaka et al., 2005; Hosaka and Watanabe, 2010; Sun et al., 2013). One of the proteins shown to be sorted under this mechanism is P-selectin. First evidence showing that this adhesion protein follows the regulated secretion pathway mediated by a sorting signal came on the 90s (Disdier et al., 1992; Koedam et al., 1992). This sorting signal, named as cytoplasmic domain, contains the transmembrane domain and cytoplasmic tail (35 amino acids from 755 to 789). The Deletion of the last 23 amino acids (cytoplasmic tail composed by C1 and C2 region) of the 35-residue cytoplasmic domain of P-selectin resulted in the direct delivery

of this protein to the plasma membrane of AtT20 cells. Targeting of coagulation factors (FIX) have been also used to show the targeting efficiency of the cytoplasmic domain in AtT20 cells (Plantier et al., 2003). On the other hand, the replacement of the cytoplasmic tail of tissue factor, a plasma membrane protein, with the cytoplasmic domain of P-selectin, redirected the chimeric molecule to the secretory granules in AtT20 cells (Disdier et al., 1992). Generation of Tyr777>Ala, Tyr777>Phe, Gly778>Ala, Phe780>Ala and Leu768/Asn769>Ala/Ala mutants significantly decreased P-selectin targeting into the secretory granules of AtT20 cells (Modderman et al., 1998), identifying with this an atypical tyrosine-based motif within the C1 and C2 region of the cytoplasmic domain needed to mediate sorting. Additionally, the transmembrane domain has been shown to be necessary in the P-selectin cytoplasmic domain, as it was shown that just the cytoplasmic tail could not induce granular targeting (Fleming et al., 1998). Additionally, the removal of the cytoplasmic tail, showed no significant alteration in P-selectin alpha granule targeting in platelets from transgenic mice, carrying a truncated P-selectin, with just the transmembrane domain (Hartwell et al., 1998). All these results described a novel function for the transmembrane domain of P-selectin in enhancing the efficiency of granular targeting and implicating protein transmembrane domains in intracellular trafficking (Fleming et al., 1998).

Interesting is that the same amino acid sequence encoded by the C1 region (amino acids 763-772) was shown to be responsible for lysosomal targeting in CHO cells, since deletion of 10 amino acids from the cytoplasmic domain of P-selectin extended the half-life of P-selectin compared to the wild type, evidencing by this the role of this region as an element of control to regulate P-selectin expression at the surface of activated endothelial cells (Green et al., 1994). In endothelial cell for example, the sorting receptor of P-selectin appears to be the Adaptor Protein 3 (AP3); however in platelets this is not true and the receptor has not been confirmed yet (Daugherty et al., 2001); demonstrating that the same sorting signal may interact with different sorting machineries in a cell type dependent manner. All these issues make more difficult of course the study and prediction of sorting signals and sorting mechanisms, highlighting then the importance of making the targeting

studies in the correct target cell to avoid a possible misreading. This was one of the reasons why we decided to work specifically and uniquely in megakaryocyte and platelets, avoiding the uses of cell lines, regardless the fact that by working with primary cells we have to deal with expensive, short term and non-homogeneous cell cultures (since all the cell may not be transduced or equally transduced and most of the time selection markers cannot be used). In our study for instance, we could nicely showe and evidence the targeting efficiency of the cytoplasmic domain (conformed by the transmembrane domain and cytoplasmic tail, TDCT) for the targeting of a GFP variant, d2eGFP and the codon optimized B-domain deleted human coagulation factor 8 in megakaryocytes. In platelets we were able to show first evidences by confocal analysis as well, but this still needs confirmatory analysis, by means of functional test in platelets from transplanted mice.

All this differences in the structural and biochemical properties of the sorting signals as well in the mechanism of protein targeting and trafficking, give us an idea about the complexity of this mechanism, indicating that the controls which regulates this process may not be just cell type specific, or protein specific, but isoform specific as well (Bie, 1996; Sobota et al., 2006), depending on the function of the protein to be accomplished, since they can be sorted to different subcellular compartments, or different secretory granules. This do not exclude until certain extend homology in the mechanisms within the different cell types, due to the evidence of sorting ectopic protein in different cellular types; but it is a fact that should be consider at the moment of selecting a sorting motif and the cell model to be selected for the study as well. In the case of platelets this may have an impact on the subtype of alpha granule (in case of their existence) to be sorted or even in the type of cellular response to be secreted, if we assumed that every subtype of alpha granule would be differentially released, as assumed in some studies. For the targeting of proteins in platelets this may have an impact, since as described before, secretory alpha granules appear to be a heterogeneous group of secretion granules according to some studies (Sehgal and Storrie, 2007; van Nispen tot Pannerden et al., 2010; Battinelli et al., 2011; Chatterjee et al., 2011; Curotto et al., 2012) suggesting that differential packaging could support differential content and release of alpha-granule proteins. Some others however, indicate a

stochastic and random protein delivery to individual granules (Kamykowski et al., 2011). This may give us the hint, that what is important to be considered is not the complete homology or similarity in the single short linear sequences itself, but rather we should focus on the three-dimensional confirmation, which is more likely to be similar between certain groups of proteins which interact with the putative targeting machinery, as we previously saw in the case of soluble cytokines.

5.8. FVIII gene therapy approaches

The attempts to cure hemophilia through gene therapy for coagulation FVIII approaches, belongs to one of the most extended experimental studies and therapies in development phase in the field of monogenetic disorders. Optimization strategies have included promoter engineering to increase transcription, codon optimization of mRNA to improve translation, amino-acid substitution to promote secretion and delivery of the engineered coagulation factor through viral vector platforms (retroviral and Adeno-Associated vectors). However, concerns about transgene expression levels achieved in plasma or target cells, immune responses to both vector and transgene product, strategies for in vivo gene transfer and the risk of insertional mutagenesis, are still open questions and issues that are being tried to be answer and standardize in the last preclinical studies. Gene therapy has the potential to overcome some of the disadvantages associated with conventional protein replacement therapy for hemophilia A. On the other hand, with the appropriate vector, it could ensure long term FVIII expression, eliminating the need for repeated replacement therapies. However, FVIII protein is a particularly challenging target for gene therapy due to the length of its coding sequence, short half-life and strong immunogenicity, making it difficult to achieve sustained therapeutic levels of gene expression in vivo. Since FVIII is normally produced in the liver, clinical studies have supported the use of liver-directed recombinant adeno-associated virus vectors (rAAV) for the treatment of hemophilia B and hemophilia A (Manno et al., 2006; Yasumoto et al., 2013; Nathwani et al., 2014; Greig et al., 2016; Greig et al., 2017; Sun et al., 2017; Brown et al., 2018). However, two main challenge have slowed the AAV-FVIII vector

development for hemophilia A when compared to similar AAV-FIX vectors designed for hemophilia B. These obstacles include the limited DNA packaging capacity of the AAV for the large FVIII transgene size (CDS 7056 bp) (Lu et al., 2008; Wu et al., 2010; Nathwani et al., 2011; McIntosh et al., 2013; Hirsch et al., 2016) and the inefficient biosynthesis of human FVIII transgene products in heterologous target cells (Marquette et al., 1995; Kaufman et al., 1997; Summers et al., 2011; Fantacini et al., 2016). Due to the large transgene size of the B-domain deleted FVIII as well as the requirement for additional non-coding viral and gene expression regulatory control elements, rAAV-FVIII vectors routinely exceed its ideal length, resulting in suboptimal transgene packaging and delivery. The packaging capacity of AAV vectors is limited to 4.7–5.0 kb in length between the inverted terminal repeats (Grieger and Samulski, 2012). This make hard the AAV design for FVIII expression, due to the strong requirement for gene expression control elements to be as short as possible, while retaining at the same time an efficient hepatocyte-restricted transcription (Wu et al., 2010; Kyostio-Moore et al., 2016). Another problem of AAV vectors is the prevalence of neutralizing antibodies in the human population, which increases the risks of immune reaction against the vector decreasing its efficiency. From the therapeutic point of view, with the AAV vectors, FVIII overexpression would be mediated by the constitutive secretory pathway in liver cells, meaning that a general increase on the plasmatic FVIII levels would be required. This fact would represent an issue in patients with inhibitors or a risk for its development.

All these limitations found in the gene therapy approaches with AAVs, increased the development of the lentiviral vector gene therapy platforms for the correction of the FVIII deficiency. Lentiviral vectors are capable of transduce a wide variety of dividing and non-dividing cells with high efficiency, especially terminally differentiated and highly specialized tissues without affecting the normal function of transduced cells (Torashima et al., 2006; Meunier and Pohl, 2009; Escors and Breckpot, 2010; Merten et al., 2016; Naldini et al., 2016; Counsell et al., 2017; Milone and O'Doherty, 2018). Stable and long-term expression of the transgene can be achieved since lentiviral vectors can integrate into the host genome. Moreover, lentiviral vectors can carry efficiently a larger transgene sequence

(up to 10 kb), increasing and extending their application in gene therapy since most of the currently transferred genes can be cloned into lentiviral vectors (Kumar et al., 2001; Cooray et al., 2012). Further advantage relies on their safety of minimal immunogenicity and very rare cases of immune reaction in the host. (Milone and O'Doherty, 2018). In terms of FVIII transgene expression, most of the studies relies on the FVIII expression in the hematopoietic system, due to the efficiency in cell transduction and relatively easy cell manipulation, not during their isolation but during their genetic modification and reinfusion into the patient as well. This modality of cell therapy offers the advantage of in most cases no need for suitable a donor, since autologous cells of the patient would be used. So far, many preclinical studies have developed different strategies to overcome FVIII deficiency, but none has been stablished as standard gene therapy. One of the major issues in FVIII gene therapy is the prevention of anti- FVIII antibodies (inhibitors). Patients with hemophilia A are commonly treated with the FVIII protein replacement therapy. However, approximately 20–30% of severe hemophilia patients will develop antibodies against FVIII. Inhibitors are usually polyclonal IgG with specificity against various epitopes on FVIII, especially the A2, A3 and C2 domains. Because the inhibitors are usually constantly present in excess of FVIII in the plasma, they can partially or completely neutralize FVIII molecules, resulting in severe bleeding episodes, by impairing the activity of the transfused or overexpressed FVIII. Therefore, FVIII engineering has being carried out since initial studies in an effort to avoid this event. On the other hand, the use of HSCs as target cell population for genetic modification may prevent inhibitor formation to transduced FVIII by induction of immune tolerance.

Within the hematopoietic system platelets stand out as one of the most interesting cells to use for gene therapy in the treatment of hemophilia. In this sense, many studies have advocated on exploring and developing different strategies for FVIII expression in platelets, due to the advantages of being a cell that directly participates in the process of coagulation. On the other hand platelet-targeted gene therapy have shown to restore hemostasis with the induction of immune tolerance mediated by $CD4^+$ T cells (Chen et al., 2017b), additionally it has also been shown that transgenic mice expressing up to 30-

fold higher platelet-FVIII levels (more than required to restore hemostasis in hemophilic mice) restored coagulation and platelets were neither hyper-activated nor hyper-activatable upon agonist activation. Moreover, in resting state or under prothrombotic conditions induced by lipopolysaccharides-mediated inflammation, supratherapeutic levels of platelet-FVIII did not appear to be thrombogenic (Schroeder et al., 2014; Baumgartner et al., 2017). Overexpression of platelet- human B-domain-deleted FVIII (hBDDFVIII) under the control of the platelet-specific alphaIIb promoter in platelets of hemophilic ($FVIII^{null}$) mice in the presence of inhibitors was stored in platelet granules and released at the site of activation, correcting the bleeding phenotype (Shi et al., 2006; Shi et al., 2007). Similar results were observed by Ohmori and collaborators when using a simian immunodeficiency virus-based vector harboring GPIb promoter to direct FVIII expression in platelets from transplanted mice (Ohmori et al., 2008) and developing megakaryocytes and platelets from transgenics mice (Yarovoi et al., 2003) driven by the hGP1BA promoter. Moreover, Lentivirus-mediated platelet-specific FVIII expression improves hemostasis in preimmunized $FVIII^{null}$ mice, evidenced by long-term FVIII expression without developing anti-FVIII memory response and survival after tail clip test (Kuether et al., 2012). In another study, it was shown that hematopoietic stem cells transduced with lentiviral vectors encoding FVIII fused to the von Willebrand Factorpropeptide-D2 domain to transport FVIII into α-granules driven by the ITGA2B gene promoter, prevents severe bleeding episodes in dogs with hemophilia A for at least 2.5 years after transplantation regardless the low FVIII: C expression (0.2-1.3 U FVIII:C per dog) (Du et al., 2013). It is also important to consider that the lack of spontaneous formation of inhibitory antibodies to human FVIII is not necessarily evidence of immune tolerance induction, but rather may be the direct consequence of 'hiding' the FVIII within platelet α-granules. Furthermore, the sub-myeloablative condition used to create a niche in the bone marrow for the engraftment of the lentiviral vector-transduced HSCs may play a role towards the inability to detect inhibitory antibodies to human BDDFVIII. Other studies have employed porcine FVIII for BMT in hemophilia A mouse model (Johnston et al., 2013) and the canine FVIII (Greene

et al., 2014), shown to be more active than the human FVII but with more apoptotic effects in the megakaryocytes.

Some other studies, in an effort to avoid patient conditioning for bone marrow transplants, have tried the development of another alternatives for gene transfer. To circumvent this issue, Ramezany and collaborators adapted a non-myeloablative conditioning regimen and directed factor VIII (FVIII) protein synthesis to B lineage cells using an insulated self-inactivating (SIN) simian immunodeficiency virus (SIVmac1A11)-derived lentiviral vector containing an immunoglobulin heavy chain enhancer-promoter. The idea behind was to use the secretory machinery of transduced naive B cell to induce secretion of small amount of FVIII (to induced tolerance and avoid inhibitor development) after reinfusion, followed by production of sufficient amounts of transgenic FVIII protein for a therapeutic effect, after delivery of small numbers of genetically-modified HSCs via a "mini-transplant" procedure. The study showed that transplantation of lentiviral vector-modified HSCs resulted in therapeutic levels of FVIII (showing correction of hemophilic phenotype after clot formation assays and survival after tail clipping) in the circulation of all transplanted mice up to 6 months after transplantation, with induction of a low anti FVIII antibody responsiveness after challenge with recombinant FVIII. Immunostaining of spleen cells showed that the majority of FVIII was synthesized by B220+ B cells and CD138+ plasma cells. Therapeutic levels of FVIII could be transferred to secondary recipients by bone marrow transplantation, confirming gene transfer into long-term repopulating HSCs (Ramezani et al., 2011). The recently developed novel methodology of direct intraosseous (IO) delivery of LVs showed efficient transduction of bone marrow cells, generating high levels of transgene expression in HSCs. IO delivery of E-F8-LV (a FVIII fused to polyethylene glycol (PEG)) and using the ubiquitous EF1α promoter, generated initially therapeutic levels of FVIII, however, a strong anti-FVIII antibody responses was detected in the circulation, neutralizing functional FVIII activity. In contrast, a single IO delivery of G-FVIII-LV (a FVIII fused to a monomeric Fc fragment of immunoglobulin G) using a megakaryocytic-specific GP1bα promoter achieved platelet-specific FVIII expression, leading to a persistent and partial correction of Hemophilia A in

treated animals. (Wang et al., 2015). These findings further support the benefits of using lineage-specific promoter and the use of specific target cells and platelets as an ideal FVIII delivery vehicle, as FVIII is stored in α-granules, is protected from neutralizing antibodies and during bleeding, activated platelets locally excrete FVIII to promote clot formation (Miao, 2016). However, in all this studies FVIII would be secreted to the extracellular space as a soluble protein. additionally, the expression of soluble FVIII may represent either a risk in patients with FVIII inhibitors or a risk for the patient to develop anti-FVIII antibodies, since some of the secreted FVIII may still be available in the plasma once the coagulation process is concluded. The fact that in some of the studies anti-FVIII antibodies have not been detected during the investigation, does not exclude the fact that in patients a long-term risk exists.

In comparison to the targeting approaches previously described, in our model, the need to achieve increases of FVIII plasmatic levels is not required to be considered, since FVIII would be stored on the secretory granules and selectively secreted on a focalized manner as a transmembrane protein. On the other hand, translocation of FVIII to the platelet cell surface as exposed coagulation factor avoids possible plasmatic soluble FVIII that could potentially give rise to an anti-FVIII antibody response and development of inhibitors. Something important and considered in previous studies, it was to address the effectivity of clot formation and to find possible alterations in genetically modified platelets expressing FVIII. In this sense, a study performed by Neyman and collaborators showed that in tests of clot formation after laser injury, the platelet-FVIII (pFVIII) is at least as effective as infused FVIII and plasmatic FVIII. They observed that platelet-delivered FVIII is more effective in correcting the bleeding events in $FVIII^{null}$ mice than an infusion of an equal dose of human FVIII, with an immediate accumulation of platelets. Likewise, platelet-FVIII improved both arterial and venule fibrin generation with an overall improvement in both vessels comparable to that, observed after a 15% human FVIII infusion, regardless some temporal and spatial differences observed in fibrin and platelet accumulation within clots, depending on how FVIII was delivered (Neyman et al., 2008).

5.9. What still needs to be done……

In our study with the targeting vectors,and based on the results observed in the confocal microscopy, we could show that we can target proteins into the secretory alpha granules of megakaryocytes and platelets. However, it is important to keep in mind that for the further development and establishment of potential therapies, using platelets as a carrier cells for FVIII delivery, functional tests still remain to be performed in order to evaluated more carefully not just the targeting itself, but its possible effects on the function of the cells. For instance, as a first approach to further corroborate the targeting, we can analyze the targeting on megakaryocyte and platelets by electronic microscopy; due to its higher resolution it is possible to observe better the cytoplasmic compartment and the content of the alpha granules. For the transmembrane-alpha granule targeting it is important to verify and evidence the absence of the ectopic protein on the membrane of the resting platelets and its further exposition on the surface after platelet activation. This is a very simple procedure, since it is done by detection of the protein on the platelets by flow cytometry before and after activation. Some studies have shown that this cellular activation with thrombin activation it is also possible to do it in the megakaryocytes (Briquet-Laugier et al., 2004) (Cramer et al., 1993). Regarding the FVIII targeting model, further transplantation assays need to be performed, to show in flow cytometry analysis the translocation of the FVIII protein to the cell surface of the platelets. This can also be corroborated by performing ELISAs from supernatants of activated and resting platelets from transplanted mice, to show the absence of the FVIII as soluble secreted protein after activation of platelets. These results could be also compared with ELISAs from supernatants of platelets from mice transplanted with the Pf4.RANTES.d2eGFP vector, which target GFP as a intragranular protein. In this case, we should only be able to measure GFP only after platelet activation (because only after activation GFP would be secreted as soluble protein to the extracellular space). These two experiments would be the confirmation of the targeting and would show the differences in the targeting approaches. To evaluate the if the targeted-FVIII is functional, in vitro coagulation tests can be performed, since they have been already stablished in the mice (Palm et al., 1997; Lemini

et al., 2007). Likewise, genetically modified HSCs can be transplanted into a FVIII knockout mice model with and without FVIII-inhibitors to evaluate if the platelet-FVIII delivery induce the development of anti FVIII antibodies (to evaluate the safety), or if this ectopic FVIII is either neutralized or blocked due to the presence of the antibodies (to evaluate effectiveness). Detection and measurements of FVIII mRNAs by means of RT-PCR and or RT-qPCR would be also useful to corroborate FVIII expression in platelets.

5.10. Safety issues: Possible limitations that still need to be addressed

For gene therapy in platelets, there are some concerns arising from the ectopic expression of coagulation factors or any other protein in the secretory granules of platelets. In the case of the alpha granular targeting, it is not known whether the platelet-specific expression of a transgene may interferes with the expression of an endogenous protein; if well this question may represent a possible issue, this could be address for example by comparing the expression (by means of RT-PCR, q-PCR, western blots) of a platelet endogenous alpha granular protein to the ectopic protein. Likewise, it is also not known how many molecules of proteins can be load in an alpha granule. Taking in consideration the possible existence of a heterogeneous group of alpha granules, another important question would be whether the use of a particular sorting signal may determine or affect the type of granule where the ectopic protein would be targeted; likewise, it is also known if proteins are classified according to the sorting signal itself or if it is a function or protein type dependent process.

Regarding FVIII gene therapy, still remains the concerns whether platelet-derived FVIII or FIX will cause thrombogenesis. In 2bF8 or 2bF9 transgenic models, for example, all mice with platelet-FVIII, including mice with a normal level of murine plasma FVIII plus pFVIII, presented normal life spans with no evidence of health problems related to thrombogenesis (Shi et al., 2003; Shi et al., 2006; Shi et al., 2008). Other studies however, have shown that in the pFVIII/FVIII^{null} mice, the clots appear to be less organized and stable, and degranulation faster compared to that in WT mice. They also observed by comparing with the WT mice, that pFVIII/FVIII^{null} mice had a significant increase, of

approximately 2.5-fold in both arteriole and venule embolization rate per study. In this study however, in comparison with the platelet-FVIII mice, FVIII^{null} mice had a severe defect in both platelet-plugs and fibrin clot formation, for both arteriole and venule models. Moreover, regardless incremental infusions of human FVIII, even at greater than or at least equal to 120% antigenic levels, these mice showed incomplete corrections and offer the risk of anti-FVIII development, an effect which is not observed in platelet-FVIII mice. On the other hand, since in gene therapy it is desired to achieve a low vector copy number (lower than 2 to reduce the risk of insertional mutagenesis), it means that under this therapy conditions, it would be rare to achieve more than 30% of positive cells, decreasing with this a possible eventual embolization or pro-thrombotic effects. Nevertheless, studies that address the risk of embolization need still to be further carried out in large animal models of FVIII to see whether this issue is valid in a more clinically relevant models. For therapy purposes, here it would be important to highlight again then the need to develop a good and efficient in vitro model for platelet development, like for example iPSC, in order to address all these questions in a human system as pre-clinical models.

Despite similarities between the clots in the pFVIII/FVIII^{null} mice and the infused FVIII^{null} mice, pFVIII clearly corrects the time for fibrin clot development, which was not seen after human FVIII infusion. It is, however, important to consider, that these findings are based on studies using human FVIII to correct a bleeding diathesis in a murine model. Factors like the known shorter half-life of human FVIII in mice, the decreased specific activity of human FVIII in mice, differences in thrombin sensitivity as well as other cross-species differences may contribute to the limited efficacy of human FVIII in this model (Neyman et al., 2008). Another factor to be elucidated is whether platelet-derived FVIII could indeed induce an immune response; the development of inhibitory antibodies to exogenous FVIII is not only a severe complication already observed during FVIII infusion in hemophilia patients, but it is also a concern in gene therapy, since under this therapy also exogenous FVIII is delivered. However, FVIII or FIX storage in platelet α-granules when transgene expression is targeted to platelets, may limit immune exposure to exogenous FVIII, reducing the incidence of inhibitor development. In this sense, some studies have

shown that neither inhibitory antibodies nor non-inhibitory antibodies were detected in FVIII-lentivirus mediated platelet-directed gene therapy (Shi et al., 2007). Moreover, studies have shown that even with only 5% of FVIII-containing platelets, the bleeding phenotype could be significantly improved in hemophilia A mice with pre-existing anti-FVIII immunity (Shi et al., 2008), showing with this the advantages of using FVIII expressing platelets as delivery system for the correction of hemophilia. The use of heterologous FVIII for the correction hemophilia and the possible side effects has also been a question mark in the development of FVIII variants. For instance, platelets expressing the canine FVIII (pcFVIII) were shown to be more effective than the human FVIII (phFVIII) on the same conditions for restoring hemostasis, nevertheless the peaks of pcFVIII antigen levels were lower and were associated with greater megakaryocyte apoptosis than the phFVIII. These new insights suggest that pFVIII gene therapy strategies should focus on enhancing activity rather than antigen levels (Greene et al., 2014). The advanced self-inactivating design of lentiviral vectors and the differences in the tendency of integration site selection between lentiviral and gammaretroviral vectors may reduce the risk of insertional mutagenesis. Retroviral vectors tend to integrate preferentially into promoter-proximal regions, whereas lentiviral vectors tend to insert into open chromatin regions in gene coding regions and intronic sequences (Palma et al., 2005). Lately, drug-inducible suicide genes have been introduced into hematopoietic stem cells along with the transgene; this strategy allows the elimination of the geneticaly modified cells in case of cell transformation. As for any other gene therapy strategy, further characterization and continued vigilance regarding the insertional mutagenesis and the function of platelet-expressed FVIII are important for the development and evolution of lentivirus mediated platelet-derived gene therapy of hemophilia (Bonini et al., 2007; Zarogoulidis et al., 2013).

OUTLOOK

In this work we were able to present an exhaustive characterization of 6 important megakaryocyte-specific promoter fragments in lentiviral vectors, in terms of specificity and activity in megakaryocytic and non-megakaryocytic cells, as well in the hematopoietic stem cells and blood cell progenitors. In our experiments of Mpl overexpression, we could demonstrate the importance of using lineage-specific promoters in the context of bone marrow transplant, by showing the severe complications in two of the control mice transplanted with lin-BM cells transduced with SFFV.Mpl and PGK.Mpl vectors, such as severe erythrocytosis, leukocytosis splenomegaly and erythroid infiltrations in the liver. Significant increase of platelet counts in thrombocytopenic Mpl knockout mice, showed that genetic modification in cells (in this particular case HSCs and platelets) can be used to modify cell function and potentially apply it for therapeutic purposes.

Due to the wide range of physiological process in which platelets participate, diverse experimental therapies can be developed not just for the generation of long-term modified megakaryocytes and platelets, but also for short-term approaches, when the therapeutic effect is just temporally required. Both modalities offer the advantages of an autologous transplant, meaning no need for a suitable donor, making the therapy safer and easier to settle. For short-term approaches HSCs can be isolated genetically modified, differentiated into megakaryocytes progenitors (MPs), selected according to its surfaces markers and infused back into the patient. This approach allows to generate genetically modified megakaryocyte that would produce platelets that can be for a short period in circulation, since MP can be expanded ex vivo and safely administered as an autologous transplant into recipients patients (Bertolini et al., 1997; Drayer et al., 2002). This modality have also been tried as a heterologous transplants, by infusing megakaryocyte progenitors obtained from cord blood (Xi et al., 2013). In the murine context, it has been shown that platelets can recover rapidly in vivo by means of injection of expanded cells with TPO, SCF, FL, and IL-6 cytokine combination for expansion of Mk progenitor cells from CD34+ cells (Wang et al., 2018). This facilitates the study and research of physiological mechanisms,

development of new diseases models as well as to explore new possible therapeutic options. Both alternatives (heterologous and autologous transplantations) represent an important approach since it can be used not just as a method for the generation of megakaryocytes from hematopoietic stem cells used for rapid platelet reconstruction in patients with thrombocytopenia or with other disorders (Steward et al., 2005; Chen et al., 2009), but it can also be applied as a reinforcement treatment or support therapy for platelet reconstitution after chemotherapies, bone marrow and cord blood transplants. The possibility of reinfusion of genetically modified platelets, would add an extra value or new function to the transplanted cells, that can be positively used for the temporal supply or administration of a particular therapeutic agent. On the other hand, in a long-term basis, where HSCs are isolated, genetically modified and finally infused back to the patient, long-term expression of transgene can be achieved and can be potentially used for the cure of hematologic monogenetic diseases.

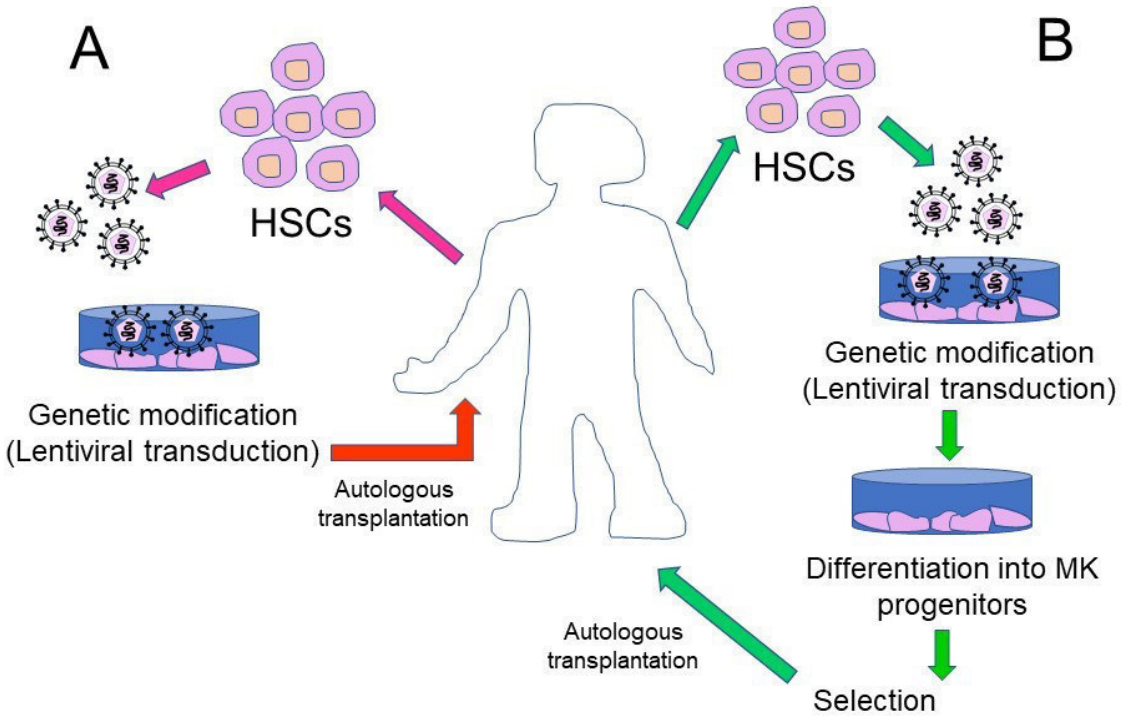


Figure 5.2 Hematopoietic stem cell therapy for the generation of genetically modified megakaryocytes and platelets. (A) Hematopoietic stem cell therapy for long-term transgene expression. Hematopoietic stem cells are isolated from the patient, cells are stimulated and transduced with the lentiviral vectors. Ex vivo genetically modified cells are finally reinfused in the conditionate patient for the stem cell transplantation. Hematopoietic cells are expected to be developed and transgene expressed in the target cells. In this case, the use of lineage specific promoters is important to reduced/avoid off-targeted transgene expression. (B) Hematopoietic stem cell therapy for the generation of short-term genetically modified megakaryocytes and platelets. In this model, HSCs cell are isolated from the patient, genetically modified by transductions, differentiated into megakaryocyte progenitors, selected by cell sorting, and reinfused back into the patient.

Experiments performed with targeting vectors showed that the inclusion of a targeting signal have indeed an effect in the protein trafficking, modifying thereby their transport and allowing a controlled storage in specific cellular compartments. On the other hand, as expected, we show that promoters have an effect on the cell lineage expression but no effect in the subcellular targeting. This is the first time that the human coagulation B-domain deleted FVIII is used to be targeted to the alpha granules membrane of megakaryocytes and platelets, following the P-selectin translocation pathway. Further applications of this targeting model can be potentially used to correct FVIII deficiency in patients with FVIII inhibitors. In this sense, by means of the fluorescence intensity analysis and digitalized-LSM images, we could demonstrate the intragranular and transmembrane targeting of the d2eGFP and coagulation FVIII, by fusing these proteins to a small peptide sequence from cytokine RANTES and the TDCT peptide of P-selectin, respectively. From the therapeutic point of view, it is a refinement and sophistication of transgene expression, this is particularly important in cases when the modulation of very specific and punctual physiological process like coagulation, angiogenesis, tissue repair, and inflammation take place. The genetic modification of megakaryocyte and platelets represent an interesting and important cell model-based approach, that will allow not just the development of new generation of cells with advanced functions, but it will help us to elucidate new mechanisms and pathways of important cellular processes, by modifying cell function and cell interactions.

Summary

Genetic Modification in Hematopoietic stem cells, using lentiviral vectors, to target protein expression to megakaryocytes and platelets

Lisette Johana Latorre Rey

October 2018

INTRODUCTION.

The blood is a fluidic tissue constituted by a cellular and a biochemical component, all immerse in a semi viscous liquid that freely circulate within the blood vessels in all the body. The cellular part is constituted by cells which differ in function, but share a common ancestor cell, also known as hematopoietic stem cell (HSCs), which differentiate itself into different hematopoietic blood progenitors in the bone marrow, by means of a process called haematopoiesis, to allow the constant and regulated blood production and reconstitution. The main groups of cells that conform the blood are: the red cells (or erythrocytes) in charge of the transport of oxygen, white cells (or leukocytes) whose main functions are focused on the development and execution of the immune response and involve cells like basophils, eosinophils, neutrophils, lymphocytes and monocytes. The third group of cells are the platelets or thrombocytes, which are developed from megakaryocytes (MKs) and whose function are more related to the maintenance of the coagulation system. More recently, they have been associated in process like vessel development and maintenance, antigen presentation and immune response. Due to their relatively easy access, blood cells are oft targets for genetic modifications. The use of blood cells as targets for genetic modification allows to deliver ectopic therapeutic proteins in a permanent or in a short term manner, depending on the life time of the cells and how differentiated or specialized are the cells used for introduced the genetic modifications. Platelets offer the additional advantage of being natural cell carriers, which release their protein cargos to the extracellular space in a controlled process following platelet activation. In this research project we aimed to generate genetically modified megakaryocytes and platelets, by targeting protein expression to their secretory alpha-granules to deliverectopic or therapeutic proteins, which would be stored and kept there until an external stimulus triggers platelet activation and platelet secretion takes place. During platelet activation, the therapeutic proteins would then be released to the extracellular space, either as a soluble protein together with the natural intragranular proteins or exposed as a transmembrane protein on the cell surface of platelets. For long-term approaches, genetic modifications must be introduced at the hematopoietic stem cell level. This brings the disadvantages that any new genetic information would be inherited by the daughter cells of all hematopoietic lineages derived from the genetically modified stem cells. Therefore, to avoid off target expression, transcriptional controls that ensure lineage-specific expression must be utilized. One way to ensure transcriptional control is the use of lineage- specific promoters.

AIMS

As first approach, we aimed to compare and characterize the lineage-specificity of expression and activity of six different promoter fragments in lentiviral vectors: the murine platelet factor 4 (mPf4) 1222 bp (-1074 to +148), human glycoprotein Ib alpha (hGP1BA) 595 bp (-265 to +330), a short and a longer fragment of the human glycoprotein 6 (hGP6 / hGP6s) 351 bp (-322 to +29) / 726 bp (-697 to +29), as well the human glycoprotein 9 (GP9) promoter 794 bp (-782 to -12). These promoter fragments were included as internal cellular promoters in self-inactivating lentiviral vectors (SIN), using an enhanced green fluorescent protein (eGFP) as gene reporter and

tested in vitro and in vivo. As ubiquitously expressing control vectors, we analyzed the expression from the hPGK promoter in the lentiviral vector or the viral spleen focus forming enhancer/promoter (SFFV) in the context of the LTR-driven gammaretroviral vector. As the second approach, for targeting of proteins to the secretory alpha granules of megakaryocytes and platelets, we followed two strategies: A) The sorting signal of the cytokine RANTES (Regulated upon Activation Normal T cell Expressed and Secreted) was fused N-terminally to the destabilized GFP, d2eGFP (RANTES. d2eGFP). This sorting domain is a small peptide sequence (VIATLKDGRK), which deliver the protein into the granules as soluble cargo. B) The transmembrane granular targeting sequence of P-selectin (the transmembrane domain and cytoplasmic tail (referred as TDCT) was fused to d2eGFP or the B domain deleted codon optimized human coagulation Factor VIII cDNA (referred as BDcohFVIII_TDCT or FVIII_TDCT). The TDCT will deliver the protein into the membrane of alpha granules. For the expression of the protein fusions, the SFFV promoter or the platelet factor 4 promoter were used as internal promoters in SIN-lentiviral vectors. These two strategies were tested in-vitro, from transduced differentiated megakaryocytes in liquid cultures, and in-vivo, by analysis of genetically modified platelets by means of Laser Scanning Confocal Microscopy (LSM) in colocalization analysis and fluorescence intensity analysis. The weight colocalization coefficient (WCC) was determined, by comparing the von Willebrand Factor and/or P-selectin as alpha granule marker, with either GFP and/or FVIII / FVIII_TDCT as targeted protein. For the analysis, transduced cells with the targeting vectors were compared with the cells transduced with the non-targeting vectors. Colocalization analysis was performed at the single cell level, based on LSM- digital images.

RESULTS: All vectors were produced with unconcentrated titers of $1x10^6$ - $1x10^7$ transducing particles/ml. To investigate the specificity of expression, lineage marker-negative cells from the BM (lin-BM) of C57BL/6 wild type mice were isolated, pre-stimulated for 24 hours and transduced twice with lentiviral vectors. Cells were then either differentiated to MKs in liquid cultures with 50ng/ml THPO, plated into semi-solid differentiated medium for colony assays, or transplanted into conditioned C57BL/6 recipient mice for the analysis of splenic blood cells, peripheral blood and bone marrow cells from transplanted mice. Reporter gene expression was measured by flow cytometry in the respective cell populations. Analysis of in vitro MKs showed that the percentage of GFP expression was higher after MK-differentiation (between 2.4 and 5-fold higher) compared to the non-differentiated transduced lineage marker negative cells. The strength of expression could also be estimated by the mean fluorescence intensity; showing that, except for the hPGK control vector where no differences was observed, the GFP intensity from MK-vectors increased with polyploidization, a measure of MK maturation at later stages of MK differentiation. Analysis of GFP expression in non-megakaryocytic cells from semi-solid cultures such as erythroid, monocytic- macrophages and granulocyte progenitors, revealed that in transduced cells with the lineage-specific vectors, the percentage of GFP was in all cases lower than with the hPGK control (<10%). Same results

were observed with transductions performed in primary mouse fibroblast and mixed cortical culture, confirming the significant strong preference of expression in the megakaryocyte lineage and a weak strength of promoter activity in off-target cells. Analysis of peripheral blood from transplanted mice showed that GFP expression was significantly higher in platelets, with a smaller background promoter activity in leukocytes and erythrocytes, in mice transplanted with the lineage-specific vectors. The highest expression was observed from the mPf4-vector, followed by hGP1BA, hGP6 and hGP6s vectors. However, higher strength of expression from Pf4 and hGP1BA vector was also associated with higher GFP expression levels in leukocytes, identifying the hGP6 vectors as the most restricted to the megakaryocyte and platelet lineage.

Analysis of peripheral blood from transplanted mice showed that GFP expression was significantly higher in platelets, with a smaller background promoter activity in leukocytes and erythrocytes, in mice transplanted with the lineage-specific vectors. The highest expression was observed from the mPf4-vector, followed by hGP1BA, hGP6 and hGP6s vectors. However, higher strength of expression from Pf4 and hGP1BA vector was also associated with higher GFP expression levels in leukocytes, identifying the hGP6 vectors as the most restricted to the megakaryocyte and platelet lineage. Analysis in bone marrow cells showed that hGP6-vectors have the lowest activity in the hematopoietic stem and progenitor cells (HSPC) with less than 10% of GFP positive stem cells. Surprisingly, the mPf4 and hGP1BA vectors were both highly active in the HSPC, in a range of 20 to 70% of GFP-positive cells. Supporting these results, mRNA expression analysis in HSPC evidenced a substantial mRNA expression of murine Pf4, human Gp1ba and Gp9. mRNA levels of Gp1ba and Gp9 were very similar, as would be expected by their function as subunits of the same receptor complex. mPf4 expression was 2.5-fold higher than Gp1ba expression, in contrast to murine Gp6 in HSPC, whose mRNA expression was almost null. GFP expression in cryosections of whole femora from mice transplanted with transduced BM cells, showed that in megakaryocytes the GFP was strongly expressed from the mPf4-vector and the hGP1BA-vector followed by the hGP6s-vectors (with almost no fluorescent signal detected in non-MK cells). The GFP expression from the hGP9 promoter was similar to the hPGK control vector; reflecting what was previously observed in peripheral blood. Mpl-deficient mice have a known defect in megakaryocyte maturation and are thrombocytopenic. Overexpression of the thrombopoietin receptor Mpl, under the control of MK-specific vectors in $Mpl^{-/-}$ lineage marker negative cells differentiated into MKs, revealed in in-vitro cultures, that maturation and polyploidization of Mks were recovered by the re-expression of Mpl, indicated by significantly more MKs with high polyploidy (>16n) at later stages of maturation, in comparison to the $Mpl^{-/-}$ MKs; as evidenced in cellular images from cytospins and flow cytometer analysis. These results were corroborated in in-vivo analysis, where Mpl-/- mice transplanted with lin-BM cells transduced with the mPf4.Mpl and hGP6.Mpl vectors, showed significantly elevated platelet counts compared to control mice transplanted with a GFP-encoding control vector (PGK-GFP).

Intragranular targeting

The targeting of proteins to the alpha granules of megakaryocyte and platelets revealed a significant difference using the weight colocalization coefficient (WCC) in MK transduced with the targeting vector RANTES.d2eGFP compared with the cells transduced with the non-targeting vector SFFV.d2eGFP (p-value <0.0001). The median of the WCC values observed from the RANTES.d2eGFP targeting vector was 0.8 (80 % of colocalization) with P-selectin stained granules, and 0.7 (70%) with von Willebrand Factor stained granules. In the case of the non-targeting vector SFFV.d2eGFP the median of the WCC observed were <0.3 (30%) both in P-selectin and von Willebrand Factor stained granules. These results agree with the dotted or granular GFP pattern observed in LSM-digital images from MK transduced with the RANTES.d2eGFP targeting vector, overlapping the signals emitted by P-selectin and von Willebrand factor stained granules. With the non-targeting SFFV.d2eGFP and mPf4p.de2GFP vectors, a more uniform and homogeneous GFP distribution was observed, extended to the nuclear and cytosolic areas. These results were corroborated in the fluorescent intensity graphs as well, evidenced by a clear overlapping of the GFP signal, emitted by cells transduced with the targeting vector RANTES.d2eGFP, with both P-selecting and von Willebrand Factor stained granules signal.

Transmembrane alpha granule targeting.

Taking advantage and based on the cellular trafficking of P-selectin during granular secretion, we fused d2eGFP and the B-domain deleted FVIII to the transmembrane domain and cytoplasmatic tail of P-selectin, as second approach for protein targeting. This targeting configuration would allow to expose the coagulation factor VIII in the membrane of the platelets as a cell surface protein, once the alpha granules have been released after platelet activation. We observed that the GFP signal of MK transduced with the P-selectin.d2eGFP fusion overlapped the signals emitted by P-selectin and von Willebrand factor stained granules, not just in LSM-digitalized images but in the fluorescens intensity analysis as well, indicating a clear signal of GFP colocalization. An evident signal overlap between the targeted FVIII (FVIII_TDCT) with the P-selectin / von Willebrand marker was observed as well, as expected. This colocalization of signals is evidenced by the strong dotted pattern in both GFP/ FVIII.TDCT with the P-selectin / von Willebrand alpha granule markers observed in images from LSM analysis and corroborated by fluorescence intensity analysis and WCC values. On the other hand, the subcellular localization of the d2eGFP, observed from the non-targeting vector mPF4p.d2eGFP, covers regions where no specific signals for the alpha granules markers were detected. A specific targeting of FVIII (expressed from the non-targeting vector, mPF4p.hBDcoFVIII) was observed, mostly in the von Willebrand factor containing granules. This was surprising since no targeting signal was included. However, it has been already described in previous studies that overexpression of FVIII in different cells lines in the presence of the von Willebrand factor, leads to a modification of the FVIII trafficking within the cell and the storage of the coagulation factor in the secretory granules as soluble protein.

Genetically modified platelets

Bone marrow transplantations into wild type C57BL/6J recipient mice were performed by transfusing lineage marker-negative cells transduced in vitro with the targeting vectors: mPf4.RANTES.d2eGFP (RANTES.d2eGFP), P-sel.Sig.pep.d2eGFP.TDCT (P-sel.d2eGFP), mPf4.hBDcoFVIII_TDCT (FVIII_TDCT) and the non-targeting control vectors mPf4.hBDcoFVIII (FVIII) / PF4.d2eGFP. 12 weeks after transplantation a leukocyte chimerism in transplanted mice between 2 and 20 percent was observed, and transgene expression in platelets varied in the range of 1 to 3 percent. Although the overall engraftment was low, transgene expression in some of the transplanted mice was enough to perform single cell analysis by LSM and obtain preliminary data about the targeting in platelets in vivo. Colocalization and fluorescens intensity analysis performed on activated platelets corroborated what was previously observed in in-vitro megakaryocytes. The no colocalization with the alpha granule marker von Willebrand factor in activated platelets, observed with the P-sel.d2eGFP and FVIII_TDCT vector, was a sign and an indication of protein translocation to the cell surface of platelets.

CONCLUSIONS AND DISCUSSION

In this work we were able to present an exhaustive characterization of 6 important megakaryocyte-specific promoter fragments in lentiviral vectors, in terms of specificity and activity in megakaryocytic and non-megakaryocytic cells, as well in the hematopoietic stem cells and blood cell progenitors. In our experiments of Mpl overexpression, we could demonstrate the importance of using lineage-specific promoters in the context of bone marrow transplant, by showing the severe complications in two of the control mice transplanted with lin-BM cells transduced with SFFV.Mpl and PGK.Mpl vectors such as severe erythrocytosis, leukocytosis splenomegaly and erythroid infiltrations in the liver. Significant increase of platelet counts in thrombocytopenic Mpl knockout mice, showed that genetic modification in cells (in this particular case HSCs and platelets) can be used to modify cell function and potentially apply it for therapeutic purposes. Likewise, experiments performed with targeting vectors showed that the inclusion of a targeting signal have indeed an effect in the protein trafficking, modifying thereby their transport and allowing a controlled storage in specific cellular compartments, which in absence of these signals, as observed with the untargeted d2eGFP, the proteins are ubiquitously expressed and distributed in the cytosol of the cell. On the other hand, as expected, we show that promoters have an effect on the cell lineage expression but no effect in the subcellular targeting. Likewise, this is the first time that the human coagulation B-domain deleted FVIII is used to be targeted to the alpha granules membrane of megakaryocytes and platelets following the P-selectin translocation pathway. Further applications of this targeting model can be potentially used to correct FVIII deficiency in patients with FVIII inhibitors. In this sense, by means of the fluorescense intensity analysis and digitalized-LSM images, we could demonstrate the intragranular and transmembrane targeting of the d2eGFP and coagulation FVIII, by fusing these proteins to a small peptide sequence from cytokine RANTES and the TDCT peptide of P-selectin,

respectively. Nevertheless, further experiments, including functional coagulation tests, ELISAs and electron microscopy should be performed to further confirm those findings. From the therapeutic point of view, this is a refinement and sophistication of transgene expression, that allows us to introduce new therapeutic proteins, whose storage and release can be tightly controlled. This is something particularly important in cases where the modulation of very specific and punctual physiological process like coagulation, angiogenesis, tissue repair, and inflammation take place. Due to the wide range of physiological process in which platelets participate, diverse experimental therapies can be developed not just for the generation of long-term modified megakaryocytes and platelets, by means of bone marrow transplants, but also for short-term approaches through the genetic modification of MK-progenitors, when the therapeutic effect is just temporally required. The genetic modification of megakaryocyte and platelets represent an interesting and important cell model-based approach, that will allow not just the development of new generation of cells with advanced functions, but it will help us to elucidate new mechanisms and pathways of important cellular processes, by modifying cell function and cell interactions.

Die Tat ist alles, nichts der Ruhm.

Johann Wolfgang von Goethe

References

Aikawa, M., Schoenbechler, M.J., Barbaro, J.F., and Sadun, E.H. (1971). Interaction of rabbit platelets and leukocytes in the release of histamine. Electron microscopic observations. The American journal of pathology *63*, 85-98.

Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M.P., Baricordi, C., Dionisio, F., Calabria, A., Giannelli, S., and Castiello, M.C., et al. (2013). Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science (New York, N.Y.) *341*, 1233151.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Alexander, W.S., Roberts, A.W., Nicola, N.A., Li, R., and Metcalf, D. (1996). Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. Blood *87*, 2162-2170.

Ambrosio, A.L., Boyle, J.A., and Di Pietro, S.M. (2012). Mechanism of platelet dense granule biogenesis: study of cargo transport and function of Rab32 and Rab38 in a model system. Blood *120*, 4072-4081.

Ambrosio, A.L., and Di Pietro, S.M. (2017). Storage pool diseases illuminate platelet dense granule biogenesis. Platelets *28*, 138-146.

Andrews, N.W. (2000). Regulated secretion of conventional lysosomes. Trends in cell biology *10*, 316-321.

Avanzi, M.P., Goldberg, F., Davila, J., Langhi, D., Chiattone, C., and Mitchell, W.B. (2014). Rho kinase inhibition drives megakaryocyte polyploidization and proplatelet formation through MYC and NFE2 downregulation. British journal of haematology *164*, 867-876.

Avecilla, S.T., Hattori, K., Heissig, B., Tejada, R., Liao, F., Shido, K., Jin, D.K., Dias, S., Zhang, F., and Hartman, T.E., et al. (2004). Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nature medicine *10*, 64-71.

Baccini, V., Roy, L., Vitrat, N., Chagraoui, H., Sabri, S., Le Couedic, J.P., Debili, N., Wendling, F., and Vainchenker, W. (2001). Role of p21(Cip1/Waf1) in cell-cycle exit of endomitotic megakaryocytes. Blood *98*, 3274-3282.

Ballmaier, M., Germeshausen, M., Schulze, H., Cherkaoui, K., Lang, S., Gaudig, A., Krukemeier, S., Eilers, M., Strauss, G., and Welte, K. (2001). c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. Blood *97*, 139-146.

Banerjee, M., Joshi, S., Zhang, J., Moncman, C.L., Yadav, S., Bouchard, B.A., Storrie, B., and Whiteheart, S.W. (2017). Cellubrevin/vesicle-associated membrane protein-3-mediated endocytosis and trafficking regulate platelet functions. Blood *130*, 2872-2883.

Banerjee, M., and Whiteheart, S.W. (2017). The ins and outs of endocytic trafficking in platelet functions. Current opinion in hematology *24*, 467-474.

Bartolomucci, A., Possenti, R., Mahata, S.K., Fischer-Colbrie, R., Loh, Y.P., and Salton, S.R.J. (2011). The extended granin family: structure, function, and biomedical implications. Endocrine reviews *32*, 755-797.

Bastian, L.S., Yagi, M., Chan, C., and Roth, G.J. (1996a). Analysis of the megakaryocyte glycoprotein IX promoter identifies positive and negative regulatory domains and functional GATA and Ets sites. The Journal of biological chemistry *271*, 18554-18560.

Bastian, L.S., Yagi, M., Chan, C., and Roth, G.J. (1996b). Analysis of the megakaryocyte glycoprotein IX promoter identifies positive and negative regulatory domains and functional GATA and Ets sites. The Journal of biological chemistry *271*, 18554-18560.

Battinelli, E.M., Markens, B.A., and Italiano, J.E. (2011). Release of angiogenesis regulatory proteins from platelet alpha granules: modulation of physiologic and pathologic angiogenesis. Blood *118*, 1359-1369.

Baum, C. (2007). Insertional mutagenesis in gene therapy and stem cell biology. Current opinion in hematology *14*, 337-342.

Baum, C., Dullmann, J., Li, Z., Fehse, B., Meyer, J., Williams, D.A., and Kalle, C. von (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. Blood *101*, 2099-2114.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. Proceedings of the National Academy of Sciences of the United States of America *89*, 2804-2808.

Baumgartner, C.K., Mattson, J.G., Weiler, H., Shi, Q., and Montgomery, R.R. (2017). Targeting factor VIII expression to platelets for hemophilia A gene therapy does not induce an apparent thrombotic risk in mice. Journal of thrombosis and haemostasis : JTH *15*, 98-109.

Bayer, M., Kantor, B., Cockrell, A., Ma, H., Zeithaml, B., Li, X., McCown, T., and Kafri, T. (2008). A large U3 deletion causes increased in vivo expression from a nonintegrating lentiviral vector. Molecular therapy : the journal of the American Society of Gene Therapy *16*, 1968-1976.

Bem, D., Smith, H., Banushi, B., Burden, J.J., White, I.J., Hanley, J., Jeremiah, N., Rieux-Laucat, F., Bettels, R., and Ariceta, G., et al. (2015). VPS33B regulates protein sorting into and maturation of α-granule progenitor organelles in mouse megakaryocytes. Blood *126*, 133-143.

Berger, G., Massé, J.M., and Cramer, E.M. (1996). Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. Blood *87*, 1385-1395.

Berman, C.L., Yeo, E.L., Wencel-Drake, J.D., Furie, B.C., Ginsberg, M.H., and Furie, B. (1986). A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. The Journal of clinical investigation *78*, 130-137.

Bertolini, F., Battaglia, M., Pedrazzoli, P., Da Prada, G.A., Lanza, A., Soligo, D., Caneva, L., Sarina, B., Murphy, S., and Thomas, T., et al. (1997). Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral blood progenitor cell transplant recipients. Blood *89*, 2679-2688.

Bessis, M. (1973). Living blood cells and their ultrastructure (Berlin, New York: Springer-Verlag).

Bie, I. de (1996). The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. The Journal of cell biology *135*, 1261-1275.

Biedler, J.L., Helson, L., and Spengler, B.A. (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer research *33*, 2643-2652.

Bizzozero, J. (1882). Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. Archiv f. pathol. Anat. *90*, 261-332.

Blagoveshchenskaya, A.D., Hannah, M.J., Allen, S., and Cutler, D.F. (2002). Selective and signal-dependent recruitment of membrane proteins to secretory granules formed by heterologously expressed von Willebrand factor. Molecular biology of the cell *13*, 1582-1593.

Blair, P., and Flaumenhaft, R. (2009). Platelet alpha-granules: basic biology and clinical correlates. Blood reviews *23*, 177-189.

Blostein, M.D., Furie, B.C., Rajotte, I., and Furie, B. (2003). The Gla domain of factor IXa binds to factor VIIIa in the tenase complex. The Journal of biological chemistry *278*, 31297-31302.

Bluteau, D., Lordier, L., Di Stefano, A., Chang, Y., Raslova, H., Debili, N., and Vainchenker, W. (2009). Regulation of megakaryocyte maturation and platelet formation. Journal of thrombosis and haemostasis : JTH *7 Suppl 1*, 227-234.

Bonfanti, R., Furie, B.C., Furie, B., and Wagner, D.D. (1989). PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells. Blood *73*, 1109-1112.

Bonini, C., Bondanza, A., Perna, S.K., Kaneko, S., Traversari, C., Ciceri, F., and Bordignon, C. (2007). The suicide gene therapy challenge: how to improve a successful gene therapy approach. Molecular therapy : the journal of the American Society of Gene Therapy *15*, 1248-1252.

Bordignon, C., Notarangelo, L.D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., and Servida, P., et al. (1995). Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. Science (New York, N.Y.) *270*, 470-475.

Boztug, K., Schmidt, M., Schwarzer, A., Banerjee, P.P., Díez, I.A., Dewey, R.A., Böhm, M., Nowrouzi, A., Ball, C.R., and Glimm, H., et al. (2010). Stem-cell gene therapy for the Wiskott-Aldrich syndrome. The New England journal of medicine *363*, 1918-1927.

Braun, C.J., Boztug, K., Paruzynski, A., Witzel, M., Schwarzer, A., Rothe, M., Modlich, U., Beier, R., Göhring, G., and Steinemann, D., et al. (2014). Gene therapy for Wiskott-Aldrich syndrome--long-term efficacy and genotoxicity. Science translational medicine *6*, 227.

Briquet-Laugier, V., Lavenu-Bombled, C., Schmitt, A., Leboeuf, M., Uzan, G., Dubart-Kupperschmitt, A., and Rosa, J.-P. (2004). Probing platelet factor 4 alpha-granule targeting. Journal of thrombosis and haemostasis : JTH *2*, 2231-2240.

Broos, K., Meyer, S.F. de, Feys, H.B., Vanhoorelbeke, K., and Deckmyn, H. (2012). Blood platelet biochemistry. Thrombosis research *129*, 245-249.

Brown, H.C., Zakas, P.M., George, S.N., Parker, E.T., Spencer, H.T., and Doering, C.B. (2018). Target-Cell-Directed Bioengineering Approaches for Gene Therapy of Hemophilia A. Molecular therapy. Methods & clinical development *9*, 57-69.

Brown, W.H. (1913). THE HISTOGENESIS OF BLOOD PLATELETS. The Journal of experimental medicine *18*, 278-286.

Bugert, P., Dugrillon, A., Günaydin, A., Eichler, H., and Klüter, H. (2003). Messenger RNA profiling of human platelets by microarray hybridization. Thrombosis and haemostasis *90*, 738-748.

Bultema, J.J., Ambrosio, A.L., Burek, C.L., and Di Pietro, S.M. (2012). BLOC-2, AP-3, and AP-1 proteins function in concert with Rab38 and Rab32 proteins to mediate protein trafficking to lysosome-related organelles. The Journal of biological chemistry *287*, 19550-19563.

Bultema, J.J., and Di Pietro, S.M. (2013). Cell type-specific Rab32 and Rab38 cooperate with the ubiquitous lysosome biogenesis machinery to synthesize specialized lysosome-related organelles. Small GTPases *4*, 16-21.

Burda, P., Laslo, P., and Stopka, T. (2010). The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. Leukemia *24*, 1249-1257.

Cabezas-Wallscheid, N., Klimmeck, D., Hansson, J., Lipka, D.B., Reyes, A., Wang, Q., Weichenhan, D., Lier, A., Paleske, L. von, and Renders, S., et al. (2014). Identification of regulatory networks in HSCs and their

immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. Cell stem cell *15*, 507-522.

Calaminus, S., Guitart, A.V., Guitart, A., Sinclair, A., Schachtner, H., Watson, S.P., Holyoake, T.L., Kranc, K.R., and Machesky, L.M. (2012). Lineage tracing of Pf4-Cre marks hematopoietic stem cells and their progeny. PloS one *7*, e51361.

Carpinelli, M.R., Hilton, D.J., Metcalf, D., Antonchuk, J.L., Hyland, C.D., Mifsud, S.L., Di Rago, L., Hilton, A.A., Willson, T.A., and Roberts, A.W., et al. (2004). Suppressor screen in Mpl-/- mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. Proceedings of the National Academy of Sciences of the United States of America *101*, 6553-6558.

Cattoglio, C., Facchini, G., Sartori, D., Antonelli, A., Miccio, A., Cassani, B., Schmidt, M., Kalle, C. von, Howe, S., and Thrasher, A.J., et al. (2007). Hot spots of retroviral integration in human CD34+ hematopoietic cells. Blood *110*, 1770-1778.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G, Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., and Casanova, J.L., et al. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science (New York, N.Y.) *288*, 669-672.

Cepko, C.L., Roberts, B.E., and Mulligan, R.C. (1984). Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell *37*, 1053-1062.

Challen, G.A., Boles, N.C., Chambers, S.M., and Goodell, M.A. (2010). Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell stem cell *6*, 265-278.

Chang, Y., Bluteau, D., Debili, N., and Vainchenker, W. (2007). From hematopoietic stem cells to platelets. Journal of thrombosis and haemostasis : JTH *5 Suppl 1*, 318-327.

Chatterjee, M., Huang, Z., Zhang, W., Jiang, L., Hultenby, K., Zhu, L., Hu, H., Nilsson, G.P., and Li, N. (2011). Distinct platelet packaging, release, and surface expression of proangiogenic and antiangiogenic factors on different platelet stimuli. Blood *117*, 3907-3911.

Chen, C.H., Lo, R.W., Urban, D., Pluthero, F.G., and Kahr, W.H.A. (2017a). α-granule biogenesis: from disease to discovery. Platelets *28*, 147-154.

Chen, T.-W., Hwang, S.-M., Chu, I.-M., Hsu, S.-C., Hsieh, T.-B., and Yao, C.-L. (2009). Characterization and transplantation of induced megakaryocytes from hematopoietic stem cells for rapid platelet recovery by a two-step serum-free procedure. Experimental hematology *37*, 1330-1339.e5.

Chen, Y., Luo, X., Schroeder, J.A., Chen, J., Baumgartner, C.K., Hu, J., and Shi, Q. (2017b). Immune tolerance induced by platelet-targeted factor VIII gene therapy in hemophilia A mice is CD4 T cell mediated. Journal of thrombosis and haemostasis : JTH *15*, 1994-2004.

Chen, Y., Schroeder, J.A., Kuether, E.L., Zhang, G., and Shi, Q. (2014). Platelet gene therapy by lentiviral gene delivery to hematopoietic stem cells restores hemostasis and induces humoral immune tolerance in FIX(null) mice. Molecular therapy : the journal of the American Society of Gene Therapy *22*, 169-177.

Chotinantakul, K., and Leeanansaksiri, W. (2012). Hematopoietic stem cell development, niches, and signaling pathways. Bone marrow research *2012*, 270425.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proceedings of the National Academy of Sciences of the United States of America *98*, 14541-14546.

Chung, C.-w., Cooke, R.M., Proudfoot, A.E.I., and Wells, T.N.C. (2002). The Three-Dimensional Solution Structure of RANTES. Biochemistry *34*, 9307-9314.

Cicalese, M.P., and Aiuti, A. (2015). Clinical applications of gene therapy for primary immunodeficiencies. Human gene therapy *26*, 210-219.

Ciferri, S., Emiliani, C., Guglielmini, G., Orlacchio, A., Nenci, G.G., and Gresele, P. (2000). Platelets release their lysosomal content in vivo in humans upon activation. Thrombosis and haemostasis *83*, 157-164.

Cool, D.R., Normant, E., Shen, F.-s., Chen, H.-C., Pannell, L., Zhang, Y., and Loh, Y. (1997). Carboxypeptidase E Is a Regulated Secretory Pathway Sorting Receptor. Genetic Obliteration Leads to Endocrine Disorders in Cpefat Mice. Cell *88*, 73-83.

Coombe, D.R. (2008). Biological implications of glycosaminoglycan interactions with haemopoietic cytokines. Immunology and cell biology *86*, 598-607.

Cooray, S., Howe, S.J., and Thrasher, A.J. (2012). Retrovirus and lentivirus vector design and methods of cell conditioning. Methods in enzymology *507*, 29-57.

Counsell, J.R., Asgarian, Z., Meng, J., Ferrer, V., Vink, C.A., Howe, S.J., Waddington, S.N., Thrasher, A.J., Muntoni, F., and Morgan, J.E., et al. (2017). Lentiviral vectors can be used for full-length dystrophin gene therapy. Scientific reports *7*, 44775.

Cramer, E.M., Caen, J.P., Drouet, L., and Breton-Gorius, J. (1986). Absence of tubular structures and immunolabeling for von Willebrand factor in the platelet alpha-granules from porcine von Willebrand disease. Blood *68*, 774-778.

Cramer, E.M., Massé, J.M., Caen, J.P., Garcia, I., Breton-Gorius, J., Debili, N., and Vainchenker, W. (1993). Effect of thrombin on maturing human megakaryocytes. The American journal of pathology *143*, 1498-1508.

Cramer, E.M., Meyer, D., Le Menn, R., and Breton-Gorius, J. (1985). Eccentric localization of von Willebrand factor in an internal structure of platelet alpha-granule resembling that of Weibel-Palade bodies. Blood *66*, 710-713.

Cronin, J., Zhang, X.-Y., and Reiser, J. (2005). Altering the tropism of lentiviral vectors through pseudotyping. Current gene therapy *5*, 387-398.

Curotto, S., Lunsford, K., Smith, W., Thomason, J., and Bulla, C. (2012). Evidence of selective packaging and different α-granule subtypes in canine platelets. Journal of comparative pathology *147*, 499-502.

Dangelmaier, C.A., and Holmsen, H. (1980). Determination of acid hydrolases in human platelets. Analytical biochemistry *104*, 182-191.

Datta, Y.H., Youssoufian, H., Marks, P.W., and Ewenstein, B.M. (1999). Targeting of a heterologous protein to a regulated secretion pathway in cultured endothelial cells. Blood *94*, 2696-2703.

Daugherty, B.L., Straley, K.S., Sanders, J.M., Phillips, J.W., Disdier, M., McEver, R.P., and Green, S.A. (2001). AP-3 adaptor functions in targeting P-selectin to secretory granules in endothelial cells. Traffic (Copenhagen, Denmark) *2*, 406-413.

Dean, G.E., Fishkes, H., Nelson, P.J., and Rudnick, G. (1984). The hydrogen ion-pumping adenosine triphosphatase of platelet dense granule membrane. Differences from F1F0- and phosphoenzyme-type ATPases. The Journal of biological chemistry *259*, 9569-9574.

Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., and Geuze, H.J. (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. Journal of cell science *113 Pt 19*, 3365-3374.

Deutsch, V., Hubel, E., Kay, S., Ohayon, T., Katz, B.-Z., Many, A., Zander, A., Naparstek, E., and Grisaru, D. (2010). Mimicking the haematopoietic niche microenvironment provides a novel strategy for expansion of

haematopoietic and megakaryocyte-progenitor cells from cord blood. British journal of haematology *149*, 137-149.

Deutsch, V.R., and Tomer, A. (2006). Megakaryocyte development and platelet production. British journal of haematology *134*, 453-466.

Deutsch, V.R., and Tomer, A. (2013). Advances in megakaryocytopoiesis and thrombopoiesis: from bench to bedside. British journal of haematology *161*, 778-793.

Deveaux, S., Filipe, A., Lemarchandel, V., Ghysdael, J., Roméo, P.H., and Mignotte, V. (1996). Analysis of the thrombopoietin receptor (MPL) promoter implicates GATA and Ets proteins in the coregulation of megakaryocyte-specific genes. Blood *87*, 4678-4685.

Dick, J.E. (2008). Stem cell concepts renew cancer research. Blood *112*, 4793-4807.

Disdier, M., Morrissey, J.H., Fugate, R.D., Bainton, D.F., and McEver, R.P. (1992). Cytoplasmic domain of P-selectin (CD62) contains the signal for sorting into the regulated secretory pathway. Molecular biology of the cell *3*, 309-321.

Dittrich, M., Birschmann, I., Pfrang, J., Herterich, S., Smolenski, A., Walter, U., and Dandekar, T. (2006). Analysis of SAGE data in human platelets: features of the transcriptome in an anucleate cell. Thrombosis and haemostasis *95*, 643-651.

Downing, S.R., and Klement, G.L. (2012). Isolation and proteomic analysis of platelets by SELDI-TOF MS. Methods in molecular biology (Clifton, N.J.) *818*, 153-170.

Drachman, J.G., Sabath, D.F., Fox, N.E., and Kaushansky, K. (1997). Thrombopoietin signal transduction in purified murine megakaryocytes. Blood *89*, 483-492.

Drayer, A.L., Smit Sibinga, C.T., Esselink, M.T., Wolf, J.T.M. de, and Vellenga, E. (2002). In vitro megakaryocyte expansion in patients with delayed platelet engraftment after autologous stem cell transplantation. Annals of hematology *81*, 192-197.

Du, L.M., Nurden, P., Nurden, A.T., Nichols, T.C., Bellinger, D.A., Jensen, E.S., Haberichter, S.L., Merricks, E., Raymer, R.A., and Fang, J., et al. (2013). Platelet-targeted gene therapy with human factor VIII establishes haemostasis in dogs with haemophilia A. Nature communications *4*, 2773.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998a). A third-generation lentivirus vector with a conditional packaging system. Journal of virology *72*, 8463-8471.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998b). A third-generation lentivirus vector with a conditional packaging system. Journal of virology *72*, 8463-8471.

Dütting, S., Gaits-Iacovoni, F., Stegner, D., Popp, M., Antkowiak, A., van Eeuwijk, J.M.M., Nurden, P., Stritt, S., Heib, T., and Aurbach, K., et al. (2017). A Cdc42/RhoA regulatory circuit downstream of glycoprotein Ib guides transendothelial platelet biogenesis. Nature communications *8*, 15838.

Ebberink, E.H.T.M., Bouwens, E.A.M., Bloem, E., Boon-Spijker, M., van den Biggelaar, M., Voorberg, J., Meijer, A.B., and Mertens, K. (2017). Factor VIII/V C-domain swaps reveal discrete C-domain roles in factor VIII function and intracellular trafficking. Haematologica *102*, 686-694.

Eeckhoute, J., Métivier, R., and Salbert, G. (2009). Defining specificity of transcription factor regulatory activities. Journal of cell science *122*, 4027-4034.

El Golli, N., Issertial, O., Rosa, J.-P., and Briquet-Laugier, V. (2005). Evidence for a granule targeting sequence within platelet factor 4. The Journal of biological chemistry *280*, 30329-30335.

Elagib, K.E., Racke, F.K., Mogass, M., Khetawat, R., Delehanty, L.L., and Goldfarb, A.N. (2003). RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood *101*, 4333-4341.

Eliades, A., Papadantonakis, N., and Ravid, K. (2010). New roles for cyclin E in megakaryocytic polyploidization. The Journal of biological chemistry *285*, 18909-18917.

Escors, D., and Breckpot, K. (2010). Lentiviral vectors in gene therapy: their current status and future potential. Archivum immunologiae et therapiae experimentalis *58*, 107-119.

Eto, K., Murphy, R., Kerrigan, S.W., Bertoni, A., Stuhlmann, H., Nakano, T., Leavitt, A.D., and Shattil, S.J. (2002). Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. Proceedings of the National Academy of Sciences of the United States of America *99*, 12819-12824.

Fang, J., Jensen, E.S., Boudreaux, M.K., Du, L.M., Hawkins, T.B., Koukouritaki, S.B., Cornetta, K., and Wilcox, D.A. (2011). Platelet gene therapy improves hemostatic function for integrin alphaIIbbeta3-deficient dogs. Proceedings of the National Academy of Sciences of the United States of America *108*, 9583-9588.

Fantacini, D.M.C., Fontes, A.M., Abreu Neto, M.S. de, Covas, D.T., and Picanço-Castro, V. (2016). The F309S mutation increases factor VIII secretion in human cell line. Revista brasileira de hematologia e hemoterapia *38*, 135-140.

Feng, Q., Shabrani, N., Thon, J.N., Huo, H., Thiel, A., Machlus, K.R., Kim, K., Brooks, J., Li, F., and Luo, C., et al. (2014). Scalable generation of universal platelets from human induced pluripotent stem cells. Stem cell reports *3*, 817-831.

Ferreira, R., Ohneda, K., Yamamoto, M., and Philipsen, S. (2005). GATA1 function, a paradigm for transcription factors in hematopoiesis. Molecular and cellular biology *25*, 1215-1227.

Finkelshtein, D., Werman, A., Novick, D., Barak, S., and Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America *110*, 7306-7311.

Fisher, R.C., and Scott, E.W. (1998). Role of PU.1 in hematopoiesis. Stem cells (Dayton, Ohio) *16*, 25-37.

Flaumenhaft, R. (2013). Platelet Secretion. In Platelets (Elsevier), pp. 343–366.

Fleming, J.C., Berger, G., Guichard, J., Cramer, E.M., and Wagner, D.D. (1998). The transmembrane domain enhances granular targeting of P-selectin. European Journal of Cell Biology *75*, 331-343.

Fock, E.-l., Yan, F., Pan, S., and Chong, B.H. (2008). NF-E2-mediated enhancement of megakaryocytic differentiation and platelet production in vitro and in vivo. Experimental hematology *36*, 78-92.

Fox, N., Priestley, G., Papayannopoulou, T., and Kaushansky, K. (2002). Thrombopoietin expands hematopoietic stem cells after transplantation. The Journal of clinical investigation *110*, 389-394.

Frecha, C., Szécsi, J., Cosset, F.-L., and Verhoeyen, E. (2008). Strategies for targeting lentiviral vectors. Current gene therapy *8*, 449-460.

Frojmovic, M.M., and Milton, J.G. (1982). Human platelet size, shape, and related functions in health and disease. Physiological reviews *62*, 185-261.

Fujimoto, T.-T., Kohata, S., Suzuki, H., Miyazaki, H., and Fujimura, K. (2003). Production of functional platelets by differentiated embryonic stem (ES) cells in vitro. Blood *102*, 4044-4051.

Furie, B., Furie, B.C., and Flaumenhaft, R. (2001). A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion. Thrombosis and haemostasis *86*, 214-221.

Furihata, K., and Kunicki, T.J. (2002). Characterization of human glycoprotein VI gene 5' regulatory and promoter regions. Arteriosclerosis, thrombosis, and vascular biology *22*, 1733-1739.

Gao, W., Shi, P., Chen, X., Zhang, L., Liu, J., Fan, X., and Luo, X. (2017). Clathrin-mediated integrin αIIbβ3 trafficking controls platelet spreading. Platelets, 1-12.

Gao, Y., Smith, E., Ker, E., Campbell, P., Cheng, E.-c., Zou, S., Lin, S., Wang, L., Halene, S., and Krause, D.S. (2012). Role of RhoA-specific guanine exchange factors in regulation of endomitosis in megakaryocytes. Developmental cell *22*, 573-584.

Garcia, J., Gunzburg, J. de, Eychène, A., Gisselbrecht, S., and Porteu, F. (2001). Thrombopoietin-mediated sustained activation of extracellular signal-regulated kinase in UT7-Mpl cells requires both Ras-Raf-1- and Rap1-B-Raf-dependent pathways. Molecular and cellular biology *21*, 2659-2670.

Gaspar, H.B., Parsley, K.L., Howe, S., King, D., Gilmour, K.C., Sinclair, J., Brouns, G., Schmidt, M., Kalle, C. von, and Barington, T., et al. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet *364*, 2181-2187.

Gaur, M., Kamata, T., Wang, S., Moran, B., Shattil, S.J., and Leavitt, A.D. (2006). Megakaryocytes derived from human embryonic stem cells: a genetically tractable system to study megakaryocytopoiesis and integrin function. Journal of thrombosis and haemostasis : JTH *4*, 436-442.

Geddis, A.E., and Kaushansky, K. (2006). Endomitotic megakaryocytes form a midzone in anaphase but have a deficiency in cleavage furrow formation. Cell cycle (Georgetown, Tex.) *5*, 538-545.

Gekas, C., and Graf, T. (2013). CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. Blood *121*, 4463-4472.

Gentner, B., Visigalli, I., Hiramatsu, H., Lechman, E., Ungari, S., Giustacchini, A., Schira, G., Amendola, M., Quattrini, A., and Martino, S., et al. (2010). Identification of hematopoietic stem cell-specific miRNAs enables gene therapy of globoid cell leukodystrophy. Science translational medicine *2*, 58.

Germeshausen, M., Ballmaier, M., and Welte, K. (2006). MPL mutations in 23 patients suffering from congenital amegakaryocytic thrombocytopenia: the type of mutation predicts the course of the disease. Human mutation *27*, 296.

Gilles, L., Guièze, R., Bluteau, D., Cordette-Lagarde, V., Lacout, C., Favier, R., Larbret, F., Debili, N., Vainchenker, W., and Raslova, H. (2008). P19INK4D links endomitotic arrest and megakaryocyte maturation and is regulated by AML-1. Blood *111*, 4081-4091.

Gitschier, J., Wood, W.I., Goralka, T.M., Wion, K.L., Chen, E.Y., Eaton, D.H., Vehar, G.A., Capon, D.J., and Lawn, R.M. (1984). Characterization of the human factor VIII gene. Nature *312*, 326-330.

Gnatenko, D.V., Dunn, J.J., McCorkle, S.R., Weissmann, D., Perrotta, P.L., and Bahou, W.F. (2003). Transcript profiling of human platelets using microarray and serial analysis of gene expression. Blood *101*, 2285-2293.

Golebiewska, E.M., and Poole, A.W. (2013). Secrets of platelet exocytosis - what do we really know about platelet secretion mechanisms? British journal of haematology.

González-Murillo, A., Lozano, M.L., Alvarez, L., Jacome, A., Almarza, E., Navarro, S., Segovia, J.C., Hanenberg, H., Guenechea, G., and Bueren, J.A., et al. (2010). Development of lentiviral vectors with optimized transcriptional activity for the gene therapy of patients with Fanconi anemia. Human gene therapy *21*, 623-630.

Goubau, C., Buyse, G.M., Di Michele, M., van Geet, C., and Freson, K. (2013). Regulated granule trafficking in platelets and neurons: a common molecular machinery. European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society *17*, 117-125.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. The Journal of general virology *36*, 59-74.

Green, S.A., Setiadi, H., McEver, R.P., and Kelly, R.B. (1994). The cytoplasmic domain of P-selectin contains a sorting determinant that mediates rapid degradation in lysosomes. The Journal of cell biology *124*, 435-448.

Greene, T.K., Lyde, R.B., Bailey, S.C., Lambert, M.P., Zhai, L., Sabatino, D.E., Camire, R.M., Arruda, V.R., and Poncz, M. (2014). Apoptotic effects of platelet factor VIII on megakaryopoiesis: implications for a modified human FVIII for platelet-based gene therapy. Journal of thrombosis and haemostasis : JTH *12*, 2102-2112.

Greig, J.A., Wang, Q., Reicherter, A.L., Bote, E., McMenamin, D., Draper, C., Chen, S.-J., Hanlon, A.L., Goode, T., and Clark, K.R., et al. (2016). 760. Optimized AAV-Mediated Human Factor VIII Gene Therapy in Hemophilia A Mice and Cynomolgus Macaques. Molecular therapy *24*, S300.

Greig, J.A., Wang, Q., Reicherter, A.L., Chen, S.-J., Hanlon, A.L., Tipper, C.H., Clark, K.R., Wadsworth, S., Wang, L., and Wilson, J.M. (2017). Characterization of Adeno-Associated Viral Vector-Mediated Human Factor VIII Gene Therapy in Hemophilia A Mice. Human gene therapy *28*, 392-402.

Grez, M., Reichenbach, J., Schwäble, J., Seger, R., Dinauer, M.C., and Thrasher, A.J. (2011). Gene therapy of chronic granulomatous disease: the engraftment dilemma. Molecular therapy : the journal of the American Society of Gene Therapy *19*, 28-35.

Grieger, J.C., and Samulski, R.J. (2012). Adeno-associated virus vectorology, manufacturing, and clinical applications. Methods in enzymology *507*, 229-254.

Grinstein, S., and Furuya, W. (1984). Intracellular distribution of acridine derivatives in platelets and their suitability for cytoplasmic pH measurements. Biochimica et biophysica acta *803*, 221-228.

Gunay-Aygun, M., Falik-Zaccai, T.C., Vilboux, T., Zivony-Elboum, Y., Gumruk, F., Cetin, M., Khayat, M., Boerkoel, C.F., Kfir, N., and Huang, Y., et al. (2011). NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules. Nature genetics *43*, 732-734.

Haas, S., Hansson, J., Klimmeck, D., Loeffler, D., Velten, L., Uckelmann, H., Wurzer, S., Prendergast, Á.M., Schnell, A., and Hexel, K., et al. (2015). Inflammation-Induced Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors. Cell stem cell.

Haberichter, S.L., Allmann, A.M., Jozwiak, M.A., Montgomery, R.R., and Gill, J.C. (2009). Genetic alteration of the D2 domain abolishes von Willebrand factor multimerization and trafficking into storage. Journal of thrombosis and haemostasis : JTH *7*, 641-650.

Haberichter, S.L., Fahs, S.A., and Montgomery, R.R. (2000). von Willebrand factor storage and multimerization: 2 independent intracellular processes. Blood *96*, 1808-1815.

Haberichter, S.L., Jacobi, P., and Montgomery, R.R. (2003). Critical independent regions in the VWF propeptide and mature VWF that enable normal VWF storage. Blood *101*, 1384-1391.

Haberichter, S.L., Jozwiak, M.A., Rosenberg, J.B., Christopherson, P.A., and Montgomery, R.R. (2002). The von Willebrand factor propeptide (VWFpp) traffics an unrelated protein to storage. Arteriosclerosis, thrombosis, and vascular biology *22*, 921-926.

Hacein-Bey-Abina, S., Fischer, A., and Cavazzana-Calvo, M. (2002). Gene therapy of X-linked severe combined immunodeficiency. International journal of hematology *76*, 295-298.

Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., and Beldjord, K., et al. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. The Journal of clinical investigation *118*, 3132-3142.

Hacein-Bey-Abina, S., Kalle, C. von, Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.-L., Fraser, C.C., and Cavazzana-Calvo, M., et al. (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. The New England journal of medicine *348*, 255-256.

Hacein-Bey-Abina, S., Pai, S.-Y., Gaspar, H.B., Armant, M., Berry, C.C., Blanche, S., Bleesing, J., Blondeau, J., Boer, H. de, and Buckland, K.F., et al. (2014). A modified γ-retrovirus vector for X-linked severe combined immunodeficiency. The New England journal of medicine *371*, 1407-1417.

Hamel, D.J., Sielaff, I., Proudfoot, A.E.I., and Handel, T.M. (2009). Chapter 4. Interactions of chemokines with glycosaminoglycans. Methods in enzymology *461*, 71-102.

Hamlett, I., Draper, J., Strouboulis, J., Iborra, F., Porcher, C., and Vyas, P. (2008). Characterization of megakaryocyte GATA1-interacting proteins: the corepressor ETO2 and GATA1 interact to regulate terminal megakaryocyte maturation. Blood *112*, 2738-2749.

Hanby, H.A., Bao, J., Noh, J.-Y., Jarocha, D., Poncz, M., Weiss, M.J., and Marks, M.S. (2017). Platelet dense granules begin to selectively accumulate mepacrine during proplatelet formation. Blood advances *1*, 1478-1490.

Handagama, P.J., George, J.N., Shuman, M.A., McEver, R.P., and Bainton, D.F. (1987). Incorporation of a circulating protein into megakaryocyte and platelet granules. Proceedings of the National Academy of Sciences of the United States of America *84*, 861-865.

Handel, T.M., Johnson, Z., Crown, S.E., Lau, E.K., and Proudfoot, A.E. (2005). Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. Annual review of biochemistry *74*, 385-410.

Harper, K., Winter, R.M., Pembrey, M.E., Hartley, D., Davies, K.E., and Tuddenham, E.G. (1984). A clinically useful DNA probe closely linked to haemophilia A. Lancet (London, England) *2*, 6-8.

Harrison, P., Wilbourn, B., Debili, N., Vainchenker, W., Breton-Gorius, J., Lawrie, A.S., Masse, J.M., Savidge, G.F., and Cramer, E.M. (1989). Uptake of plasma fibrinogen into the alpha granules of human megakaryocytes and platelets. The Journal of clinical investigation *84*, 1320-1324.

Hartley, J.W., and Rowe, W.P. (1975). Clonal cells lines from a feral mouse embryo which lack host-range restrictions for murine leukemia viruses. Virology *65*, 128-134.

Hartwell, D.W., Mayadas, T.N., Berger, G., Frenette, P.S., Rayburn, H., Hynes, R.O., and Wagner, D.D. (1998). Role of P-selectin cytoplasmic domain in granular targeting in vivo and in early inflammatory responses. The Journal of cell biology *143*, 1129-1141.

Hartwig, J.H., and Italiano, J.E. (2006). Cytoskeletal mechanisms for platelet production. Blood cells, molecules & diseases *36*, 99-103.

Hashimoto, Y., and Ware, J. (1995a). Identification of essential GATA and Ets binding motifs within the promoter of the platelet glycoprotein Ib alpha gene. The Journal of biological chemistry *270*, 24532-24539.

Hashimoto, Y., and Ware, J. (1995b). Identification of essential GATA and Ets binding motifs within the promoter of the platelet glycoprotein Ib alpha gene. The Journal of biological chemistry *270*, 24532-24539.

Hayward, C.P., Song, Z., Zheng, S., Fung, R., Pai, M., Massé, J.M., and Cramer, E.M. (1999). Multimerin processing by cells with and without pathways for regulated protein secretion. Blood *94*, 1337-1347.

Heckl, D., Wicke, D.C., Brugman, M.H., Meyer, J., Schambach, A., Büsche, G., Ballmaier, M., Baum, C., and Modlich, U. (2011). Lentiviral gene transfer regenerates hematopoietic stem cells in a mouse model for Mpl-deficient aplastic anemia. Blood *117*, 3737-3747.

Heijnen, H.F., Debili, N., Vainchencker, W., Breton-Gorius, J., Geuze, H.J., and Sixma, J.J. (1998). Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. Blood *91*, 2313-2325.

Heijnen, H.F., Schiel, A.E., Fijnheer, R., Geuze, H.J., and Sixma, J.J. (1999). Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. Blood *94*, 3791-3799.

Herter, J.M., Rossaint, J., and Zarbock, A. (2014). Platelets in inflammation and immunity. Journal of thrombosis and haemostasis : JTH *12*, 1764-1775.

Hirsch, M.L., Wolf, S.J., and Samulski, R.J. (2016). Delivering Transgenic DNA Exceeding the Carrying Capacity of AAV Vectors. Methods in molecular biology (Clifton, N.J.) *1382*, 21-39.

Hoeben, R.C., Fallaux, F.J., Cramer, S.J., van den Wollenberg, D.J., van Ormondt, H., Briët, E., and van der Eb, A.J. (1995). Expression of the blood-clotting factor-VIII cDNA is repressed by a transcriptional silencer located in its coding region. Blood *85*, 2447-2454.

Hoffman, M. (2003). A cell-based model of coagulation and the role of factor VIIa. Blood reviews *17 Suppl 1*, S1-5.

Holmes, M.L., Bartle, N., Eisbacher, M., and Chong, B.H. (2002). Cloning and analysis of the thrombopoietin-induced megakaryocyte-specific glycoprotein VI promoter and its regulation by GATA-1, Fli-1, and Sp1. The Journal of biological chemistry *277*, 48333-48341.

Holmsen, H., and Weiss, H.J. (1979). Secretable storage pools in platelets. Annual review of medicine *30*, 119-134.

Hop, C., Guilliatt, A., Daly, M., Leeuw, H.P. de, Brinkman, H.-J.M., Peake, I.R., van Mourik, J.A., and Pannekoek, H. (2000). Assembly of Multimeric von Willebrand Factor Directs Sorting of P-Selectin. Arteriosclerosis, thrombosis, and vascular biology *20*, 1763-1768.

Hosaka, M., and WATANABE, T. (2010). Secretogranin III. A Bridge between Core Hormone Aggregates and the Secretory Granule Membrane. Endocr J *57*, 275-286.

Hosaka, M., WATANABE, T., Sakai, Y., Kato, T., and Takeuchi, T. (2005). Interaction between secretogranin III and carboxypeptidase E facilitates prohormone sorting within secretory granules. Journal of cell science *118*, 4785-4795.

Howe, S.J., Mansour, M.R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M.H., Pike-Overzet, K., Chatters, S.J., and Ridder, D. de, et al. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. The Journal of clinical investigation *118*, 3143-3150.

Howell, W.H. (1890). Observations upon the occurrence, structure, and function of the giant cells of the marrow. J. Morphol. *4*, 117-130.

Huang, H., Yu, M., Akie, T.E., Moran, T.B., Woo, A.J., Tu, N., Waldon, Z., Lin, Y.Y., Steen, H., and Cantor, A.B. (2009). Differentiation-dependent interactions between RUNX-1 and FLI-1 during megakaryocyte development. Molecular and cellular biology *29*, 4103-4115.

Huang, H.-S., and Chang, H.-H. (2012). Platelets in inflammation and immune modulations: functions beyond hemostasis. Archivum immunologiae et therapiae experimentalis *60*, 443-451.

Huang, R.-H., Wang, Y., Roth, R., Yu, X., Purvis, A.R., Heuser, J.E., Egelman, E.H., and Sadler, J.E. (2008). Assembly of Weibel-Palade body-like tubules from N-terminal domains of von Willebrand factor. Proceedings of the National Academy of Sciences of the United States of America *105*, 482-487.

Huang, Y., Joshi, S., Xiang, B., Kanaho, Y., Li, Z., Bouchard, B.A., Moncman, C.L., and Whiteheart, S.W. (2016). Arf6 controls platelet spreading and clot retraction via integrin αIIbβ3 trafficking. Blood *127*, 1459-1467.

Huizing, M., Helip-Wooley, A., Westbroek, W., Gunay-Aygun, M., and Gahl, W.A. (2008). Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annual review of genomics and human genetics *9*, 359-386.

Huston, M.W., van Til, N.P., Visser, T.P., Arshad, S., Brugman, M.H., Cattoglio, C., Nowrouzi, A., Li, Y., Schambach, A., and Schmidt, M., et al. (2011). Correction of murine SCID-X1 by lentiviral gene therapy using a codon-optimized IL2RG gene and minimal pretransplant conditioning. Molecular therapy : the journal of the American Society of Gene Therapy *19*, 1867-1877.

Ihara, K., Ishii, E., Eguchi, M., Takada, H., Suminoe, A., Good, R.A., and Hara, T. (1999). Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. Proceedings of the National Academy of Sciences of the United States of America *96*, 3132-3136.

Ilyina, E., Milius, R., and Mayo, K.H. (1994). Synthetic peptide probe folding initiation sites in platelet factor-4. Stable chain reversal found within the hydrophobic sequence LIATLKNGRKISL. Biochemistry *33*, 13436-13444.

Israels, S.J., Gerrard, J.M., Jacques, Y.V., McNicol, A., Cham, B., Nishibori, M., and Bainton, D.F. (1992). Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). Blood *80*, 143-152.

Israels, S.J., McMillan, E.M., Robertson, C., Singhory, S., and McNicol, A. (1996). The lysosomal granule membrane protein, LAMP-2, is also present in platelet dense granule membranes. Thrombosis and haemostasis *75*, 623-629.

Italiano, J.E., and Battinelli, E.M. (2009). Selective sorting of alpha-granule proteins. Journal of thrombosis and haemostasis : JTH *7 Suppl 1*, 173-176.

Italiano, J.E., Lecine, P., Shivdasani, R.A., and Hartwig, J.H. (1999). Blood Platelets Are Assembled Principally at the Ends of Proplatelet Processes Produced by Differentiated Megakaryocytes. J Cell Biol *147*, 1299-1312.

Italiano, J.E., Richardson, J.L., Patel-Hett, S., Battinelli, E., Zaslavsky, A., Short, S., Ryeom, S., Folkman, J., and Klement, G.L. (2008). Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood *111*, 1227-1233.

Ivics, Z., Li, M.A., Mátés, L., Boeke, J.D., Nagy, A., Bradley, A., and Izsvák, Z. (2009). Transposon-mediated genome manipulation in vertebrates. Nature methods *6*, 415-422.

Jaenicke, R. (1991). Protein folding. Local structures, domains, subunits, and assemblies. Biochemistry *30*, 3147-3161.

Jenne, C.N., Urrutia, R., and Kubes, P. (2013). Platelets: bridging hemostasis, inflammation, and immunity. International journal of laboratory hematology *35*, 254-261.

Jennings, L.K. (2009). Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thrombosis and haemostasis *102*, 248-257.

Ji, L., Wu, H.-T., Qin, X.-Y., and Lan, R. (2017). Dissecting carboxypeptidase E: properties, functions and pathophysiological roles in disease. Endocrine connections *6*, R18-R38.

Johnston, G.I., Cook, R.G., and McEver, R.P. (1989). Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell *56*, 1033-1044.

Johnston, J.M., Denning, G., Doering, C.B., and Spencer, H.T. (2013). Generation of an optimized lentiviral vector encoding a high-expression factor VIII transgene for gene therapy of hemophilia A. Gene therapy *20*, 607-615.

Jonnalagadda, D., Izu, L.T., and Whiteheart, S.W. (2012). Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. Blood *120*, 5209-5216.

Kahr, W.H.A., Hinckley, J., Li, L., Schwertz, H., Christensen, H., Rowley, J.W., Pluthero, F.G., Urban, D., Fabbro, S., and Nixon, B., et al. (2011). Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. Nature genetics *43*, 738-740.

Kahr, W.H.A., Lo, R.W., Li, L., Pluthero, F.G., Christensen, H., Ni, R., Vaezzadeh, N., Hawkins, C.E., Weyrich, A.S., and Di Paola, J., et al. (2013). Abnormal megakaryocyte development and platelet function in Nbeal2(-/-) mice. Blood *122*, 3349-3358.

Kamykowski, J., Carlton, P., Sehgal, S., and Storrie, B. (2011). Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet α-granules. Blood *118*, 1370-1373.

Kanaji, S., Kuether, E.L., Fahs, S.A., Schroeder, J.A., Ware, J., Montgomery, R.R., and Shi, Q. (2012). Correction of murine Bernard-Soulier syndrome by lentivirus-mediated gene therapy. Molecular therapy : the journal of the American Society of Gene Therapy *20*, 625-632.

Kato, T., Ogami, K., Shimada, Y., Iwamatsu, A., Sohma, Y., Akahori, H., Horie, K., Kokubo, A., Kudo, Y., and Maeda, E. (1995). Purification and characterization of thrombopoietin. Journal of biochemistry *118*, 229-236.

Kaufman, R.J., Pipe, S.W., Tagliavacca, L., Swaroop, M., and Moussalli, M. (1997). Biosynthesis, assembly and secretion of coagulation factor VIII. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis *8 Suppl 2*, S3-14.

Kaushansky, K. (2009). Molecular mechanisms of thrombopoietin signaling. Journal of thrombosis and haemostasis : JTH *7 Suppl 1*, 235-238.

Kaushansky, K., Lok, S., Holly, R.D., Broudy, V.C., Lin, N., Bailey, M.C., Forstrom, J.W., Buddle, M.M., Oort, P.J., and Hagen, F.S. (1994). Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature *369*, 568-571.

Kay, M.A., Glorioso, J.C., and Naldini, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nature medicine *7*, 33-40.

Kerns, H.M., Ryu, B.Y., Stirling, B.V., Sather, B.D., Astrakhan, A., Humblet-Baron, S., Liggitt, D., and Rawlings, D.J. (2010). B cell-specific lentiviral gene therapy leads to sustained B-cell functional recovery in a murine model of X-linked agammaglobulinemia. Blood *115*, 2146-2155.

Kimura, S., Roberts, A.W., Metcalf, D., and Alexander, W.S. (1998). Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. Proceedings of the National Academy of Sciences *95*, 1195-1200.

Kirito, K., Fox, N., and Kaushansky, K. (2003). Thrombopoietin stimulates Hoxb4 expression: an explanation for the favorable effects of TPO on hematopoietic stem cells. Blood *102*, 3172-3178.

Kitaguchi, T., Murata, M., Anbo, H., Moriki, T., and Ikeda, Y. (1997). Characterization of the gene encoding mouse platelet glycoprotien Ib beta. Thrombosis research *87*, 235-244.

Koedam, J.A., Cramer, E.M., Briend, E., Furie, B., Furie, B.C., and Wagner, D.D. (1992). P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. The Journal of cell biology *116*, 617-625.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Koulnis, M., Pop, R., Porpiglia, E., Shearstone, J.R., Hidalgo, D., and Socolovsky, M. (2011). Identification and analysis of mouse erythroid progenitors using the CD71/TER119 flow-cytometric assay. Journal of visualized experiments : JoVE.

Kraunus, J., Schaumann, D H S, Meyer, J., Modlich, U., Fehse, B., Brandenburg, G., Laer, D. von, Klump, H., Schambach, A., and Bohne, J., et al. (2004). Self-inactivating retroviral vectors with improved RNA processing. Gene therapy *11*, 1568-1578.

Krumsiek, J., Marr, C., Schroeder, T., and Theis, F.J. (2011). Hierarchical differentiation of myeloid progenitors is encoded in the transcription factor network. PloS one *6*, e22649.

Kuether, E.L., Schroeder, J.A., Fahs, S.A., Cooley, B.C., Chen, Y., Montgomery, R.R., Wilcox, D.A., and Shi, Q. (2012). Lentivirus-mediated platelet gene therapy of murine hemophilia A with pre-existing anti-factor VIII immunity. Journal of thrombosis and haemostasis : JTH *10*, 1570-1580.

Kufrin, D., Eslin, D.E., Bdeir, K., Murciano, J.-C., Kuo, A., Kowalska, M.A., Degen, J.L., Sachais, B.S., Cines, D.B., and Poncz, M. (2003). Antithrombotic thrombocytes: ectopic expression of urokinase-type plasminogen activator in platelets. Blood *102*, 926-933.

Kumar, M., Keller, B., Makalou, N., and Sutton, R.E. (2001). Systematic determination of the packaging limit of lentiviral vectors. Human gene therapy *12*, 1893-1905.

Kustikova, O., Fehse, B., Modlich, U., Yang, M., Düllmann, J., Kamino, K., Neuhoff, N. von, Schlegelberger, B., Li, Z., and Baum, C. (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. Science (New York, N.Y.) *308*, 1171-1174.

Kustikova, O.S., Geiger, H., Li, Z., Brugman, M.H., Chambers, S.M., Shaw, C.A., Pike-Overzet, K., Ridder, D. de, Staal, F.J.T., and Keudell, G. von, et al. (2007). Retroviral vector insertion sites associated with dominant hematopoietic clones mark "stemness" pathways. Blood *109*, 1897-1907.

Kustikova, O.S., Wahlers, A., Kuhlcke, K., Stahle, B., Zander, A.R., Baum, C., and Fehse, B. (2003). Dose finding with retroviral vectors: correlation of retroviral vector copy numbers in single cells with gene transfer efficiency in a cell population. Blood *102*, 3934-3937.

Kuter, D.J., Beeler, D.L., and Rosenberg, R.D. (1994). The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production. Proceedings of the National Academy of Sciences of the United States of America *91*, 11104-11108.

Kyostio-Moore, S., Berthelette, P., Piraino, S., Sookdeo, C., Nambiar, B., Jackson, R., Burnham, B., O'Riordan, C.R., Cheng, S.H., and Armentano, D. (2016). The impact of minimally oversized adeno-associated viral vectors encoding human factor VIII on vector potency in vivo. Molecular therapy. Methods & clinical development *3*, 16006.

Lachmann, N., Jagielska, J., Heckl, D., Brennig, S., Pfaff, N., Maetzig, T., Modlich, U., Cantz, T., Gentner, B., and Schambach, A., et al. (2012). MicroRNA-150-regulated vectors allow lymphocyte-sparing transgene expression in hematopoietic gene therapy. Gene therapy *19*, 915-924.

Latorre-Rey, L.J., Wintterle, S., Dütting, S., Kohlscheen, S., Abel, T., Schenk, F., Wingert, S., Rieger, M.A., Nieswandt, B., and Heinz, N., et al. (2017). Targeting expression to megakaryocytes and platelets by lineage-specific lentiviral vectors. Journal of thrombosis and haemostasis : JTH *15*, 341-355.

Lecine, P., Italiano, J.E., Kim, S.W., Villeval, J.L., and Shivdasani, R.A. (2000). Hematopoietic-specific beta 1 tubulin participates in a pathway of platelet biogenesis dependent on the transcription factor NF-E2. Blood *96*, 1366-1373.

Lemini, C., Jaimez, R., and Franco, Y. (2007). Gender and inter-species influence on coagulation tests of rats and mice. Thrombosis research *120*, 415-419.

Lenting, P.J., van Mourik, J.A., and Mertens, K. (1998). The life cycle of coagulation factor VIII in view of its structure and function. Blood *92*, 3983-3996.

Lentjes, M.H.F.M., Niessen, H.E.C., Akiyama, Y., Bruïne, A.P. de, Melotte, V., and van Engeland, M. (2016). The emerging role of GATA transcription factors in development and disease. Expert reviews in molecular medicine *18*, e3.

Leoncini, G., Balestrero, F., and Maresca, M. (1985). Lysosomal enzymes in human platelets. Cell biochemistry and function *3*, 121-125.

Lepage, A., Uzan, G., Touche, N., Morales, M., Cazenave, J.P., Lanza, F., and La Salle, C. de (1999). Functional characterization of the human platelet glycoprotein V gene promoter: A specific marker of late megakaryocytic differentiation. Blood *94*, 3366-3380.

Levin, M.C., Lidberg, U., Jirholt, P., Adiels, M., Wramstedt, A., Gustafsson, K., Greaves, D.R., Li, S., Fazio, S., and Linton, M.F., et al. (2012). Evaluation of macrophage-specific promoters using lentiviral delivery in mice. Gene therapy *19*, 1041-1047.

Levine, M. (2010). Transcriptional enhancers in animal development and evolution. Current biology : CB *20*, R754-63.

Lévy, C., Verhoeyen, E., and Cosset, F.-L. (2015). Surface engineering of lentiviral vectors for gene transfer into gene therapy target cells. Current opinion in pharmacology *24*, 79-85.

Li, Z., Düllmann, J., Schiedlmeier, B., Schmidt, M., Kalle, C. von, Meyer, J., Forster, M., Stocking, C., Wahlers, A., and Frank, O., et al. (2002). Murine leukemia induced by retroviral gene marking. Science (New York, N.Y.) *296*, 497.

Liu, Y., Wang, Y., Gao, Y., Forbes, J.A., Qayyum, R., Becker, L., Cheng, L., and Wang, Z.Z. (2015). Efficient generation of megakaryocytes from human induced pluripotent stem cells using food and drug administration-approved pharmacological reagents. Stem cells translational medicine *4*, 309-319.

Lo, B., Li, L., Gissen, P., Christensen, H., McKiernan, P.J., Ye, C., Abdelhaleem, M., Hayes, J.A., Williams, M.D., and Chitayat, D., et al. (2005). Requirement of VPS33B, a member of the Sec1/Munc18 protein family, in megakaryocyte and platelet alpha-granule biogenesis. Blood *106*, 4159-4166.

Lordier, L., Jalil, A., Aurade, F., Larbret, F., Larghero, J., Debili, N., Vainchenker, W., and Chang, Y. (2008). Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signaling. Blood *112*, 3164-3174.

Lordier, L., Pan, J., Naim, V., Jalil, A., Badirou, I., Rameau, P., Larghero, J., Debili, N., Rosselli, F., and Vainchenker, W., et al. (2012). Presence of a defect in karyokinesis during megakaryocyte endomitosis. Cell cycle (Georgetown, Tex.) *11*, 4385-4389.

Lu, H., Chen, L., Wang, J., Huack, B., Sarkar, R., Zhou, S., Xu, R., Ding, Q., Wang, X., and Wang, H., et al. (2008). Complete correction of hemophilia A with adeno-associated viral vectors containing a full-size expression cassette. Human gene therapy *19*, 648-654.

Luchsinger, L.L., Almeida, M.J. de, Corrigan, D.J., Mumau, M., and Snoeck, H.-W. (2016). Mitofusin 2 maintains haematopoietic stem cells with extensive lymphoid potential. Nature *529*, 528-531.

Lui-Roberts, W.W.Y., Collinson, L.M., Hewlett, L.J., Michaux, G., and Cutler, D.F. (2005). An AP-1/clathrin coat plays a novel and essential role in forming the Weibel-Palade bodies of endothelial cells. The Journal of cell biology *170*, 627-636.

Macaulay, I.C., Carr, P., Gusnanto, A., Ouwehand, W.H., Des Fitzgerald, and Watkins, N.A. (2005). Platelet genomics and proteomics in human health and disease. The Journal of clinical investigation *115*, 3370-3377.

Madan, B., Seo, S.Y., and Lee, S.-G. (2014). Structural and sequence features of two residue turns in beta-hairpins. Proteins *82*, 1721-1733.

Maetzig, T., Galla, M., Baum, C., and Schambach, A. (2011). Gammaretroviral vectors: biology, technology and application. Viruses *3*, 677-713.

Malkowski, M.G., Wu, J.Y., Lazar, J.B., Johnson, P.H., and Edwards, B.F.P. (1995). The Crystal Structure of Recombinant Human Neutrophil-activating Peptide-2 (M6L) at 1.9-Å Resolution. J. Biol. Chem. *270*, 7077-7087.

Manno, C.S., Pierce, G.F., Arruda, V.R., Glader, B., Ragni, M., Rasko, J.J., Rasko, J., Ozelo, M.C., Hoots, K., and Blatt, P., et al. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nature medicine *12*, 342-347.

Manz, M.G., Traver, D., Miyamoto, T., Weissman, I.L., and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood *97*, 3333-3341.

Marks, M.S., Heijnen, H.F.G., and Raposo, G. (2013). Lysosome-related organelles: unusual compartments become mainstream. Current opinion in cell biology *25*, 495-505.

Marquette, K.A., Pittman, D.D., and Kaufman, R.J. (1995). A 110-amino acid region within the A1-domain of coagulation factor VIII inhibits secretion from mammalian cells. The Journal of biological chemistry *270*, 10297-10303.

Martin, P., and Papayannopoulou, T. (1982). HEL cells. A new human erythroleukemia cell line with spontaneous and induced globin expression. Science *216*, 1233-1235.

Matatall, K.A., Shen, C.-C., Challen, G.A., and King, K.Y. (2014). Type II interferon promotes differentiation of myeloid-biased hematopoietic stem cells. Stem cells (Dayton, Ohio) *32*, 3023-3030.

Matsubara, Y., Ono, Y., Suzuki, H., Arai, F., Suda, T., Murata, M., and Ikeda, Y. (2013). OP9 bone marrow stroma cells differentiate into megakaryocytes and platelets. PloS one *8*, e58123.

Matuskova, M., and Durinikov, E. (2016). Retroviral Vectors in Gene Therapy. In Advances in Molecular Retrovirology, S.K. Saxena, ed. (InTech).

Maynard, D.M., Heijnen, H.F.G., Horne, M.K., White, J.G., and Gahl, W.A. (2007). Proteomic analysis of platelet alpha-granules using mass spectrometry. Journal of thrombosis and haemostasis : JTH *5*, 1945-1955.

Mayo, K.H., Roongta, V., Ilyina, E., Milius, R., Barker, S., Quinlan, C., La Rosa, G., and Daly, T.J. (1995). NMR solution structure of the 32-kDa platelet factor 4 ELR-motif N-terminal chimera: a symmetric tetramer. Biochemistry *34*, 11399-11409.

Mazharian, A., Watson, S.P., and Séverin, S. (2009). Critical role for ERK1/2 in bone marrow and fetal liver-derived primary megakaryocyte differentiation, motility, and proplatelet formation. Experimental hematology *37*, 1238-1249.e5.

McCormack, M.P., Hall, M.A., Schoenwaelder, S.M., Zhao, Q., Ellis, S., Prentice, J.A., Clarke, A.J., Slater, N.J., Salmon, J.M., and Jackson, S.P., et al. (2006). A critical role for the transcription factor Scl in platelet production during stress thrombopoiesis. Blood *108*, 2248-2256.

McEver, R.P., Beckstead, J.H., Moore, K.L., Marshall-Carlson, L., and Bainton, D.F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. The Journal of clinical investigation *84*, 92-99.

McIntosh, J., Lenting, P.J., Rosales, C., Lee, D., Rabbanian, S., Raj, D., Patel, N., Tuddenham, E.G.D., Christophe, O.D., and McVey, J.H., et al. (2013). Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood *121*, 3335-3344.

McMichael, M. (2012). New models of hemostasis. Topics in companion animal medicine *27*, 40-45.

Meng, R., Wang, Y., Yao, Y., Zhang, Z., Harper, D.C., Heijnen, H.F.G., Sitaram, A., Li, W., Raposo, G., and Weiss, M.J., et al. (2012). SLC35D3 delivery from megakaryocyte early endosomes is required for platelet dense granule biogenesis and is differentially defective in Hermansky-Pudlak syndrome models. Blood *120*, 404-414.

Merten, O.-W., Hebben, M., and Bovolenta, C. (2016). Production of lentiviral vectors. Molecular therapy. Methods & clinical development *3*, 16017.

Metcalf, D., Carpinelli, M.R., Hyland, C., Mifsud, S., Dirago, L., Nicola, N.A., Hilton, D.J., and Alexander, W.S. (2005). Anomalous megakaryocytopoiesis in mice with mutations in the c-Myb gene. Blood *105*, 3480-3487.

Metzger, B.P.H., Duveau, F., Yuan, D.C., Tryban, S., Yang, B., and Wittkopp, P.J. (2016). Contrasting Frequencies and Effects of cis- and trans-Regulatory Mutations Affecting Gene Expression. Molecular biology and evolution *33*, 1131-1146.

Meunier, A., and Pohl, M. (2009). Lentiviral vectors for gene transfer into the spinal cord glial cells. Gene therapy *16*, 476-482.

Miao, C.H. (2016). Hemophilia A gene therapy via intraosseous delivery of factor VIII-lentiviral vectors. Thrombosis journal *14*, 41.

Miller, A.D., and Rosman, G.J. (1989). Improved retroviral vectors for gene transfer and expression. BioTechniques *7*, 980.

Milone, M.C., and O'Doherty, U. (2018). Clinical use of lentiviral vectors. Leukemia *32*, 1529-1541.

Miwako, I., Schröter, T., and Schmid, S.L. (2003). Clathrin- and dynamin-dependent coated vesicle formation from isolated plasma membranes. Traffic (Copenhagen, Denmark) *4*, 376-389.

Miyawaki, K., Arinobu, Y., Iwasaki, H., Kohno, K., Tsuzuki, H., Iino, T., Shima, T., Kikushige, Y., Takenaka, K., and Miyamoto, T., et al. (2015). CD41 marks the initial myelo-erythroid lineage specification in adult mouse hematopoiesis: redefinition of murine common myeloid progenitor. Stem cells (Dayton, Ohio) *33*, 976-987.

Modderman, P.W., Beuling, E.A., Govers, L.A., Calafat, J., Janssen, H., dem Borne, A.E. von, and Sonnenberg, A. (1998). Determinants in the cytoplasmic domain of P-selectin required for sorting to secretory granules. The Biochemical journal *336 (Pt 1)*, 153-161.

Modlich, U., Kustikova, O.S., Schmidt, M., Rudolph, C., Meyer, J., Li, Z., Kamino, K., Neuhoff, N. von, Schlegelberger, B., and Kuehlcke, K., et al. (2005). Leukemias following retroviral transfer of multidrug resistance 1 (MDR1) are driven by combinatorial insertional mutagenesis. Blood *105*, 4235-4246.

Modlich, U., Schambach, A., Brugman, M.H., Wicke, D.C., Knoess, S., Li, Z., Maetzig, T., Rudolph, C., Schlegelberger, B., and Baum, C. (2008). Leukemia induction after a single retroviral vector insertion in Evi1 or Prdm16. Leukemia *22*, 1519-1528.

Molineux, G., Pojda, Z., Hampson, I.N., Lord, B.I., and Dexter, T.M. (1990). Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. Blood *76*, 2153-2158.

Morris, K.M., Aden, D.P., Knowles, B.B., and Colten, H.R. (1982). Complement biosynthesis by the human hepatoma-derived cell line HepG2. The Journal of clinical investigation *70*, 906-913.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661-673.

Mouthon, M.A., Bernard, O., Mitjavila, M.T., Romeo, P.H., Vainchenker, W., and Mathieu-Mahul, D. (1993). Expression of tal-1 and GATA-binding proteins during human hematopoiesis. Blood *81*, 647-655.

Mulloy, B., and Rider, C.C. (2006). Cytokines and proteoglycans: an introductory overview. Biochemical Society transactions *34*, 409-413.

Nagai, R., Matsuura, E., Hoshika, Y., Nakata, E., Nagura, H., Watanabe, A., Komatsu, N., Okada, Y., and Doi, T. (2006). RUNX1 suppression induces megakaryocytic differentiation of UT-7/GM cells. Biochemical and biophysical research communications *345*, 78-84.

Nakamura, S., Takayama, N., Hirata, S., Seo, H., Endo, H., Ochi, K., Fujita, K.-i., Koike, T., Harimoto, K.-i., and Dohda, T., et al. (2014). Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells. Cell stem cell *14*, 535-548.

Nakatsu, F., and Ohno, H. (2003). Adaptor protein complexes as the key regulators of protein sorting in the post-Golgi network. Cell structure and function *28*, 419-429.

Naldini, L., Blömer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., and Trono, D. (1996a). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science (New York, N.Y.) *272*, 263-267.

Naldini, L., Blömer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., and Trono, D. (1996b). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science (New York, N.Y.) *272*, 263-267.

Naldini, L., Trono, D., and Verma, I.M. (2016). Lentiviral vectors, two decades later. Science (New York, N.Y.) *353*, 1101-1102.

Nathwani, A.C., Reiss, U.M., Tuddenham, Edward G D, Rosales, C., Chowdary, P., McIntosh, J., Della Peruta, M., Lheriteau, E., Patel, N., and Raj, D., et al. (2014). Long-term safety and efficacy of factor IX gene therapy in hemophilia B. The New England journal of medicine *371*, 1994-2004.

Nathwani, A.C., Rosales, C., McIntosh, J., Rastegarlari, G., Nathwani, D., Raj, D., Nawathe, S., Waddington, S.N., Bronson, R., and Jackson, S., et al. (2011). Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. Molecular therapy : the journal of the American Society of Gene Therapy *19*, 876-885.

Nawijn, M.C., Alendar, A., and Berns, A. (2011). For better or for worse: the role of Pim oncogenes in tumorigenesis. Nature reviews. Cancer *11*, 23-34.

Negri, D.R., Michelini, Z., Bona, R., Blasi, M., Filati, P., Leone, P., Rossi, A., Franco, M., and Cara, A. (2011). Integrase-defective lentiviral-vector-based vaccine: a new vector for induction of T cell immunity. Expert opinion on biological therapy *11*, 739-750.

Neyman, M., Gewirtz, J., and Poncz, M. (2008). Analysis of the spatial and temporal characteristics of platelet-delivered factor VIII-based clots. Blood *112*, 1101-1108.

Nguyen, H.G., Yu, G., Makitalo, M., Yang, D., Xie, H.-X., Jones, M.R., and Ravid, K. (2005). Conditional overexpression of transgenes in megakaryocytes and platelets in vivo. Blood *106*, 1559-1564.

Nightingale, T., and Cutler, D. (2013). The secretion of von Willebrand factor from endothelial cells; an increasingly complicated story. Journal of thrombosis and haemostasis : JTH *11 Suppl 1*, 192-201.

Nishikii, H., Kanazawa, Y., Umemoto, T., Goltsev, Y., Matsuzaki, Y., Matsushita, K., Yamato, M., Nolan, G.P., Negrin, R., and Chiba, S. (2015). Unipotent megakaryopoietic pathway bridging hematopoietic stem cells and mature megakaryocytes. Stem cells (Dayton, Ohio).

Nishimura, T., Hatoya, S., Kanegi, R., Sugiura, K., Wijewardana, V., Kuwamura, M., Tanaka, M., Yamate, J., Izawa, T., and Takahashi, M., et al. (2013). Generation of functional platelets from canine induced pluripotent stem cells. Stem cells and development *22*, 2026-2035.

Nowrouzi, A., Glimm, H., Kalle, C. von, and Schmidt, M. (2011). Retroviral vectors: post entry events and genomic alterations. Viruses *3*, 429-455.

Ohmori, T., Ishiwata, A., Kashiwakura, Y., Madoiwa, S., Mitomo, K., Suzuki, H., Hasegawa, M., Mimuro, J., and Sakata, Y. (2008). Phenotypic correction of hemophilia A by ectopic expression of activated factor VII in platelets. Molecular therapy : the journal of the American Society of Gene Therapy *16*, 1359-1365.

Ohmori, T., Mimuro, J., Takano, K., Madoiwa, S., Kashiwakura, Y., Ishiwata, A., Niimura, M., Mitomo, K., Tabata, T., and Hasegawa, M., et al. (2006). Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibalpha promoter: in vivo model for platelet-targeting gene therapy. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *20*, 1522-1524.

Ohno, H. (2006). Physiological roles of clathrin adaptor AP complexes: lessons from mutant animals. Journal of biochemistry *139*, 943-948.

Okada, Y., Nobori, H., Shimizu, M., Watanabe, M., Yonekura, M., Nakai, T., Kamikawa, Y., Wakimura, A., Funahashi, N., and Naruse, H., et al. (2011). Multiple ETS family proteins regulate PF4 gene expression by binding to the same ETS binding site. PloS one *6*, e24837.

Orkin, S.H. (2000). Diversification of haematopoietic stem cells to specific lineages. Nature reviews. Genetics *1*, 57-64.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science (New York, N.Y.) *273*, 242-245.

Ott, M.G., Schmidt, M., Schwarzwaelder, K., Stein, S., Siler, U., Koehl, U., Glimm, H., Kühlcke, K., Schilz, A., and Kunkel, H., et al. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nature medicine *12*, 401-409.

Ozaki, Y., Suzuki-Inoue, K., and Inoue, O. (2013). Platelet receptors activated via mulitmerization: glycoprotein VI, GPIb-IX-V, and CLEC-2. Journal of thrombosis and haemostasis : JTH *11 Suppl 1*, 330-339.

Palm, M., Frankenberg, L., Johansson, M., and Jalkesten, E. (1997). Evaluation of coagulation tests in mouse plasma. Comparative Haematology International *7*, 243-246.

Palma, M. de, Montini, E., Santoni de Sio, F.R., Benedicenti, F., Gentile, A., Medico, E., and Naldini, L. (2005). Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. Blood *105*, 2307-2315.

Park, S.Y., and Guo, X. (2014). Adaptor protein complexes and intracellular transport. Bioscience reports *34*.

Passegué, E., Jamieson, C.H.M., Ailles, L.E., and Weissman, I.L. (2003). Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proceedings of the National Academy of Sciences of the United States of America *100 Suppl 1*, 11842-11849.

Patel, S.R., Hartwig, J.H., and Italiano, J.E. (2005a). The biogenesis of platelets from megakaryocyte proplatelets. The Journal of clinical investigation *115*, 3348-3354.

Patel, S.R., Richardson, J.L., Schulze, H., Kahle, E., Galjart, N., Drabek, K., Shivdasani, R.A., Hartwig, J.H., and Italiano, J.E. (2005b). Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. Blood *106*, 4076-4085.

Peters, C.G., Michelson, A.D., and Flaumenhaft, R. (2012). Granule exocytosis is required for platelet spreading: differential sorting of α-granules expressing VAMP-7. Blood *120*, 199-206.

Pittman, D.D., Alderman, E.M., Tomkinson, K.N., Wang, J.H., Giles, A.R., and Kaufman, R.J. (1993). Biochemical, immunological, and in vivo functional characterization of B-domain-deleted factor VIII. Blood *81*, 2925-2935.

Plantier, J.-L., Enjolras, N., Rodriguez, M.-H.E., Massé, J.-M., Cramer, E.M., and Négrier, C. (2003). The P-selectin cytoplasmic domain directs the cellular storage of a recombinant chimeric factor IX. Journal of thrombosis and haemostasis : JTH *1*, 292-299.

Polasek, J. (1989). Lysosomal concept of platelet secretion--revisited. European journal of haematology. Supplementum *50*, 3-24.

Polasek, J. (2005). Platelet secretory granules or secretory lysosomes? Platelets *16*, 500-501.

Prandini, M.H., Martin, F., Thevenon, D., and Uzan, G. (1996). The tissue-specific transcriptional regulation of the megakaryocytic glycoprotein IIb gene is controlled by interactions between a repressor and positive cis-acting elements. Blood *88*, 2062-2070.

Prandini, M.H., Uzan, G., Martin, F., Thevenon, D., and Marguerie, G. (1992). Characterization of a specific erythromegakaryocytic enhancer within the glycoprotein IIb promoter. The Journal of biological chemistry *267*, 10370-10374.

Pulikkan, J.A., Madera, D., Xue, L., Bradley, P., Landrette, S.F., Kuo, Y.-H., Abbas, S., Zhu, L.J., Valk, P., and Castilla, L.H. (2012). Thrombopoietin/MPL participates in initiating and maintaining RUNX1-ETO acute myeloid leukemia via PI3K/AKT signaling. Blood *120*, 868-879.

Ramezani, A., Zweier-Renn, L.A., and Hawley, R.G. (2011). Factor VIII delivered by haematopoietic stem cell-derived B cells corrects the phenotype of haemophilia A mice. Thrombosis and haemostasis *105*, 676-687.

Ranzani, M., Annunziato, S., Adams, D.J., and Montini, E. (2013). Cancer gene discovery: exploiting insertional mutagenesis. Molecular cancer research : MCR *11*, 1141-1158.

Rasheed, S., Nelson-Rees, W.A., Toth, E.M., Arnstein, P., and Gardner, M.B. (1974). Characterization of a newly derived human sarcoma cell line (HT-1080). Cancer *33*, 1027-1033.

Ravid, K., Beeler, D.L., Rabin, M.S., Ruley, H.E., and Rosenberg, R.D. (1991a). Selective targeting of gene products with the megakaryocyte platelet factor 4 promoter. Proceedings of the National Academy of Sciences of the United States of America *88*, 1521-1525.

Ravid, K., Beeler, D.L., Rabin, M.S., Ruley, H.E., and Rosenberg, R.D. (1991b). Selective targeting of gene products with the megakaryocyte platelet factor 4 promoter. Proceedings of the National Academy of Sciences of the United States of America *88*, 1521-1525.

Ravid, K., Lu, J., Zimmet, J.M., and Jones, M.R. (2002). Roads to polyploidy: the megakaryocyte example. Journal of cellular physiology *190*, 7-20.

Reich, N.C., and Liu, L. (2006). Tracking STAT nuclear traffic. Nature reviews. Immunology *6*, 602-612.

Rendu, F., and Brohard-Bohn, B. (2001). The platelet release reaction: granules' constituents, secretion and functions. Platelets *12*, 261-273.

Richardson, J.L., Shivdasani, R.A., Boers, C., Hartwig, J.H., and Italiano, J.E. (2005). Mechanisms of organelle transport and capture along proplatelets during platelet production. Blood *106*, 4066-4075.

Richardson, U.I. (1983). ACTH secretion in mouse pituitary tumor cells in culture: inhibition of CRF-stimulated hormone release by somatostatin. Life sciences *33*, 1981-1988.

Rollins, B.J. (1997). Chemokines. Blood *90*, 909-928.

Romani de Wit, T., Leeuw, H.P. de, Rondaij, M.G., Laaf, R.T. de, Sellink, E., Brinkman, H.-J., Voorberg, J., and van Mourik, J.A. (2003). Von Willebrand factor targets IL-8 to Weibel–Palade bodies in an endothelial cell line. Experimental cell research *286*, 67-74.

Rosenberg, J.B., Foster, P.A., Kaufman, R.J., Vokac, E.A., Moussalli, M., Kroner, P.A., and Montgomery, R.R. (1998). Intracellular trafficking of factor VIII to von Willebrand factor storage granules. The Journal of clinical investigation *101*, 613-624.

Rosenberg, J.B., Haberichter, S.L., Jozwiak, M.A., Vokac, E.A., Kroner, P.A., Fahs, S.A., Kawai, Y., and Montgomery, R.R. (2002). The role of the D1 domain of the von Willebrand factor propeptide in multimerization of VWF. Blood *100*, 1699-1706.

Rowley, J.W., Schwertz, H., and Weyrich, A.S. (2012). Platelet mRNA: the meaning behind the message. Current opinion in hematology *19*, 385-391.

Ruiz, F.A., Lea, C.R., Oldfield, E., and Docampo, R. (2004). Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. The Journal of biological chemistry *279*, 44250-44257.

Sander, H.J., Slot, J.W., Bouma, B.N., Bolhuis, P.A., Pepper, D.S., and Sixma, J.J. (1983). Immunocytochemical localization of fibrinogen, platelet factor 4, and beta thromboglobulin in thin frozen sections of human blood platelets. The Journal of clinical investigation *72*, 1277-1287.

Sanjuan-Pla, A., Macaulay, I.C., Jensen, C.T., Woll, P.S., Luis, T.C., Mead, A., Moore, S., Carella, C., Matsuoka, S., and Bouriez Jones, T., et al. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature *502*, 232-236.

Santilli, G., Almarza, E., Brendel, C., Choi, U., Beilin, C., Blundell, M.P., Haria, S., Parsley, K.L., Kinnon, C., and Malech, H.L., et al. (2011). Biochemical correction of X-CGD by a novel chimeric promoter regulating high levels of transgene expression in myeloid cells. Molecular therapy : the journal of the American Society of Gene Therapy *19*, 122-132.

Sato, N., Kiyokawa, N., Taguchi, T., Suzuki, T., Sekino, T., Ohmi, K., Itagaki, M., Sato, T., Lepage, A., and Lanza, F., et al. (2000). Functional conservation of platelet glycoprotein V promoter between mouse and human megakaryocytes. Experimental hematology *28*, 802-814.

Saur, S.J., Sangkhae, V., Geddis, A.E., Kaushansky, K., and Hitchcock, I.S. (2010). Ubiquitination and degradation of the thrombopoietin receptor c-Mpl. Blood *115*, 1254-1263.

Schaeffer, R.D., and Daggett, V. (2011). Protein folds and protein folding. Protein engineering, design & selection : PEDS *24*, 11-19.

Schambach, A., Bohne, J., Chandra, S., Will, E., Margison, G.P., Williams, D.A., and Baum, C. (2006a). Equal potency of gammaretroviral and lentiviral SIN vectors for expression of O6-methylguanine-DNA methyltransferase in hematopoietic cells. Molecular therapy : the journal of the American Society of Gene Therapy *13*, 391-400.

Schambach, A., Mueller, D., Galla, M., Verstegen, M.M.A., Wagemaker, G., Loew, R., Baum, C., and Bohne, J. (2006b). Overcoming promoter competition in packaging cells improves production of self-inactivating retroviral vectors. Gene therapy *13*, 1524-1533.

Schroeder, J.A., Chen, Y., Fang, J., Wilcox, D.A., and Shi, Q. (2014). In vivo enrichment of genetically manipulated platelets corrects the murine hemophilic phenotype and induces immune tolerance even using a low multiplicity of infection. Journal of thrombosis and haemostasis : JTH *12*, 1283-1293.

Sehgal, S., and Storrie, B. (2007). Evidence that differential packaging of the major platelet granule proteins von Willebrand factor and fibrinogen can support their differential release. Journal of thrombosis and haemostasis : JTH *5*, 2009-2016.

Selten, G., Cuypers, H.T., Zijlstra, M., Melief, C., and Berns, A. (1984). Involvement of c-myc in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. The EMBO journal *3*, 3215-3222.

Senzel, L., Gnatenko, D.V., and Bahou, W.F. (2009). The platelet proteome. Current opinion in hematology *16*, 329-333.

Sharda, A., and Flaumenhaft, R. (2018). The life cycle of platelet granules. F1000Research *7*, 236.

Shaw, J.P., Johnson, Z., Borlat, F., Zwahlen, C., Kungl, A., Roulin, K., Harrenga, A., Wells, T.N.C., and Proudfoot, A.E.I. (2004). The X-ray structure of RANTES: heparin-derived disaccharides allows the rational design of chemokine inhibitors. Structure (London, England : 1993) *12*, 2081-2093.

Shi, Q., Fahs, S.A., Wilcox, D.A., Kuether, E.L., Morateck, P.A., Mareno, N., Weiler, H., and Montgomery, R.R. (2008). Syngeneic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia A mice with preexisting FVIII immunity. Blood *112*, 2713-2721.

Shi, Q., Kuether, E.L., Chen, Y., Schroeder, J.A., Fahs, S.A., and Montgomery, R.R. (2014). Platelet gene therapy corrects the hemophilic phenotype in immunocompromised hemophilia A mice transplanted with genetically manipulated human cord blood stem cells. Blood *123*, 395-403.

Shi, Q., Wilcox, D.A., Fahs, S.A., Fang, J., Johnson, B.D., DU, L.M., Desai, D., and Montgomery, R.R. (2007). Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. Journal of thrombosis and haemostasis : JTH *5*, 352-361.

Shi, Q., Wilcox, D.A., Fahs, S.A., Kroner, P.A., and Montgomery, R.R. (2003). Expression of human factor VIII under control of the platelet-specific alphaIIb promoter in megakaryocytic cell line as well as storage together with VWF. Molecular genetics and metabolism *79*, 25-33.

Shi, Q., Wilcox, D.A., Fahs, S.A., Weiler, H., Wells, C.W., Cooley, B.C., Desai, D., Morateck, P.A., Gorski, J., and Montgomery, R.R. (2006). Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. The Journal of clinical investigation *116*, 1974-1982.

Shi, Q., Wilcox, D.A., Morateck, P.A., Fahs, S.A., Kenny, D., and Montgomery, R.R. (2004). Targeting platelet GPIbalpha transgene expression to human megakaryocytes and forming a complete complex with endogenous GPIbbeta and GPIX. Journal of thrombosis and haemostasis : JTH *2*, 1989-1997.

Shirakawa, R., Yoshioka, A., Horiuchi, H., Nishioka, H., Tabuchi, A., and Kita, T. (2000). Small GTPase Rab4 regulates Ca2+-induced alpha-granule secretion in platelets. The Journal of biological chemistry *275*, 33844-33849.

Shivdasani, R.A., Rosenblatt, M.F., Zucker-Franklin, D., Jackson, C.W., Hunt, P., Saris, C.J., and Orkin, S.H. (1995). Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell *81*, 695-704.

Singhal, R., Deng, X., Chenchik, A.A., and Kandel, E.S. (2011). Long-distance effects of insertional mutagenesis. PloS one *6*, e15832.

Sitaram, A., Dennis, M.K., Chaudhuri, R., Jesus-Rojas, W. de, Tenza, D., Setty, S.R.G., Wood, C.S., Sviderskaya, E.V., Bennett, D.C., and Raposo, G., et al. (2012). Differential recognition of a dileucine-based sorting signal by AP-1 and AP-3 reveals a requirement for both BLOC-1 and AP-3 in delivery of OCA2 to melanosomes. Molecular biology of the cell *23*, 3178-3192.

Smith, S.A. (2009). The cell-based model of coagulation. Journal of veterinary emergency and critical care (San Antonio, Tex. : 2001) *19*, 3-10.

Sobota, J.A., Ferraro, F., Bäck, N., Eipper, B.A., and Mains, R.E. (2006). Not all secretory granules are created equal: Partitioning of soluble content proteins. Molecular biology of the cell *17*, 5038-5052.

Socolovsky, M., Nam, H., Fleming, M.D., Haase, V.H., Brugnara, C., and Lodish, H.F. (2001). Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. Blood *98*, 3261-3273.

Soeda, T., Nogami, K., Nishiya, K., Takeyama, M., Ogiwara, K., Sakata, Y., Yoshioka, A., and Shima, M. (2009). The factor VIIIa C2 domain (residues 2228-2240) interacts with the factor IXa Gla domain in the factor Xase complex. The Journal of biological chemistry *284*, 3379-3388.

Solar, G.P., Kerr, W.G., Zeigler, F.C., Hess, D., Donahue, C., Sauvage, F.J. de, and Eaton, D.L. (1998). Role of c-mpl in early hematopoiesis. Blood *92*, 4-10.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science (New York, N.Y.) *241*, 58-62.

Spicer, S.S., Greene, W.B., and Hardin, J.H. (1969). Ultrastructural localization of acid mucosubstance and antimonate-precipitable cation in human and rabbit platelets and megakaryocytes. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society *17*, 781-792.

Stegner, D., and Nieswandt, B. (2011). Platelet receptor signaling in thrombus formation. Journal of molecular medicine (Berlin, Germany) *89*, 109-121.

Stein, S., Ott, M.G., Schultze-Strasser, S., Jauch, A., Burwinkel, B., Kinner, A., Schmidt, M., Krämer, A., Schwäble, J., and Glimm, H., et al. (2010). Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nature medicine *16*, 198-204.

Stenberg, P.E., and Levin, J. (1989). Mechanisms of platelet production. Blood cells *15*, 23-47.

Stenberg, P.E., McEver, R.P., Shuman, M.A., Jacques, Y.V., and Bainton, D.F. (1985). A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. The Journal of cell biology *101*, 880-886.

Stevenson, W.S., Morel-Kopp, M.-C., Chen, Q., Liang, H.P., Bromhead, C.J., Wright, S., Turakulov, R., Ng, A.P., Roberts, A.W., and Bahlo, M., et al. (2013). GFI1B mutation causes a bleeding disorder with abnormal platelet function. Journal of thrombosis and haemostasis : JTH *11*, 2039-2047.

Steward, C.G., Blair, A., Moppett, J., Clarke, E., Virgo, P., Lankester, A., Burger, S.R., Sauer, M.G., Flanagan, A.M., and Pamphilon, D.H., et al. (2005). High peripheral blood progenitor cell counts enable autologous backup before stem cell transplantation for malignant infantile osteopetrosis. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *11*, 115-121.

Summers, R.J., Meeks, S.L., Healey, J.F., Brown, H.C., Parker, E.T., Kempton, C.L., Doering, C.B., and Lollar, P. (2011). Factor VIII A3 domain substitution N1922S results in hemophilia A due to domain-specific misfolding and hyposecretion of functional protein. Blood *117*, 3190-3198.

Sun, J., Hua, B., Chen, X., Samulski, R.J., and Li, C. (2017). Gene Delivery of Activated Factor VII Using Alternative Adeno-Associated Virus Serotype Improves Hemostasis in Hemophiliac Mice with FVIII Inhibitors and Adeno-Associated Virus Neutralizing Antibodies. Human gene therapy *28*, 654-666.

Sun, M., WATANABE, T., Bochimoto, H., Sakai, Y., Torii, S., Takeuchi, T., and Hosaka, M. (2013). Multiple sorting systems for secretory granules ensure the regulated secretion of peptide hormones. Traffic (Copenhagen, Denmark) *14*, 205-218.

Sun, S., Zimmet, J.M., Toselli, P., Thompson, A., Jackson, C.W., and Ravid, K. (2001). Overexpression of cyclin D1 moderately increases ploidy in megakaryocytes. Haematologica *86*, 17-23.

Sunohara, M., Morikawa, S., Sato, T., Miyado, M., Sato, I., Sato, T., and Fuse, A. (2008). Promoter regulatory motifs involved in c-mpl gene expression induced by PMA. Cell biology international *32*, 692-697.

Suske, G. (1999). The Sp-family of transcription factors. Gene *238*, 291-300.

Suzuki, H., Katagiri, Y., Tsukita, S., Tanoue, K., and Yamazaki, H. (1990). Localization of adhesive proteins in two newly subdivided zones in electron-lucent matrix of human platelet ? -granules. Histochemistry *94*.

Svenson, J.L., Chike-Harris, K., Amria, M.Y., and Nowling, T.K. (2010). The mouse and human Fli1 genes are similarly regulated by Ets factors in T cells. Genes and immunity *11*, 161-172.

Tabin, C.J., Hoffmann, J.W., Goff, S.P., and Weinberg, R.A. (1982). Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex virus thymidine kinase gene. Molecular and cellular biology *2*, 426-436.

Tablin, F., Castro, M., and Leven, R.M. (1990). Blood platelet formation in vitro. The role of the cytoskeleton in megakaryocyte fragmentation. Journal of cell science *97 (Pt 1)*, 59-70.

Takayama, M., Fujita, R., Suzuki, M., Okuyama, R., Aiba, S., Motohashi, H., and Yamamoto, M. (2010). Genetic analysis of hierarchical regulation for Gata1 and NF-E2 p45 gene expression in megakaryopoiesis. Molecular and cellular biology *30*, 2668-2680.

Takayama, N., Nishikii, H., Usui, J., Tsukui, H., Sawaguchi, A., Hiroyama, T., Eto, K., and Nakauchi, H. (2008). Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. Blood *111*, 5298-5306.

Takeuchi, T., and Hosaka, M. (2008). Sorting Mechanism of Peptide Hormones and Biogenesis Mechanism of Secretory Granules by Secretogranin III, a Cholesterol-Binding Protein, in Endocrine Cells. CDR *4*, 31-38.

Tanguy, E., Carmon, O., Wang, Q., Jeandel, L., Chasserot-Golaz, S., Montero-Hadjadje, M., and Vitale, N. (2016). Lipids implicated in the journey of a secretory granule: from biogenesis to fusion. Journal of neurochemistry *137*, 904-912.

Taupenot, L., Harper, K.L., and O'Connor, D.T. (2005). Role of H+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function. The Journal of biological chemistry *280*, 3885-3897.

Teofili, L., and Larocca, L.M. (2011). Advances in understanding the pathogenesis of familial thrombocythaemia. British journal of haematology *152*, 701-712.

Thomas, M.R., and Storey, R.F. (2015). The role of platelets in inflammation. Thrombosis and haemostasis *114*, 449-458.

Thomas, P., and Smart, T.G. (2005). HEK293 cell line: a vehicle for the expression of recombinant proteins. Journal of pharmacological and toxicological methods *51*, 187-200.

Thompson, A., Zhao, Z., Ladd, D., Zimmet, J., and Ravid, K. (1996). A new transgenic mouse model for the study of cell cycle control in megakaryocytes. Stem cells (Dayton, Ohio) *14 Suppl 1*, 181-187.

Thompson, A.R. (2003). Structure and function of the factor VIII gene and protein. Seminars in thrombosis and hemostasis *29*, 11-22.

Thon, J.N., Montalvo, A., Patel-Hett, S., Devine, M.T., Richardson, J.L., Ehrlicher, A., Larson, M.K., Hoffmeister, K., Hartwig, J.H., and Italiano, J.E. (2010). Cytoskeletal mechanics of proplatelet maturation and platelet release. The Journal of cell biology *191*, 861-874.

Thon, J.N., Peters, C.G., Machlus, K.R., Aslam, R., Rowley, J., Macleod, H., Devine, M.T., Fuchs, T.A., Weyrich, A.S., and Semple, J.W., et al. (2012). T granules in human platelets function in TLR9 organization and signaling. The Journal of cell biology *198*, 561-574.

Tiedt, R., Schomber, T., Hao-Shen, H., and Skoda, R.C. (2007). Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. Blood *109*, 1503-1506.

TILL, J.E., and McCULLOCH, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation research *14*, 213-222.

TOCANTINS, L.M. (1938). THE MAMMALIAN BLOOD PLATELET IN HEALTH AND DISEASE. Medicine *17*, 155.

Todeschini, A.-L., Georges, A., and Veitia, R.A. (2014). Transcription factors: specific DNA binding and specific gene regulation. Trends in genetics : TIG *30*, 211-219.

Toole, J.J., Pittman, D.D., Orr, E.C., Murtha, P., Wasley, L.C., and Kaufman, R.J. (1986). A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. Proceedings of the National Academy of Sciences of the United States of America *83*, 5939-5942.

Torashima, T., Okoyama, S., Nishizaki, T., and Hirai, H. (2006). In vivo transduction of murine cerebellar Purkinje cells by HIV-derived lentiviral vectors. Brain research *1082*, 11-22.

Toscano, M.G., Frecha, C., Benabdellah, K., Cobo, M., Blundell, M., Thrasher, A.J., Garcia-Olivares, E., Molina, I.J., and Martin, F. (2008). Hematopoietic-specific lentiviral vectors circumvent cellular toxicity due to ectopic expression of Wiskott-Aldrich syndrome protein. Human gene therapy *19*, 179-197.

Totani, L., and Evangelista, V. (2010). Platelet-leukocyte interactions in cardiovascular disease and beyond. Arteriosclerosis, thrombosis, and vascular biology *30*, 2357-2361.

Traver, D., Akashi, K., Manz, M., Merad, M., Miyamoto, T., Engleman, E.G., and Weissman, I.L. (2000). Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. Science (New York, N.Y.) *290*, 2152-2154.

Ungewickell, E.J., and Hinrichsen, L. (2007). Endocytosis: clathrin-mediated membrane budding. Current opinion in cell biology *19*, 417-425.

Urban, D., Li, L., Christensen, H., Pluthero, F.G., Chen, S.Z., Puhacz, M., Garg, P.M., Lanka, K.K., Cummings, J.J., and Kramer, H., et al. (2012). The VPS33B-binding protein VPS16B is required in megakaryocyte and platelet α-granule biogenesis. Blood *120*, 5032-5040.

Uren, A.G., Kool, J., Berns, A., and van Lohuizen, M. (2005). Retroviral insertional mutagenesis: past, present and future. Oncogene *24*, 7656-7672.

Uzan, G., Prenant, M., Prandini, M.H., Martin, F., and Marguerie, G. (1991). Tissue-specific expression of the platelet GPIIb gene. The Journal of biological chemistry *266*, 8932-8939.

van den Biggelaar, M., Bouwens, E.A.M., Voorberg, J., and Mertens, K. (2011). Storage of factor VIII variants with impaired von Willebrand factor binding in Weibel-Palade bodies in endothelial cells. PloS one *6*, e24163.

van den Biggelaar, M., Meijer, A.B., Voorberg, J., and Mertens, K. (2009). Intracellular cotrafficking of factor VIII and von Willebrand factor type 2N variants to storage organelles. Blood *113*, 3102-3109.

van den Oudenrijn, S, Bruin, M., Folman, C.C., Peters, M., Faulkner, L.B., Haas, M. de, and von dem Borne, A E (2000). Hm5074 441.448 // Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. British journal of haematology *110*, 441-448.

van Nispen tot Pannerden, H., Haas, F. de, Geerts, W., Posthuma, G., van Dijk, S., and Heijnen, H.F.G. (2010). The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes. Blood *116*, 1147-1156.

Vehar, G.A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D.P., Rotblat, F., Oppermann, H., Keck, R., Wood, W.I., and Harkins, R.N., et al. (1984). Structure of human factor VIII. Nature *312*, 337-342.

Velten, L., Haas, S.F., Raffel, S., Blaszkiewicz, S., Islam, S., Hennig, B.P., Hirche, C., Lutz, C., Buss, E.C., and Nowak, D., et al. (2017). Human haematopoietic stem cell lineage commitment is a continuous process. Nature cell biology *19*, 271-281.

Verhoeyen, E., and Cosset, F.-L. (2004). Surface-engineering of lentiviral vectors. The journal of gene medicine *6 Suppl 1*, S83-94.

Waehler, R., Russell, S.J., and Curiel, D.T. (2007). Engineering targeted viral vectors for gene therapy. Nature reviews. Genetics *8*, 573-587.

Wagner, D.D., Mayadas, T., and Marder, V.J. (1986). Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. The Journal of cell biology *102*, 1320-1324.

Wagner, D.D., Saffaripour, S., Bonfanti, R., Sadler, J.E., Cramer, E.M., Chapman, B., and Mayadas, T.N. (1991). Induction of specific storage organelles by von Willebrand factor propolypeptide. Cell *64*, 403-413.

Wahlers, A., Zipfel, P.F., Schwieger, M., Ostertag, W., and Baum, C. (2002). In vivo analysis of retroviral enhancer mutations in hematopoietic cells: SP1/EGR1 and ETS/GATA motifs contribute to long terminal repeat specificity. Journal of virology *76*, 303-312.

Walther, W., and Stein, U. (2000). Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs *60*, 249-271.

Wang, H., Ge, W., Zhuang, Y., Fu, J., Li, D., and Ju, X. (2018). Fast recovery of platelet production in NOD/SCID mice after transplantation with ex vivo expansion of megakaryocyte from cord blood CD34+ cells. Journal of cancer research and therapeutics *14*, 233-239.

Wang, X., Shin, S.C., Chiang, A.F., Khan, I., Pan, D., Rawlings, D.J., and Miao, C.H. (2015). Intraosseous delivery of lentiviral vectors targeting factor VIII expression in platelets corrects murine hemophilia A. Molecular therapy : the journal of the American Society of Gene Therapy.

Wanisch, K., and Yáñez-Muñoz, R.J. (2009). Integration-deficient lentiviral vectors: a slow coming of age. Molecular therapy : the journal of the American Society of Gene Therapy *17*, 1316-1332.

Ward, D.M., Griffiths, G.M., Stinchcombe, J.C., and Kaplan, J. (2000). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. Traffic (Copenhagen, Denmark) *1*, 816-822.

Ware, J., Russell, S.R., Marchese, P., and Ruggeri, Z.M. (1993). Expression of human platelet glycoprotein Ib alpha in transgenic mice. The Journal of biological chemistry *268*, 8376-8382.

Wei, C.M., Gibson, M., Spear, P.G., and Scolnick, E.M. (1981). Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. Journal of virology *39*, 935-944.

Wei, M.L. (2006). Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. Pigment cell research *19*, 19-42.

Weyrich, A.S. (2014). Platelets: more than a sack of glue. Hematology. American Society of Hematology. Education Program *2014*, 400-403.

Weyrich, A.S., Schwertz, H., Kraiss, L.W., and Zimmerman, G.A. (2009). Protein synthesis by platelets: historical and new perspectives. Journal of thrombosis and haemostasis : JTH *7*, 241-246.

White, J.G. (2013). Platelet Structure. In Platelets (Elsevier), pp. 117–144.

Wicke, D.C., Meyer, J., Buesche, G., Heckl, D., Kreipe, H., Li, Z., Welte, K.H., Ballmaier, M., Baum, C., and Modlich, U. (2010). Gene therapy of MPL deficiency: challenging balance between leukemia and pancytopenia. Molecular therapy : the journal of the American Society of Gene Therapy *18*, 343-352.

Wilcox, D.A., Olsen, J.C., Ishizawa, L., Bray, P.F., French, D.L., Steeber, D.A., Bell, W.R., Griffith, M., and White, G.C. (2000). Megakaryocyte-targeted synthesis of the integrin beta(3)-subunit results in the phenotypic correction of Glanzmann thrombasthenia. Blood *95*, 3645-3651.

Wilcox, D.A., Olsen, J.C., Ishizawa, L., Griffith, M., and White, G.C. (1999). Integrin alpha IIb promoter-targeted expression of gene products in megakaryocytes derived from retrovirus-transduced human hematopoietic cells. Proceedings of the National Academy of Sciences *96*, 9654-9659.

Wise, R.J., Pittman, D.D., Handin, R.I., Kaufman, R.J., and Orkin, S.H. (1988). The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. Cell *52*, 229-236.

WRIGHT, J.H. (1906). The Origin and Nature of the Blood Plates. The Boston Medical and Surgical Journal *154*, 643-645.

Wu, Z., Yang, H., and Colosi, P. (2010). Effect of genome size on AAV vector packaging. Molecular therapy : the journal of the American Society of Gene Therapy *18*, 80-86.

Wunderlich, Z., and Mirny, L.A. (2009). Different gene regulation strategies revealed by analysis of binding motifs. Trends in genetics : TIG *25*, 434-440.

Xi, J., Zhu, H., Liu, D., Nan, X., Zheng, W., Liu, K., Shi, W., Chen, L., Lv, Y., and Yan, F., et al. (2013). Infusion of megakaryocytic progenitor products generated from cord blood hematopoietic stem/progenitor cells: results of the phase 1 study. PloS one *8*, e54941.

Xu, G., Kanezaki, R., Toki, T., Watanabe, S., Takahashi, Y., Terui, K., Kitabayashi, I., and Ito, E. (2006). Physical association of the patient-specific GATA1 mutants with RUNX1 in acute megakaryoblastic leukemia accompanying Down syndrome. Leukemia *20*, 1002-1008.

Yarovoi, H.V., Kufrin, D., Eslin, D.E., Thornton, M.A., Haberichter, S.L., Shi, Q., Zhu, H., Camire, R., Fakharzadeh, S.S., and Kowalska, M.A., et al. (2003). Factor VIII ectopically expressed in platelets: efficacy in hemophilia A treatment. Blood *102*, 4006-4013.

Yasumoto, A., Madoiwa, S., Kashiwakura, Y., Ishiwata, A., Ohmori, T., Mizukami, H., Ozawa, K., Sakata, Y., and Mimuro, J. (2013). Overexpression of factor VII ameliorates bleeding diathesis of factor VIII-deficient mice with inhibitors. Thrombosis research *131*, 444-449.

Yoshihara, H., Arai, F., Hosokawa, K., Hagiwara, T., Takubo, K., Nakamura, Y., Gomei, Y., Iwasaki, H., Matsuoka, S., and Miyamoto, K., et al. (2007). Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell stem cell *1*, 685-697.

Youssefian, T., and Cramer, E.M. (2000). Megakaryocyte dense granule components are sorted in multivesicular bodies. Blood *95*, 4004-4007.

Youssefian, T., Massé, J.M., Rendu, F., Guichard, J., and Cramer, E.M. (1997). Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb-IIIa. Blood *89*, 4047-4057.

Yu, S.F., Rüden, T. von, Kantoff, P.W., Garber, C., Seiberg, M., Rüther, U., Anderson, W.F., Wagner, E.F., and Gilboa, E. (1986). Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proceedings of the National Academy of Sciences of the United States of America *83*, 3194-3198.

Zarogoulidis, P., Darwiche, K., Sakkas, A., Yarmus, L., Huang, H., Li, Q., Freitag, L., Zarogoulidis, K., and Malecki, M. (2013). Suicide Gene Therapy for Cancer - Current Strategies. Journal of genetic syndromes & gene therapy *4*.

Zhang, C.-F., Dhanvantari, S., Lou, H., and Loh, Y.P. (2003). Sorting of carboxypeptidase E to the regulated secretory pathway requires interaction of its transmembrane domain with lipid rafts. The Biochemical journal *369*, 453-460.

Zhang, G., Shi, Q., Fahs, S.A., Kuether, E.L., Walsh, C.E., and Montgomery, R.R. (2010). Factor IX ectopically expressed in platelets can be stored in alpha-granules and corrects the phenotype of hemophilia B mice. Blood *116*, 1235-1243.

Zhang, X., Chen, L., Bancroft, D.P., Lai, C.K., and Maione, T.E. (1994). Crystal structure of recombinant human platelet factor 4. Biochemistry *33*, 8361-8366.

Zhang, X., and Godbey, W.T. (2006). Viral vectors for gene delivery in tissue engineering. Advanced drug delivery reviews *58*, 515-534.

Zhu, J., and Emerson, S.G. (2002). Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene *21*, 3295-3313.

Zimmerman, G.A., and Weyrich, A.S. (2008). Signal-dependent protein synthesis by activated platelets: new pathways to altered phenotype and function. Arteriosclerosis, thrombosis, and vascular biology *28*, s17-24.

Zucker-Franklin, D. (1981). Endocytosis by human platelets: metabolic and freeze-fracture studies. The Journal of cell biology *91*, 706-715.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. Journal of virology *72*, 9873-9880.

Publications

Publication I

Journal of Thrombosis and Haemostasis, **15**: 341–355 DOI: 10.1111/jth.13582

ORIGINAL ARTICLE

Targeting expression to megakaryocytes and platelets by lineage-specific lentiviral vectors

L. J. LATORRE-REY,* S. WINTTERLE,† S. DÜTTING,‡ S. KOHLSCHEEN,* T. ABEL,§ F. SCHENK,* S. WINGERT,¶ M. A. RIEGER,¶ B. NIESWANDT,‡ N. HEINZ*[1] and U. MODLICH*

**Research Groups for Gene Modification in Stem Cells, LOEWE Center for Cell and Gene Therapy Frankfurt/Main, Paul-Ehrlich-Institute, Langen; †Institute of Experimental Hematology, Hannover Medical School, Hannover; ‡Department of Experimental Biomedicine-Vascular Medicine, University Hospital and Rudolf Virchow Center, University of Würzburg, Würzburg; §Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institute, Langen; and ¶LOEWE Center for Cell and Gene Therapy and Department of Medicine, Hematology/Oncology, Goethe University Frankfurt, Frankfurt am Main, Germany*

To cite this article: Latorre-Rey LJ, Wintterle S, Dütting S, Kohlscheen S, Abel T, Schenk F, Wingert S, Rieger MA, Nieswandt B, Heinz N, Modlich U. Targeting expression to megakaryocytes and platelets by lineage-specific lentiviral vectors. *J Thromb Haemost* 2017; **15**: 341–55.

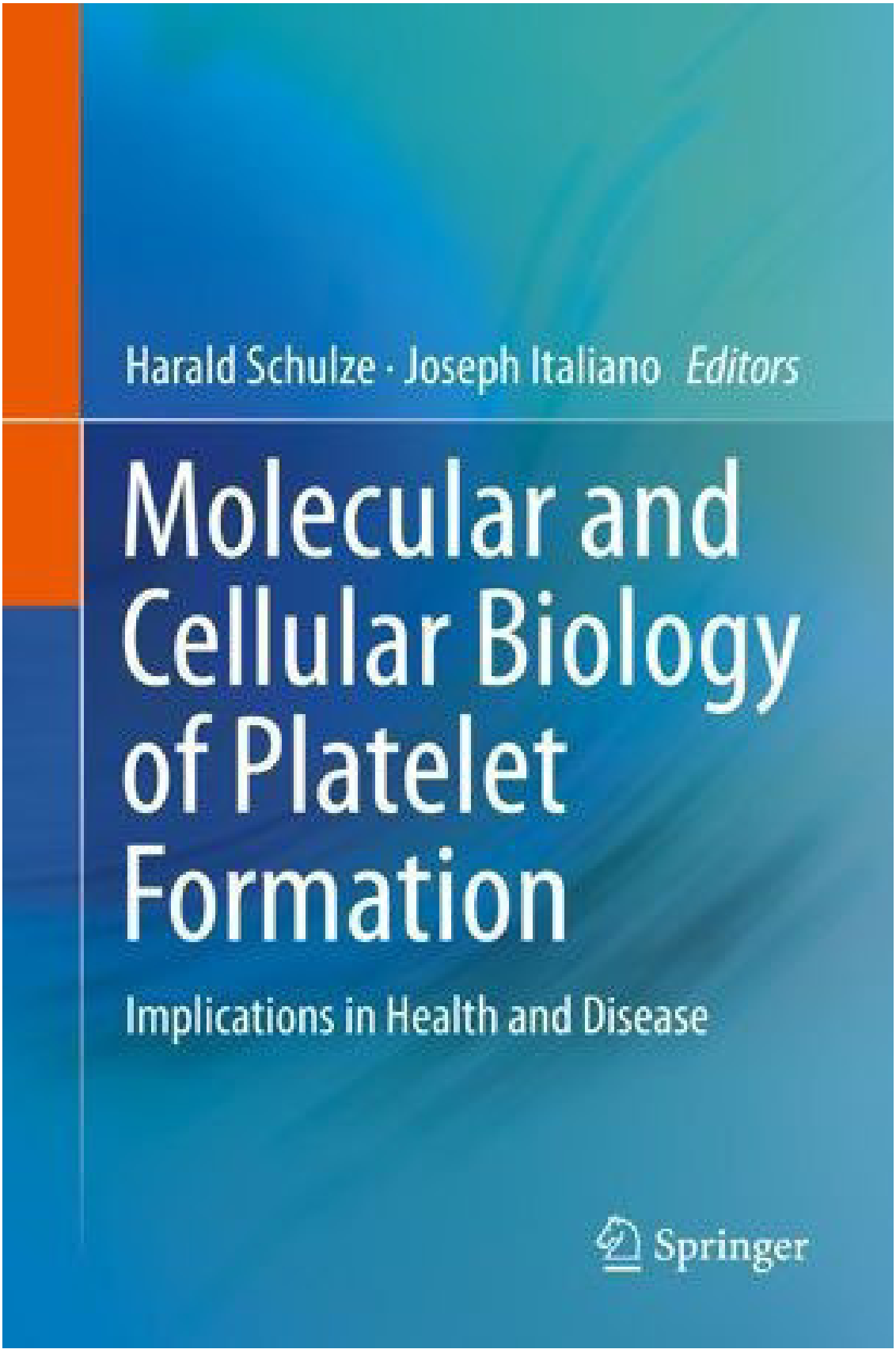
Essentials

- Platelet phenotypes can be modified by lentiviral transduction of hematopoietic stem cells.
- Megakaryocyte-specific lentiviral vectors were tested *in vitro* and *in vivo* for restricted expression.
- The glycoprotein 6 vector expressed almost exclusively in megakaryocytes.
- The platelet factor 4 vector was the strongest but with activity in hematopoietic stem cells.

Summary. *Background:* Lentiviral transduction and transplantation of hematopoietic stem cells (HSCs) can be utilized to modify the phenotype of megakaryocytes and platelets. As the genetic modification in HSCs is transmitted onto all hematopoietic progenies, transgene expression from the vector should be restricted to megakaryocytes to avoid un-physiologic effects by ectopic transgene expression. This can be achieved by lentiviral vectors that control expression by lineage-specific promoters. *Methods:* In this study, we introduced promoters of megakaryocyte/platelet-specific genes, namely human glycoprotein 6 (*hGP6*) and *hGP9*, into third generation lentiviral vectors and analyzed their functionality *in vitro* and *in vivo* in bone marrow transplantation assays. Their specificity and efficiency of expression was compared with lentiviral vectors utilizing the promoters of murine platelet factor 4 (*mPf4*) and *hGP1BA*, both with strong activity in megakaryocytes (MKs) used in earlier studies, and the ubiquitously expressing phosphoglycerate kinase (hPGK) and spleen focus forming virus (SFFV) enhancer/promoters. *Results:* Expression from the mPf4 vector in MKs and platelets was the strongest similar to expression from the viral SFFV promoter, however, the mPf4 vector, also exhibited considerable off-target expression in hematopoietic stem and progenitor cells. In contrast, the newly generated *hGP6* vector was highly specific to megakaryocytes and platelets. The specificity was also retained when reducing the promoter size to 350 bp, making it a valuable new tool for lentiviral expression in MKs/platelets. *Conclusion:* MK-specific vectors express preferentially in the megakaryocyte lineage. These vectors can be applied to develop murine models to study megakaryocyte and platelet function, or for gene therapy targeting proteins to platelets.

Keywords: bone marrow transplantation; gene transfer techniques; lentivirus; megakaryocytes; platelets.

Publication II



Strategies for the Gene Modification of Megakaryopoiesis and Platelets

Lisette Johana Latorre Rey and Ute Modlich

Research Group for Gene Modification in Stem Cells, LOEWE Centre for Cell and Gene Therapy Frankfurt, Paul-Ehrlich-Institute, Langen, Germany

1. Gene modifications in megakaryocytes and platelets

Platelets are the central players controlling blood hemostasis. Deregulation of their function leads to various bleeding disorders in human patients with sometimes life threatening symptoms. To develop therapeutic approaches for these patients, we have to understand the mechanisms behind the diseases.

Murine models can be employed to study the physiological role of disease-related proteins. One of the widely used models is the gene knockout. Alternatively, genes can be introduced into the genome as transgenic cassettes, or can be inserted into the locus of a cellular geneby a knock-in approach. Due to new gene targeting methods that developed in the last years, gene modification can be introduced much faster than the classical homologous recombination.

Germline modification can be laborious and time consuming. In many cases, scientists wish for faster models and target the adult cells rather than developing a new mouse model with the appropriate modification. However, as platelets are anuclear cells and do not contain a DNA genome, their genetic modification is difficult. Therefore, the gene has to be introduced at earlier stages such as the MK progenitors or the hematopoietic stem cells (HSC). For the full reconstitution of the hematopoietic system after transplantation, long-term HSC have to be modified. In addition, the modification has to be stable and transmitted faithfully onto the daughter cells after cell division. Therefore, in general for these approaches integrating vectors, such as the retroviral vectors, will be employed.

In this chapter we will introduce the different gene modification strategies and models that can be employed to modify megakaryopoiesis and platelets. Many of these approaches are widely used in biological science, however, we will elucidate their use with a special emphasis on their performance in platelet biology.

2. Germline modifications: knockout models

To study the function of genes and pathways in development or disease, model systems with gene deletion (gene knockout) are wildly used. The knockout approach is based on the removal of an important segment of the gene that leads to the disruption of the open reading frame, with the consequent abolition of the expression of a functional gene product. The deletion can be permanent (constitutive) or inducible (conditional) when the effect is desired in a temporal and tissue specific manner. The knockout technology has allowed for the development of numerous mouse strains and cell lines in which it is possible to study gene functions, signaling pathways and disease modeling. These genetic modifications are introduced mainly at the embryonic stem cells level. Huge efforts have been invested with the aim of engineering more accurate models since the first knockouts were published.

Modifications introduced in embryonic stem (ES) cells can generate a genetically modified mouse due to their capacity to colonize the germ line and to develop a complete organism (mouse) post-implantation in a foster mother (Robertson, 1991). Mainly male ES cell lines (XY) are modified, since the XY karyotype appears to be more stable in culture and generate many more offspring than female (XY) karyotype (Mansour, 1990; Kuno et al., 2015). During the modification and prior to implantation, ES cells are most commonly culture on mitotically inactive feeder cells (MEFs) in order to maintain their germline properties after genetic manipulation, although there are some feeder-independent ES lines that show high germline competence (Ying et al., 2008; Limaye et al., 2009).

Genetic modification of ES cells in classical knockouts is performed by homologous recombination (Smithies et al., 1985; Wong and Capecchi, 1986; Capecchi, 1989); by taking advantage of the cellular repair machinery, it is possible to align and target a donor engineered plasmid with the corresponding region of homology within the genome, inducing recombination and the replacement of the wild type genetic locus, by (usually) a drug resistance gene, leading to the removal of an essential DNA region of a gene, disrupting the gen´s open reading frame (Hall et al., 2009) (**Figure 1A**). Targeting constructs usually contain the coding sequence of a positive drug selection maker to enrich for the population that has successfully recombined with the target sequence. This resistance gene is mostly the neomycin resistance gene (neo^R), which is an aminoglycoside phosphotransferase that interferes with protein synthesis in eukaryotic cells. In the targeting plasmid, the recombinant DNA segment is flanked by 2 arms which are homologous with the target sequence of the gene to be deleted (Southern and Berg, 1982). Although only 2kb are required, 6 to 14 kb of homology regions are typically used (Melton, 2002). However, in cases where the mutation to be introduced leads to the replacement of an

essential exon by the neo^R without including a polyadenylation signal, downstream exons of the gene could be also transcribed and spliced. To avoid this problem, neo^R is often transcribed in the opposite direction to the wild type gene. In these cases, a strong promoter drives the expression of the neo^R. The inverted orientation of the neo^R also ensures that the strong promoter will have no transcriptional effect on any genes downstream of the knockout (Müller, 1999).

To increase the accuracy of selection, in most cases the donor plasmids also include a negative selection marker outside of the homology arms in order to discard random integrated clones (Mansour et al. 1988)(**Figure 1A**). For example, the *herpes simplex virus* thymidine kinase (HSV-tk) can be used for negative selection as it phosphorylates drugs like gancyclovir or FIAU (1-[2′-deoxy-2′-fluoro- β-D-arabinofuranosyl]-5-iodouracil) into cytotoxic compounds in HSV-TK positive cells thereby eliminating random integrated recombinant clones (Hall et al., 2009).

Many methods can be used for the introduction of the DNA into the ES cells, including calcium-phosphate precipitation, electroporation and nuclear microinjection (Mansour, 1990). However, regardless the low rate of stable recombinants obtained by electroporation (10^{-5}-10^{-2}), it is the most often used technique, because large amounts of cell can be transfected. In contrast, nuclear microinjection has the advantages of being the more efficient method (10-20% of the cells become stable transformed) (Mansour, 1990), and leading to a better targeting ratio (1:15 targeted recombinants to random integration (Hall et al., 2009)).To identify clones with the desired recombination event, screenings usually by Southern blot and PCR analysis are needed. Clones with random integration of the complete vector are excluded upon drug treatment. Once a stem cell with the designated genetic alteration is identified, the ES cell clone is then expanded and injected into a blastocyst and implanted into a foster mother. The mice obtained from the mating of chimeric mice with the wild-type mice, will be either wild-type or heterozygous for the targeted mutation, and these heterozygous mice must be bred to homozygosity in the descendent (Babinet and Cohen-Tannoudji, 2001).

3. Conditional knockout systems

In some cases, constitutive knockout mouse models die at early fetal-embryonic stages or off springs are not viable because the permanent deletion of the respective gene may be detrimental for early stages in development. To overcome this problem, gene deletion has to take place at later stages of development or even in the adult mouse. To achieve this, knockout systems were developed in which a spatial-temporal targeting is possible. This new knockout

systems are based on the work of Sternberg and Hamilton who by studying the bacteriophage PI site-specific recombination, identified the two important elements for the recombination event in a bacterial free system (Sternberg and Hamilton, 1981): a site called *loxP*, a 34 base-pair sequence containing two 13 base-pair inverted repeats, separated by an eight base-pair spacer region (Hoess and Abremski, 1984) which must be present in both sites of the recombining cassette. The other element identified was an enzyme that mediates the recombination event codified by the PI gene called *Cre* (*cyclization recombination or cause recombination*). Cre Recombinase is a protein of 343 amino with two distinct domains. The amino terminal domain contains residues 20−129 and it is form by 5 alpha helixes, two of these make contact with the loxP sites. The carboxy terminal domain of the enzyme consists amino acids 132–341 and it harbors the active site of the enzyme (Guo et al., 1997). Under this premise, any cassette or gene flanked by two loxP sites and in the presence of the Cre enzyme would recombine and be excised (Deng, 2012) (**Figure 1B**). In this approach, cassettes desired to recombine very often contain a selection marker and mark the germline-modified mice with floxed alleles (in this moment phenotypically wild type). These mice have to be bred to Cre expressing mice to allow the generation of the knockout and open up the possibility of tissue-specific and inducible knockouts. To achieve this, the Cre has to be expressed in a tissue-specific manner, e.g. expression of the Cre is controlled by a tissue specific promoter or knocked into the locus of a tissue-specific gene, or in an inducible manner when expressed by an inducible promoter.

Another attractive feature of the Cre system is that several types of recombination events can be produced depending on whether the *loxP* are placed in the same DNA molecule (recombination in Cis) or in different molecules (recombination in trans). Also the *loxP* site orientation is important, since it is possible to generate insertions, inversions, translocations, and single point mutations. If the two *loxP* sites have the same orientation, the DNA region situated between these *loxP* sites is deleted during recombination. If the orientation of the two *loxP* sites is opposed to each other, recombination leads to the inversion of the region comprised between the two sites; when the *loxP* sites are both integrated in different place in the genome, recombination in trans induces chromosomal rearrangements. More rare events but also possible, is the generation of insertions, if one *loxP* site is integrated in the genome and the other is carried by a circular plasmid; in this case, an insertion of the sequence carried by the plasmid can be generated. However, these rearrangements are not so easy to generate, and therefore require accurate selection methods (Babinet and Cohen-Tannoudji, 2001).

Although less often used than the Cre recombination system, others recombinases are characterized. In this sense O'Gorman and collaborators in 1991 described a recombination system based on the site-specific recombinase, FLP-1 from *Saccharomyces cerevisiae*

(O'Gorman et al., 1991) which can also mediate recombination events in embryonic stem cells (Dymecki, 1996; Buchholz et al., 1998). The Flp-recombinase (flippase) consists of a 13-kDa NH_2-terminal domain (p13) and a larger 32-kDa COOH-terminal domain (p32) with the major determinants for DNA binding, and was originally isolated by Broach and Hicks (Broach and Hicks, 1980). The nucleotide sequence of the flippase recognition target (*FRT)* site is 34-bp-long. The site contains two palindromic sequences of 13 bp each, separated by a central and asymmetric sequence of 8 bp, or spacer, which defines the orientation of the site; The recombinase binds the palindromic sequences whereas the spacer is the site of DNA break, exchange and ligation (Cox, 1983; Turan et al., 2011).

Similar to the Cre-LoxP recombinase system, the position and orientation of the two *FRT* sites will also determine the outcome of the FLP-mediated recombination. FLP excises DNA fragments as a circular molecule, when the DNA target region is located between two *FRT* sites having the same orientation. The excision reaction is reversible, however; the reintegration of the excised circle into the linear product is kinetically less favorable than excision of the circle; and excision products may accumulate with time (Turan et al., 2011). Inversions of DNA segments can be generated, when they are located between two *FRT* sites in opposite orientation. FLP can also mediates recombination between two *FRT* sites located on different or non-homologous chromosomes; if this is the case, FLP will exchanges the chromosomal sequences located downstream of the sites, leading to a reciprocal translocation. To insert a circle into a linear chromosome or to generate an inversion event, *FRT* variants can be also engineered to allow a single and irreversible single strand recombination (SSR) event (Lacroix et al., 2011).

By sequencing of the 7 kb *immC* region from four P1-related phages, Sauer and McDermont identified a novel DNA tyrosine recombinase from the phage D6, which is closely related to the P1 Cre recombinase, including the capacity of recombination in mammalian cells, but differs from Cre in their DNA recognition site (Sauer and McDermott, 2004). In this case, the Dre recombinase presents DNA specificity for a 32 bp DNA site, called *rox,* and no host bacterial proteins are required for efficient Dre-mediated site-specific DNA recombination. Later on Anastassiadis and collaborators developed a more sophisticated Dre system, called “Dre-PBD” which provided an efficient and tight controlled system for the inducible expression of Dre recombinase activity in mammalian cells. In their study, they generated a Dre recombinase fused to the PBD (progesterone ligand binding domain) in a targeting construct, using a PBD that had been truncated at the C-terminus in order to induce the recombination event in a

RU486- dependent manner in both fibroblast and ES reporter cells (Anastassiadis et al., 2009; Anastassiadis et al., 2013).

4. Spatial-temporal and tissue-specific knockouts to model platelet disease

Cre-expression can be tissue-specific or temporally controlled based on the expression from inducible promoters (e.g. tet-responsive promoter) or by the generation of ligand-dependent chimeric Cre recombinases (e.g. tamoxifen-inducible) (**Figure 2**).

One inducible model was developed by Feil and collaborators (Feil et al., 1996; Feil et al., 1997) who fused the Cre recombinase to the mutated human estrogen receptor (ER) ligand-binding domain (LBD), and therefore, expression of Cre can be regulated by the addition of tamoxifen (Feil et al., 1997). Tamoxifen is administered to the mice either by injection or via their food. This inducible system, for example, has been used to study the conditional ablation of Gata-1 (Gutiérrez et al., 2008). Another model is based on the combination of the Cre-*loxP* system with tetracycline-mediated regulation of gene expression (**Figure 2B**). This approach allowed the spatial and temporal controlled expression of the Cre recombinase. In this inducible model, Utomo and collaborators, for example,used the *retinoblastoma* (RB) or the *whey acidic protein* (WAP) promoters to drive expression of the rtTA (for tissue specific expression), while the Cre expression was controlled by the tetO-hCMV promoter (for temporal induction). Without doxycycline, rtTA is inactive and unable to induce transcription of Cre. In the presence of doxycycline, rtTA binds to the tetO-hCMV promoter leading to Cre expression (Utomo et al., 1999). Making use of the transgenic mouse expressing the rtTA from the CMV promoter, the efficient expression of a miR downregulating the Bcl-XL antiapoptotic gene induced thrombocytopenias in mice (Takiguchi et al., 2010). Bcl-XL is known to be essential for maintaining platelet survival (Kile, 2014) The rtTA can also be expressed in a MK specific fashion (Nguyen et al., 2005). In one mouse model, the rtTA is expressed by a 1.1 kb fragment of the rat Pf4 promoter previously described by Ravid and colleagues (Ravid et al., 1991a) (**Figure 2D**). Using the Pf4-rtTa mouse, e.g. the role of survivin in megakaryopoiesis and platelets was studied (McCrann et al., 2008).

In another inducible approach developed by Kühn and colleagues, the expression of the Cre recombinase was placed under the control of the Mx1 promoter. The *myxovirus resistance 1* (Mx1) promoter can be transiently activated in many tissues upon administration of interferon (IFN-α and IFN-β) (Hug et al., 1988) or by a synthetic double-stranded RNA polyinosinic-polycytidylic acid, also called pI-pC or poly(I:C), which induces the cells' response to viral infections and with this induces type-1 interferones. Therefore, those mice carrying a floxed

target gene and a Mx1-cre transgene, will only develop the targeted modification after the administration of interferons or the poly(I:C) compound (Kuhn et al., 1995) (**Figure 2A**).

Besides the transgenic approaches Cre can also be delivered by viral vectors. Anton and Graham developed a replication deficient human adenovirus type 5 vector expressing the Cre recombinase gene under the control of the human cytomegalovirus immediate-early promoter (CMV, vector named AdCre). Transient expression of Cre after co-infection of 293 cells with the AdCre andadenoviral vector carrying the luciferase cDNA whose expression was blocked by the presence of an external floxed DNA sequence, turned on luciferase expression after excision of this floxed sequence (Anton and Graham, 1995). Following this initial demonstration of the concept, AdCre has been exploited for in vivo Cre delivery into different tissues (Akagi et al., 1997), or for ex vivo delivery, for example, into T-cells (Zha et al., 2008).

The most wildly used application of the Cre inducible system for the study of gene function in platelets is the transgenic mouse expressing Cre under the control of the murine platelet factor 4 (Pf4) promoter generated by (Tiedt et al., 2007) (**Figure 2C**). In this mouse, a codon-optimized Cre was inserted in the position of the first exon and expressed from the murine Pf4 promoter that was included as transgenic cassette into the mouse genome. The upstream and downstream sequence consists of about 100 kb in total, also containing four reading frames of further genes (*Cxcl5*, *Cxcl7 (Ppbp, pro-platelet basic protein),*Cxcl3 and *Cxcl15*). Functionality and specificity of the Cre recombinase in the Pf4-Cre mouse was tested by breeding to the Rosa26 LacZ reporter strain (Soriano, 1999) and to the *β1*-flox/flox mouse (Graus-Porta et al., 2001) . Tiedt et al. demonstrate the megakaryocyte-specific LacZ expression and the prevention of the lethal phenotype of the complete (constitutive) integrin β1 knockout by the lineage-specific deletion of the integrin β1 only in megakaryopoiesis . In contrast, Calaminus and collaborators analyzed the specificity of the Pf4-Cre mouse by intercrossing to a reporter line (Rosa26-tdRFP;Pf4-Cre) and reported significant levels of recombination in both fetal liver and bone marrow HSCs. They also demonstrate that Cre activity under the Pf4 extends to other blood cell lineage (myeloid and lymphoid) and up to 50% in the HSC compartment (Calaminus et al., 2012). However, in a very similar approach by Ng et al., analyzing Pf4 Cre mice crossed with mice carrying a floxed Rosa EYFP locus, did not detect recombination outside the megakaryocytic lineage (Ng et al., 2014). Most recently, Pertuy and colleagues (Pertuy et al., 2015) detected intestinal epithelial cells in the distal colon to have recombined their floxed genomic locus under the Pf4 Cre conditions. Also, 80% of the Apc flox/flox;Pf4-cre mice developed APC depletion-induced tumors. In the blood, they detected recombined cells only very rarely (~0.3%). Positive cells were found as a subset of tissue-infiltrating leukocytes which were positive for F4/80, and therefore part of the monocyte/macrophage lineage. The activation

of immune cells from the spleen with PMA also increased the number of cell that underwent Cre mediated recombination, as measured by the GFP reporter expression.

In addition to Pf4, also CD41 (integrin alpha 2b, glycoprotein IIb) is a suitable marker for adult megakaryocytes and platelets and a CD41-Cre transgenic mouse was generated. In a first approach, ~5 kb sequence of the murine *GPIIb* gene, (2.5 kb of the upstream and 2.8 kb containing the first exon and intron sequences) was used to express nls-Cre in transgenic mice (Emambokus and Frampton, 2003). In agreement with CD41 expression during hematopoietic development, recombination could be detected in the yolk sac (day 8.5), AGM and dorsal aorta (day 9.5) and the fetal liver which at day 12.5 contained ~60% recombined hematopoietic cells. Surprisingly, in the adult hematopoiesis in two month old mice, recombined cells contribute with below 5%. This was a very surprising observation and could be explained in two ways (1) only a subset of the CD41 positive fetal HSC give rise to the definitive adult HSC and/or (2) a second wave of fetal HSC arises that does not undergo a CD41 positive stage. In the same study, a Cre knock in into the first exon of the endogenous alpha 2b locus was performed which unexpectedly did not mark the embryonic and fetal hematopoiesis to the same extent. In an independent approach to generate a CD41-Cre mouse (Nowakowski et al., 2011), using a 2.7 kb upstream fragment of the murine alpha 2b gene to drive expression of Cre with a nuclear localization signal (nls-Cre), a restriction of recombination events to the adult megakaryocytic lineage was reported, however, fetal hematopoiesis was not analyzed in depth in this study. Another transgenic mouse utilized a 2.7 kb fragment of the alpha 2b promoter to drive the thymidine kinase (tk) gene which could delete tk positive cells by the application of ganciclovir. In this study, thrombocytopenia could be induced by ganciclovir underlining the functionality of the CD41 promoter fragment in adult megakaryopoiesis (Tropel et al., 1997). In the meantime, also a CD41 knock in mouse was generated expressing the yellow fluorescent protein (YFP) from the endogenous CD41 locus and confirming the specific expression of CD41 in megakaryopoiesis in the adult mouse (Zhang et al., 2007b).

5. Transgenic models

In the transgenic approaches, genes are introduced into the genome for overexpression. In general, they are not at their physiological location in the genome and expression is not controlled by their physiological promoter. In some cases a promoter fragment of the same gene is used. Therefore, mice are provided by an additional gene copy, sometimes even with multiple gene copies. Constructs for generating transgenic mice usually consist of a promoter/enhancer region with transcriptional start site, introns (for a higher percentage of transgene expression in

mouse lines), protein coding sequence (cDNA) from the gene of interest and a polyA sequence. The protein coding sequence (cDNA) has to contain the translation start site, the ATG start codon, sometimes with a Kozak consensus sequence placed in front. The entire transgene element is usually excised from the vector backbone before zygote injection. The inclusion of protein tag sequences to the transgene, like VSV-g, 6xHis, HA, V5, or Myc epitopes can be helpful to detect the transgene expression as specific antibodies to these epitopes are available. This strategy allows differentiating endogenous versus transgenic expression.

Transgenic mice are generated by microinjecting the transgenic construct into a fertilized oocyte, zygote or spermatogoneal stem cells, by the use of a retro- and lentivirus vectors (Ikawa et al., 2003; Kanatsu-Shinohara et al., 2004; Chandrashekran et al., 2014) or by transfecting a transgenic construct into mouse embryonic stem (ES) cells and then injected into mouse blastocysts (van Keuren et al., 2009). Transgenic approaches can also be used to analyze tissue-specific or developmental stage-specific gene expression by introducing reporter genes, such as β-galactosidase (*lacZ*) or green fluorescent protein (*GFP*), under the control of a specific gene promoter, whose expression can be followed or tracked during development (Cho et al., 2009).

One extensively used approach when studying megakaryopoiesis, is the transgenic mouse with megakaryocyte-specific transgene expression driven by the platelet factor 4 promoter (Pf4) developed by Ravid and colleagues as already was introduced in this chapter, section 4 (rat Pf4 promoter (Ravid et al., 1991a)). The targeting plasmid was constructed by fusing 1104 bases of the 5' upstream sequence of the rat Pf4 promoter, the cap site to +20bp, Kozak sequence and the coding DNA. In contrast to what has been shown in more recent studies using the murine Pf4 promoter consisting of a longer sequence (Calaminus et al., 2012), transgenic mice generated with the rat Pf4 promoter, expressed the β-galactosidase gene exclusively in the platelets and megakaryocytes, but not in the erythrocytes and leukocytes blood cell lineages in the adult mouse. Analysis in other tissues also revealed the lack of transgene expression in in brain, heart, intestine, kidney, liver, lung, and skeletal muscle in the adult mouse.

Few years later, in 1996 Thompson and collaborators expressed a c-Myc ERT fusion from the Pf4 promoter (Thompson et al., 1996). They found the overexpression of c-Myc in megakaryocytes of transgenic mice to increase the fraction of proliferating megakaryocytes, but not to change the erythroid or myelomonocytic lineages. When they tested whether the changes in megakaryocytes differentiation were associated with changes in Cyclin A and D3 expression, (since Cyclin A is essential for the entry into S phase and Cyclin D3 is essential for the development of polyploid megakaryocytes), they observed that Cyclin D3 was downregulated

while the level of Cyclin A was slightly upregulated; concluding that cultured megakaryocytes overexpressing c-Myc were induced to proliferate, but have a limited potential to fully differentiate.

Transgenic mouse models have also been used to study hemophilia, e.g. hemophilia A. Yarovoi and collaborators generated transgenic FVIII expressing mice, with the aim to correct the FVIII circulating levels in hemophilic mice, by targeting the expression of coagulation FVIII into the alpha granules of the platelets (Yarovoi et al., 2003). This is an alternative approach to avoid the immune responses and development of inhibitors against recombinant FVIII in hemophilic patients. They used the GP1b alpha promoter to drive the expression of B-domain-deleted-FVIII fused with the alpha granule sorting signal targeting of the von Willebrand Factor (vWF). They were able to show that in the transgenic mouse model, targeted-FVIII can be ectopically expressed not just in developing megakaryocytes, but also in circulating platelets within their alpha granules. This report is the first evidence, that platelets can be potentially used as carrier cells for the transport of therapeutic proteins, since from these modified platelets the transgenic coagulation factor VIII was released and corrected the bleeding time in $FVIII^{null}$ mice.

In a therapeutic approach, Kufrin and colleagues evaluated the effect of expressing fibrinolytic proteins in platelets as a new alternative to prevent hemorrhage and thrombosis, by using the Pf4 promoter to ensure megakaryocyte specific expression (Kufrin et al., 2003). To generate the transgene mice, they used 1.2 kb from the murine Pf4 promoter followed by the 11 coding exons sequence of the urokinase plasminogen activator (uPA) and the 3′-UTR and polyadenylation signal from the human growth hormone (hGH) gene. Transgenic mice presented altered platelet biology and bleeding diathesis, similar to is observed in patients with Quebec platelet disorder; mice were also resistant to develop occlusive arterial thrombi in a $FeCl_3$-induced carotid artery thrombosis model. The transfusion of urokinase-expressing platelets into wild-type mice prevented also the thrombi formation. This model demonstrates the possibility to genetically modify developing megakaryocytes in such a way that platelet function is effectively switched from pro-thrombotic to antithrombotic phenotype.

6. Mouse transgenesis with the Sleeping Beauty Transposon system (SBTS)

Another alternative for the generation of transgenic mice is the modifications of ES cells by the sleeping beauty transposon system. In contrast to retroviral vector which have the problem to be recognized as foreign genetic material and are often silenced (Jähner et al., 1982; Lund et al., 1996; Lois et al., 2002), transposon-mediated transgenesis does encounter this problem less frequently.

Transposons were discovered in 1940 by Barbara McClintock who later on would earn the Novel prize for the discovery. These elements are known as DNA segments which are able to move or “Jump” within the genome by using the host cell machinery. Ubiquitously found in nature, these elements are part of the genome of basically all living organisms; but they were not used as a vehicle for gene delivery, until 1997, when Ivics and collaborators demonstrated that, by eliminating the inactivating mutations of one ancient transposon system, sleeping beauty, a Tc1-like transposon from fish, they could reactivate the transposase and transposition in human cells was possible (Ivics et al., 1997).

The SBTS technology offers a non-viral system for gene delivery as an alternative for transient and long term expression. The transposable element (sleeping beauty) contains a single gene encoding the transposase, which is flanked by terminal inverted repeats (IR/DRs), each one containing two binding sites for the transposase. For gene therapy purposes, the transposase gene within the element can be replaced by a therapeutic gene. The sleeping beauty transposon in a plasmid provides only transient expression of a transgene from a promoter, unless the transgene is transposed for integration into the host genome. Therefore for long term expression, the SBTS consists of two components: a transposon containing a gene-expression cassette, and the coding sequence for the transposase enzyme which can be placed either on the same (in cis) or on a different plasmid than the transposon (in trans). The transposase binds the IR/DR domains flanking the transgene and forms a synaptic complex, in which the transposon forms a loop and the inverted repeats are paired. Finally, the transposon is excised from the donor plasmid and integrates into the genome, thereby, the SBTS works on a cut and paste mechanism (Izsvák and Ivics, 2004).

There are several methods for delivery of the transposon system into a cell in culture (with electroporation or transfection) or in a tissue (by hydrodynamic injection for example) (Aronovich et al., 2011). For animal transgenesis, pronuclear microinjection of sleeping beauty transposons is performed. In recent papers, Ivics and Izsvac describe a methods for the use on the SBTS system by the co-injection of the synthetic mRNA encoding the hyperactive transposase SB100X, together with a circular DNA plasmid carrying the transposon into the pronuclei of fertilized oocytes (Ivics et al., 2014).

7. Knock in mouse models

Knock in models are based on the targeted insertion of a new gene in a particular locus, by using homologous recombination in embryonic stem cells. In this case instead of a loss of function by deleting an important segment of a gene, a gain of function from a new coding sequence is desired. In addition, targeted mutations can be also achieved by the replacement of the wild type sequence by a mutated version within a targeting construct. If the knock in of a mutant gene was to be inducible or tissue-specific, a stop cassette flanked by loxP recombination sites is inserted in front of the mutant gene of interest thereby preventing its expression. By expression of the recombinase (Cre, Flp, Dre) the stop cassette is excised and the mutant cDNA is expressed (Rappaport and Johnson, 2014).

One well known example in platelet research is the expression of the human glycoprotein IB alpha (*GPIBA*) gene from the murine locus. By disrupting the coding sequence of the murine murine *GPIba* gene, Ware and collaborators generated a murine model resembling the hallmark characteristics of the human Bernard-Soulier syndrome. In the next step, by replacing the missing murine gene with the human version of the *GPIba* gene, they were able to rescue the murine Bernard-Soulier phenotype, and by this strategy demonstrated a direct link between the expression of a GPIb-IX-V complex with a normal megakaryopoiesis and platelet morphogenesis (Ware et al., 2000).

Zhang and colleagues developed a CD41-YFP mouse containing YFP-labeled megakaryocytes and platelets. In this case the yellow fluorescent protein (eYFP) gene was inserted just after the start site of the *GPIIb* gene by homologous recombination into embryonic stem cells. Suggesting that this model could be potentially used not just for in vivo imaging of megakaryocytes and platelets, but also for studies on platelet aging and function (Zhang et al., 2007b, 2007b). In a different model, by inserting the cDNA of the green fluorescent protein (GFP) into the *growth factor independent 1b* (*Gfi1b*) gene locus, Vassen and collaborators were able to track the expression pattern of Gfi1b in erythroid cells, megakaryocytes, and their progenitor cells (Vassen et al., 2007).

8. In vivo models using viral vectors for gene transfer

For gene delivery into adult stem cells which cannot be cultured for prolonged times, transfer vectors have been developed that are derived from viruses and take advantage of the viral characteristics to infect target cells more easily than the non-viral transfer systems. For the stable integration of genetic material and therefore the long term modification of cells, retroviral vectors are the transfer system of choice. They transduce their target cells by receptor mediated binding and uptake and integrate their genome utilizing their viral integrase. By deletion of the viral coding genes from the wildtype viral genome, vectors were generated that cannot replicate and give space to transfer coding sequences of other genes of interest into cells (**Figure 3**).

The most advanced retroviral vectors are derived from the gammaretroviral murine leukemia viruses (Williams et al., 1984; Miller and Rosman, 1989; Maetzig et al., 2011) and the human immunodeficiency virus (HIV) (Naldini et al., 1996; Dropulić, 2011), known as the lentiviral vectors. Other retroviral vector systems are developed from the *equine infectious anemia viruses* (EIAV) (Mitrophanous et al., 1999; Dropulić, 2011), the *simian immunodeficiency viruses* (SIV) (Nègre et al., 2002), Foamyviruses (Trobridge et al., 2002) and alpharetroviruses (Suerth et al., 2010). The simplest genome carries the gammaretrovirus and all their genes are shared by the other members of the retrovirus family: (1) the *group specific antigen* (gag), which encodes for the matrix, capsid und nucleocapsid, (2) the *pol* gene (codes for the protease, reverse transcriptase und integrase) and (3) the viral envelope (*env*). In the case of the complex lentivirus, additional genes are encoded in the viral RNA such as the *rev* gene and other accessory genes. The interaction of the Rev with the *rev responsive element* (RRE) contained in the viral RNA mediates the nuclear RNA export (Malim et al., 1989; Pollard and Malim, 1998). To produce retroviral vectors, the viral genes are placed on so called “helper plasmids” which for the production of viral vector particles are co-transfected into the packaging cell lines (Miller, 1990; Yu et al., 2003). Packaging cells are cells lines that by expressing all necessary viral proteins produce the viral particles and release them into the cell culture supernatant.

Retroviral particles have a diameter of 100 nm, and consist of an enveloped nucleocapsid and two copies of single stranded RNA. The viral RNA contains a 5‘ cap structure and a 3‘ polyA tail. After infection of the target cells, the RNA is converted into double stranded DNA by the reverse transcriptase and with help of the integrase inserted into the host genome.

The integrated provirus contains two *long terminal repeats* (LTRs) on each site. In the LTRs there are the viral enhancer and promoter (in the *unique* U3 region), the cap und polyadenylation signals (in the R-region) and the *attachment site* (*att*). The retroviral 5‘ untranslated region

consists of the R- and U5- region, attachment site (*att*), the primer binding site (PBS) and the packaging signal (ψ). R-region, att, PBS and ψ are equally essential for the replicating virus and the vector as they mediate the packaging of the viral RNA (ψ), the initiation of the reverse transcription (PBS) and the integration (*att*). In contrast, enhancer and promoter sequences in the U3 regions can be deleted and instead a promoter can be inserted in an internal position just upstream of the cDNA. Retroviral vectors with this architecture are named self-inactivating (SIN) vectors because during the production the viral RNA is transcribed from the 5'LTR but the deletion in the 3'LTR is copied to the 5' position during the RT and therefore, the U3 region is deleted in the integrated viral vector genome (Yu et al., 1986; Zufferey et al., 1998; Kraunus et al., 2004). The SIN configuration minimizes the chance of replication-competent viral particles to occur during the treatment with gene vectors and allows the use of lineage-specific or inducible promoters in an internal position to express the transgene.

Retroviral vectors enter the cells by binding to specific cell surface molecules which are predefined by the viral envelope protein. Therefore, the tropisms of viral vectors can be modulated by the envelope protein (Bartosch and Cosset, 2004). For example, the murine ecotropic retrovirus binds the mCAT receptor and the amphotropic virus the PIT-2 receptor. HIV-derived lentivirus recognizes CD4 and CXCR4/5 on the surface of T-cells but by pseudotyping with the *vesicular stomatitis virus glycoprotein* (VSVG) which binds the LDL receptor (Finkelshtein et al., 2013) the tropisms for transduction is very broad.

After entering the cell, the viral particle undergoes uncoating and the viral RNA is released but hidden in the pre-integration complex (PIC). The reverse transcriptase contained in the particle mediates the generation of the double stranded DNA. The PIC travels along micro tubules to the nuclear membrane. In the case of lentiviral vectors the DNA can enter the nucleus via the nuclear pore while gammaretroviral vectors rely on the breakdown of the nuclear membrane during mitosis (Miller et al., 1990). As HSCs are or only slowly cycling, lentiviral vectors are favored over gammaretroviral vectors for HSC transduction (Guenechea et al., 2000). The integration of the double stranded viral DNA is mediated by the integrase. The attachment to the genomic DNA is determined by specific tethering factors which bind to the integrases (LEDGF for lentiviruses (Cherepanov et al., 2003), BET proteins for the gammaretroviruses (Gupta et al., 2013; Rijck et al., 2013; Sharma et al., 2013). These specific interactions explain the typical integration site preferences of the different members of the retoviruses; gammaretrovirus prefers to integrate into promoter regions and CpG islands while lentivirus integrates within genes. Both of the them tend to target actively transcribed gene regions (Schröder et al., 2002). In contrast

alpha and foamy viruses integrate more randomly and therefore less often close to, or in genes (Mitchell et al., 2004; Trobridge et al., 2006).

To avoid integration-associated adverse reactions, lentiviral vector can also be used in a non-integrative form, however, the lentiviral genome will then not persist but will be diluted with increasing cell divisions. These lentiviral vectors contain an integrase with a mutation in the catalytic domain (D64, D116, E152) (Philippe et al., 2006; Yáñez-Muñoz et al., 2006). Therefore, after entering the nucleus the lentiviral genomes stays in a non-integrated, episomal form, as one or two LTR circles (Wanisch and Yáñez-Muñoz, 2009). These circles also form during the normal live cycle of lentiviruses but are considered to be non-productive and will also not integrate at later times. Still, the transgene can be transcribed and produced and therefore, integration-deficient lentiviral vectors (iDLVs) are very interesting delivery tools for transient expression, as they deliver the DNA to the cells via the lentiviral entry mechanism, e.g. for the delivery of reprogramming or transdifferentiating factors, transposase, zing finger nucleases, Cre or Flp (Lombardo et al., 2007; Staunstrup and Mikkelsen, 2011).

9. Models for megakaryopoiesis and platelets using retroviral vectors

As platelets do not contain a nucleus and a cellular genome, the application of gene transfer methods to platelets directly is limited. Therefore, genetic modifications must be performed at earlier stages in platelet differentiation, either in the progenitor cells or in the HSC. By transplantation of HSC into conditioned recipients, the hematopoiesis of the recipient mouse can be modified. Depending on the donor chimerism and transduction rates before transplantation, gammaretro/lentiviral gene transfer to HSC allows the expression of ectopic proteins throughout the entire hematopoiesis.

For the analysis of gene functions in megakaryopoiesis and platelets, however, it may be important to restrict expression to the megakaryocytic lineage. By using tissue-specific promoters in retro/lentiviral vectors it is possible to direct expression to a certain blood lineage (**Figure 4**). In the case of megakaryopoiesis, promoter fragments of MK-specific genes can be used. This concept has been demonstrated by using fragments of the platelet factor 4 (Pf4) (Greene et al., 2010; Greene et al., 2014), GPIIb (Zhang et al., 2010) or GPIba promoters (Ohmori et al., 2007; Ohmori et al., 2008), as internal promoters in retro- or lentiviral vectors, summarized in the **Table**.

The specificity from the MK promoters is mediated by typical transcription factor binding sites and regulatory regions. One study performed by (Lemarchandel et al., 1993), identified 2

important consensus sequences within the human glycoprotein IIB promoter (GPIIB), also identified later in the platelet factor 4 promoter (Pf4) (Ravid et al., 1991b; Minami et al., 1998), in the glycoprotein IX promoter (GP9) (Bastian et al., 1996) and in the glycoprotein VI promoter (GP6) (Furihata and Kunicki, 2002). One of these sequences, a -55 GATA binding site, and the second one, located at -40 contains an Ets consensus sequence. This 2 binding elements were also identified in the glycoprotein 1b alpha promoter (GP1bα) (Hashimoto and Ware, 1995). Lemarchandel and collaborators were able to show that point mutation in these 2 regions, affects dramatically the activity of the promoter, but not their tissue specificity. A further study performed by (Okada et al., 2003), identified Meis1/Pbx as important transcription factors for the megakaryocyte-specific expression of the *platelet factor 4* gene.

As all MK-specific promoter share similar binding sites, ideally, one consensus sequences for expression in MK could be determined and a synthetic promoter generated similar to the approach published for hepatocyte-specific expression (Nair et al., 2014). However, the promoter regulates only transcription but RNA abundance is also controlled by posttranscriptional regulation via microRNAs. This concept has been successfully exploited by Brown, Gentner and Naldini (Brown et al., 2007; Gentner and Naldini, 2012) by introducing miR target sequences into the RNA transcribed from the vector after integration. They could demonstrate the successful detargeting of protein expression from antigen-presenting cells or HSC after lentiviral transduction (Annoni et al., 2009; Gentner et al., 2010). With high numbers of integrated vector copies, and as results high levels of transcribed RNA containing the miR target sites, these could even delete miR from the system by excessing miR binding, inducing a miR knockout situation (Gentner et al., 2009).

The importance of lineage-restricted expression was convincingly demonstrated in our study correcting the thrombocytopenia caused by Mpl deficiency in the Mpl-/- mouse. Human patients with MPL deficiency present with the hematological disorder named congenital amegakaroycytic thrombocytopenia (CAMT, (van den Oudenrijn, S et al., 2000; Ballmaier et al., 2001). The gammaretro- or lentiviral overexpression of Mpl by ubiquitous promoters (viral promoter or the phosphoglycerate kinase (PGK) promoter) severely disturbed the balance between Mpl and its ligand thrombopoietin. As a consequence, Thpo levels were reduced, causing severe thrombocytopenia when expressed in wildtype mice (Wicke et al., 2010). This was against the expectation, as Mpl is also a proto-oncogene and therefore, high levels of expression will also cause activation of the downstream pathways and induce cell proliferation. The hyperproliferative phase, however, was only transient and mice did not succumbed due to myeloproliferative disorders but because of severe pancytopenia. Only the restricted expression

of Mpl by the Mpl promoters and also the GPIba promoter allowed the correction of the thrombocytopenia without side effects. As Thpo/Mpl signaling is also essential for the maintenance of HSC, the phenotypic picture of CAMT is the aplastic anemia. Mpl expression from the GPIba promoter, human or mouse Mpl promoter also rescued the HSC defect (Heckl et al., 2011). In this respect, the GPIba promoter demonstrated surprisingly high activity in HSC similar to the MPL promoter.

A 2 kb fragment of the murine Mpl promoter was characterized (Ziegler et al., 2002) and has also been used to express Mpl in the Mpl-/- mouse by transgenic approaches (Lannutti et al., 2009; Tiedt et al., 2009). Due to the transfer technology generating the transgenic mice, in the study by Lanutti et al. 38 copies of the expression cassette were detected. Nevertheless, Mpl expression levels were lower than the endogenous levels in wildtype mice, but surprisingly, platelet counts were elevated (thrombocytosis). This can be explained by the high Thpo levels in Mpl-/- mice that strongly activate Mpl also when expressed at low levels. All these studies in the Mpl-/- mouse model highlight the importance to achieve physiological transgene expression levels to guarantee that phenotypes in murine models reflect the biological relevant situation.

10. Modification of megakaryopoiesis by adeno-associated vectors

The adeno-associated vectors (AAV) are developed from non-pathogenic human parvoviruses. They are small, non-enveloped viral vectors that carry a single stranded DNA genome of about 5 kb (for review (Büning et al., 2008)). The AAV particles enter the cells by receptor-mediated uptake and their genomes persist as non-integrated episomes. For productive infectious cycles the wildtype AAV depends on the co-infection with helper viruses such as adenoviruses. For the generation of vectors, similar to the retroviral system, all AAV specific genes are removed and supplied to the producer cells by plasmid cotransfection. In the residual AAV genome only the inverted terminal repeats (ITR) are left which do not contain enhancer/promoter sequences but have to flank the genomic information for productive packaging.

AAV vectors come in different serotypes that determine the tropism of infection and some cells are rather resistant to AAV transduction including megakaryoblastic cell lines. However, by the use of a bispecific F(ab')2 complex to megakaryocyte-specific receptors, transduction of megakaryoblastic cells lines and primary MK could be achieved (Bartlett et al., 1999). AAV vectors can also display proteins on their capsid mediating vector binding to the cell surface receptor and subsequent uptake (Münch et al., 2013). In general the gene transfer by AAV vectors to dividing cells is transient and as the genome does not integrate and therefore, not normally applied to HSC. By in vivo vector delivery and targeting of post-mitotic hepatocytes,

however, thrombopoietic growth factors or coagulation factors can be expressed with long term impact on the in vivo phenotype. This concept is exploited for the treatment of hemophilia and discussed below.

11. Study of gene function in human platelets in humanized mouse models

Species-specific differences make it desirable to analyze gene function directly in human rather than murine cells. To study gene function, human HSC can be transduced and differentiated. Clinical trials aiming for the correction of monogenetic diseases of the blood system rely on the long term modification by stable integration of the therapeutic gene and also the long term engraftment of the modified HSC. Therefore, in vitro transduction protocols for human CD34+ cells are well developed and the transplantation and engraftment in humanized mouse models extensively studied (Woods et al., 2000; Santoni de Sio, Francesca and Naldini, 2009). One hurdle is the efficient transduction of CD34+ cells without the loss of their repopulation abilities. To induce proliferation by maintaining stemness in vitro, combination of cytokines are applied (stem cell factor (SCF), thrombopoietin (THPO), Fms-like tyrosine kinase 3 ligand (FLT3L) (Sauvageau et al., 2004)), or SCF, THPO, Angptl5 and insulin growth factor binding protein 3 (Zhang et al., 2008) or small drugs added, e.g. the aryl hydrocarbon receptor antagonist Stemreginin (SR1) (Boitano et al., 2010) or prostaglandin E2, all of which also support in vitro genetic modifications of CD34+ cells (Genovese et al., 2014). CD34+ cell transduction can further be improved by the use of more suitable envelope proteins such as the baboon retroviral envelope (Girard-Gagnepain et al., 2014) or by displaying specific scFv on the envelope (Kays et al., 2014; Brendel et al., 2015) or by the inhibition of pathways responsible for blocking lentiviral transduction (Petrillo et al., 2015).

There are different mouse strains available as immune-deficient recipients allowing the engraftment of human cell and the establishment of human hematopoiesis in vivo including megakaryopoiesis and platelet release. The most widely used models are the NOD/SCID and NOD/SCID, IL2rg-/- (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}, NSG) mouse strains (Shultz et al., 2005). There are other mice developed on different genetic backgrounds, the NOD.cg-Prkdc^{scid}Il2rg^{tm1Sug} (NOG), and Rag-/-, IL2rg-/- (C;129S4- Rag2$^{tm1.1Flv}$, BRG) mice (Shultz et al., 2007). In general, all models allow the analysis of human hematopoiesis, but with different lineage contributions of human cells. So far NSG mice allowed the best engraftment of B, T and NK cells and also the highest human chimerism in the BM (Drake et al., 2012).

Human platelets in the mouse are, however, difficult to obtain for reasons that are not well understood. In a recent study aiming for platelet directed expression of FVIII, the highest levels

of human platelets the murine blood of NSG mice was detected 2-3 weeks after human HSPC transplantation (~50%) and the levels declined substantially after 5 weeks dropping to 1% human contribution to the platelets after 7 months (Shi et al., 2014). The major thrombopoietic cytokine Thpo is not completely cross reactive between human and mouse with lower activity of the murine Thpo on the human MPL receptor. Replacing the murine *Thpo* gene by human *THPO* by a knock in strategy in BRG mice supports human HSC engraftment and maintenance due to MPL signaling in HSC. However, *THPO* knock in mice are thrombocytopenic due to the lower levels (heterozygous) or complete lack (homozygous) of the murine Thpo and because the human THPO levels appear not to be sufficiently high to support human thrombopoiesis or replace the murine cytokine on murine platelets (Rongvaux et al., 2011). This finding demonstrates that the all murine environment may lack some of the important regulating factors for human megakaryopoiesis and platelet release.

12. Gene therapy approaches for bleedings disorders

Hematopoietic stem cell gene therapy has been employed in the clinic for the treatment of monogenetic hematopoietic disorders with severe disease courses with great therapeutic success (Cavazzana-Calvo et al., 2000; Gaspar et al., 2004; Ott et al., 2006; Aiuti et al., 2013; Hacein-Bey-Abina et al., 2014) This therapy is based on the isolation of autologous HSC, their in vitro genetic modification and subsequent re-transplantation into the patient. For the long term correction of hematopoiesis also the gene correction has to persist. Therefore, currently retro- or lentiviral vectors are the transfer systems of choice. Transduction with these vectors delivers the defective gene as an additional copy to the genome.

Although the concept of HSC-directed gene therapy is well established, the therapy-associated risks for the patients are still very high. These include the risk of insufficient engraftment of HSC that underwent in vitro cytokine stimulation and transductions, the risk associated with the pre-transplant conditioning and the long term risk of developing leukemia due to insertional mutagenesis (Cavazzana-Calvo et al., 2013; Kaufmann et al., 2014). Therefore, at present the transplantation of gene modified HSC is only applied in patients with no other treatment options. However, in principle gene therapy could also cure bleeding disorders such as Glanzmann Thrombasthenia or Bernard-Soulier-Syndrome. Gene Therapy for the treatment of Wiskott Aldrich Syndrome has been performed (Boztug et al., 2010; Braun et al., 2014) and is discussed in detail in the chapter by Braun and Klein in this book.

Before gene therapy can be translated into the clinics the efficacy of the therapy has to be demonstrated in preclinical animal models. Using lentiviral vectors for expression of human GPIBA and transplantation of transduced HSC, the thrombopathia in the murine GPIba-/- mouse, a model for Bernard-Soulier-Syndrome, was corrected (Kanaji et al., 2012). The treatment improved the platelet counts, shortened bleeding times and reduced the platelet volume. In this study, expression from the self-inactivating lentiviral vector was driven by the human aIIb integrin promoter, providing specificity of expression to the megakaryocytes and platelets. The correction was similarly efficient as the transgenic expression of the human *GPIBa* in *GPIba*-/- mice (Ware et al., 2000). In another study, gene therapy was applied to treat Glanzman Thrombasthenia in the Great Pyrenees dog model (Fang et al., 2011). In these dogs, the inherited disorder is based on a 14 bp insertion in exon 13 of the *GPIIb* (integrin alpha 2b) gene (Lipscomb et al., 2000). The dogs develop symptoms similar to human Glanzman patients and are, therefore, a very good model to study the human disease. In the study by Fang and colleagues, the transplantation of transduced peripheral blood CD34+ cells followed a non-myeloablative pretransplant conditioning with 100 cGy total body irradiation or 4 mg/kg busulfan. The lower dose regime was developed due to bleeding complications observed in previous transplantation trials in these aIIb integrin deficient dogs. As a consequence of the non-myeloablative precondition, the donor chimerism after transplantation was expected to by low, but could be enriched by the selection for the co-expressed drug resistance gene MGMTP140K by the treatment with O^6-benzylguanine and carmustine. The contribution of gene corrected platelets was enriched to 10% which stayed stable over the observation period of up to 5 years in one of the dogs. Unfortunately, no information on the enrichment of donor cells in the BM or HSC level was provided.

The concept of enrichment of transduced cells by drug selection has been developed over the last 20 years (Moritz et al., 1995; Milsom and Williams, 2007) and now successfully conduced in a clinical trial to treat glioblastoma patients who underwent autologous blood stem cell transplantation (Adair et al., 2014). The enrichment of gene modified cells post transplantation will allow reducing the toxicity of precondition regimes and may, therefore, open up the possibility to perform gene therapy treatment in patients with milder diseases phenotypes, such as bleeding disorders. However, it has to be kept in mind that also the cytotoxic drug selection is accompanied with substantial side effects to the patients. In addition, the treatment with allogenic BM transplantation is one therapeutic option for Glanzman patients with severe disease presentation (Connor et al., 2008; Wiegering et al., 2013; Walz et al., 2014) or also for

Bernard-Soulier patients (Rieger et al., 2006), however, this is still the exception to the normal treatment.

The transplantation of gene modified HSC targeting expression into platelets has also been explored for the treatment of hemophilia A and B. Platelet-specific expression of FVIII of FIX would supply the necessary clotting factors to the side where the coagulation process takes place. To ensure the storage of the factors in the secretory alpha granules of the platelets, fusion proteins with the vWF protein have been generated. Beside gene therapy, hemophilia is well treatable by the substitution of the missing clotting factors with FVIII and FIX, however, is some cases patients develop auto-antibodies against the synthetic factors, especially FVIII. In these cases, the conventional treatment is ineffective and platelet-directed delivery by gene therapy may solve these issues as the clotting factors are hidden within the platelet granules and therefore not recognized by the immune system. However, also the non-targeted expression in all blood cells from an ubiquitously active promoter in the lentiviral context can cure hemophilia by transduction and transplantation of HSC (Johnston et al., 2013); this concept has been followed for the treatment of hemophilia A in an open clinical trial run at the Emory University School of Medicine in Atlanta.

In the past, however, gene therapy approaches for the treatment of hemophilia have mostly been directed towards gene transfer to the liver using AAV or lentiviral vectors (High et al., 2014). In a clinical trial run by the St. Jude Children's Hospital, Memphis, USA and in University Hospital London, UK, a single application of AAV type vector, serotype 8, coding for the human *FIX* gene resulted in expression of ~6% of the normal FIX levels in the blood and a cure of disease symptoms making the patients independent of any further replacement therapies (Nathwani et al., 2011; Nathwani et al., 2014). After AAV vector gene transfer, the genomic information is kept in a non-integrated, episomal form. This prevents any risk of interfering with the host genome, but it also does not persist long term, as the episomes will not replicate with the cellular genome. As hepatocytes rarely divide, the episomal information may still persist. In an approach making use of non-integrating lentiviral vectors this has further been proven (Mátrai et al., 2011). Immune recognition of the transduced liver cells or the viral capsid proteins are the major obstacles at the moment that have to be overcome by improved vector technology.

13. Advanced technologies for targeted genetic modifications and gene knockouts

In the recent years, methodologies have developed that allow the side-specific modification in the genome by zink finger nucleases (ZFN), transcription activator like (TAL) effector nucleases (TALEN) (Boch et al., 2009; Moscou and Bogdanove, 2009; Christian et al., 2010) or, most recent, the CRISPR/Cas9 technology (Jinek et al., 2012; Mussolino and Cathomen, 2012). These technologies have in common that they target the genome by recognition sequences, in the case of ZFN and TALEN the DNA binding domains and in the CRISPR/CAs9 system, the binding is modulated by the guide RNA (Doudna and Charpentier, 2014). After binding to the target site, a DNA double strand break is induced that then can be repaired by non-homologous end joining which result in mutations in the sequence, or by homologous recombination which allows the insertion of a new sequence. In this case, the homologous region has to be provided as separate DNA template to the cells.

The CRISPR system was developed from the type II CRISPR/Cas system in bacteria where it serves as part of the adaptive immunity against viruses and plasmids. In this original system the endonuclease Cas9 is guided to the DNA location by two RNAs (tracrRNA:crRNA) which in the synthetic system is joint to a single guide RNA. In the CRISPR system, therefore, the recognition is mediated by nucleotides, unlike the ZNF and TALEN systems which bind the DNA by protein-nucleotide interaction (DNA binding by zink finger proteins or TAL proteins). This major difference also explains the easy use of the CRISPR/Cas9 system as the recognition sequence (in the guide RNA) can be just copied from the genome sequence around the desired location following the Watson and Crick base pairing. However, also the TAL proteins work on the *two amino acids:one nucleotide* base and can be rather flexibly designed. For the DNA cleavage, endonucleases are employed. In the case of the ZNF and TALEN, the endonuclease FokI is used that assembles to the active dimer from two inactive monomers that have to meet at the desired site which is mediated by two independent TALE or zink finger arrays. In contrast, the Cas9 endonuclease is recruited by only one single guide RNA, and therefore, the specificity is lower compared to the “double” guided strategy. To overcome this limitation, also a double Cas9 nickase system has been developed (Fu et al., 2014; Guilinger et al., 2014; Tsai et al., 2014). By delivering more than one guide RNA together with the Cas9 endonuclease, multiple sites can be targeted in one cell, so called multiplexing. Ideally, all the player for this application can be encoded on one construct (Kabadi et al., 2014; Sakuma et al., 2014). By using lentiviral vectors for the delivery of the CRISPR/Cas9 into murine HSC, the multiplex approach has been successfully employed for a genetic screen of deletion mutants in murine leukemia (Heckl et al., 2014).

Shared by all the three systems is the barrier to bring in all the players into the cells, when a homologous recombination in the targeted locus is required, this also applies to the homologous DNA, in addition to the endonuclease and the zink finger or TALE proteins in these two systems, or the guide RNA on the other. In some cells, such as pluripotent cells, electroporation could be performed. Also in these cells, correct recombinant can be selected. However, in primary stem cells, such as HSC, this is a difficult task. In a recent study by the group of L. Naldini, a proof-of-concept study demonstrated the successful correction of the *IL2RG* gene by a ZFN-mediated approach in human HSC and their subsequent transplantation into NSG mice (Genovese et al., 2014). They developed a protocol based on the delivery by integration deficient lentiviral vectors (iDLV) and RNA electroporation. Similarly this has been employed for the delivery into T-cells (Mock et al., 2014). Still, the use of targeted genome modification for mouse models based on the transplantation of in vitro modified primary HSC is challenging; however, for introduction of mutations in pluripotent stem cells, for the generation of mouse models with germ line modifications or iPSC-based disease models, these new technologies opens up new avenues in research.

14. Overcoming unwanted effects of genetic modification

Every genetic modification alters the genome and can induce unwanted (side) effects. In clinical gene therapy, this can lead to severe adverse events with life-threatening complications (Williams and Thrasher, 2014), however, also when working with animal models, the researcher wishes for physiological models without skewed phenotypes. In both cases, gene transfer technologies have advanced very far and offer good strategies to overcome limitations.

The integration of an expression cassette into the genome consisting of enhancer/promoters can interfere with the regulation of neighboring genes by insertional mutagenesis. In this respect, this is independent of the transfer system used. However, with a higher likelihood to integrate into gene dense regions, into actively transcribed genes and close to promoters which is characteristic for gammaretroviral vectors, the risk is increasing. As the vectors are derived from replicating viruses that selected on the basis of good expression to ensure rapid spread, these regions in the genome are favored. The potentially most dangerous interaction from a vector is mediated by its enhancer/promoter by upregulating gene expression from cellular promoters (“enhancer interaction”). In the case of proto-oncogenes this has led to the development of leukemias in human gene therapy patients (Hacein-Bey-Abina et al., 2008; Howe et al., 2008; Stein et al., 2010; Braun et al., 2014) and animal models (Modlich et al., 2005; Seggewiss et al., 2006; Modlich et al., 2008). In fact, gammaretroviruses and gammaretroviral vectors are potent

tools for gene fishing approached by selecting insertional mutants (Mikkers and Berns, 2003; Touw and Erkeland, 2007). By deleting the viral enhancer/promoters and moving the promoter into the internal position, the insertional mutagenic (genotoxic) potential is reduced (Modlich et al., 2009). Lentiviral vectors have a slightly different integration preference and therefore less often interact with cellular promoters due to a further distance to them. On the other hand, lentiviral vectors tend to integrate into introns of genes and can by splicing-mediated interaction alter the transcription of the gene. The downregulation of gene expression was observed after the integration of a lentiviral SIN vector with the megakaryocyte-specific GPIba promoter as internal promoter into the 8th intron of the early B-cell factor 1 (Ebf1) gene (Heckl et al., 2012). Haplo-insufficiency of Ebf1 caused B-cell leukemia with a block in B-cell development. The GPIba promoter is widely used for megakaryocyte-specific expression. The promoter fragment (-258 to +330) spanning the entire first exon and first intron contains splice sites from the exon-intron boundaries. Splice interference generates transcripts with insufficient polyadenylation which are, therefore, subjected to non-sense mediated RNA decay. To overcome this problem, SIN vectors with cellular promoters devoid of unnecessary splice sites should be employed. Also, some other vector platform may target integration into less active chromatin, for example the alpharetroviral vectors or the non-viral sleeping beauty transposon system.

Another drawback in gene modification can be silencing of transgene expression by epigenetic effects (Ellis, 2005; Antoniou et al., 2013). This is especially problematic when introducing modifications into pluripotent cells. One option to avoid position effects is the targeting of so-called safe harbors, one of which is the AAVS1 locus, by the use of the ZNF or TALEN approaches. The adeno-associated virus integration site 1 is located on chromosome 19, in the first intron of the PPP1R12C gene which codes for the protein phosphatase 1 regulatory subunit 12. This region has open chromatin and is flanked by insulator elements that shield the inserted gene from activation from the outside. Gene expression from transgenic cassettes inserted into this locus was found to be active and not to have any effects on the host cell. Alternatively, other locations in the genome could be defined as “safe”, for example the murine Rosa26 locus. Recently the group of M. Sadelain has defined criteria for a safe harbor as follows (1) the distance to the next 5’ end of a gene to be at least 50 kb and (2) >300 kb away from a cancer-related gene and (3) microRNA, (4) to be located outside a transcription unit and (5) ultraconserved region in the human genome (Papapetrou et al., 2011). When accepting these criteria, any cells with genetic modifications that fits this definition would be safely modified.
Alternatively, the expression cassette can be flanked by insulators. The best studied insulator is chicken hypersensitive site 4, derived from the chicken globin locus (Chung et al., 1993;

Ghirlando et al., 2012). Unfortunately, so far insulators cannot completely shut off interactions with the genome, but research in defining new and more accurate insulators are ongoing (Liu et al., 2015). Also genetic elements are being investigates that allow for the faithful expression from an inserted transgenic cassette by the use of the ubiquitous chromatin opening (UCOE) element (Zhang et al., 2007a). Until recently, this element was rather large and also consisting of additional splice sites but more recent research has optimized the UCOE element (Müller-Kuller et al., 2015).

15. Pluripotent cells that mimic human thrombopathias

The discovery of the technology to reprogram somatic cells to pluripotent cells, so called induced pluripotent cells (iPSC) by the seminal work of S. Yamanaka (Takahashi and Yamanaka, 2006) has opened new options for the development of disease models. iPSC can be differentiated to megakaryocytes and platelets and all the intermediate steps can be studied in vitro. This also makes it possible to study gene functions directly in human cells. This is especially helpful if no suitable murine models exist, for example when the murine phenotype does not or only partially reflect the human disease. One example is the murine Was knockout mouse which shows the leukopenia and defects in T-cell activation similar to human WAS patients but do show altered B-cell responses, do not develop eczema or hematopoietic malignancies (Snapper et al., 1998). In addition, Was mice have chronic colitis that is rarely found in human WAS patients. In respect to the defects in platelets, Was knockout mice have only a modest thrombocytopenia and no reduced platelet volumes. By utilizing iPS cells reprogrammed from human patients, the WAS deficient phenotype and underlying mechanisms can be studies (Ingrungruanglert et al., 2014). Similarly, iPS cells of patients with Glanzmann thrombasthenia have been generated, differentiated into platelets and their functionality compared to platelets from patient blood (Orban et al., 2015). By a gene correction approach delivering the defective *alpha 2b* gene via recombination into the AAVS1safe harbor locus, alphaIIb expression could be reestablished (Sullivan et al., 2014). In this study, alpha 2b was expressed by the GPIba promoter reaching the same levels of expression than in equally differentiated wildtype iPS cells. Although these are very promising results, the regulation by the transgenic expression cassette can only partially mimic the physiological regulation. In a study to correct MPL-deficiency in human MPL-/- iPS cells (CAMT iPS cells), MPL was expressed by a classical gammaretroviral vectors with functional LTRs (Hirata et al., 2013). The expression levels in this approach did not accurately resemble the normal levels and in agreement with that the authors describe erythroid biased differentiation in MPL overexpressing MPL-/- iPS cells. When aiming to correct defects in iPS

cells or to overexpress genes, the controlled expression during all steps of differentiation, also avoiding potential silencing, has to be guaranteed and will be a challenge for the future (Ackermann et al., 2014).

Table: Examples of studies utilizing gammaretro- or lentiviral gene transfer

Gene	Vect.	Promoter	Model	Outcome	Reference
Hemophilia A models expressing FVIII					
human B-domain deleted FVIII	SIV-LV	CMV	CB-CD34+, transplantation into Nod/Scid mice	~ 10% human engraftment, efficient FVIII expression (1.2-3.5 ng/ml, 1-3% of normal levels	(Kikuchi et al., 2004)
eGFP, human B-domain deleted FVIII	SIV-LV	hGPIba (-258 to +330) hGPIIb (-554 to +33)	in vitro cell lines, -CD34+ in vitro MK differentiation, BMT in hemophilia A mouse model[1]	Platelet-specific expression, FVIII expression detected	(Ohmori et al., 2006)
human B-domain deleted FVIII	LV	hGPIIb (-889 to +35)	BMT in hemophilia A mouse model	Sustained FVIII expression, correction of hemorrhagic symptoms, no inhibitors	(Shi et al., 2007)
human inactivation resistant FVIII (IR8), human and canine B-domain deleted FVIII (hBF8, cBF8)	LV	mPf4 (-120 to +82)	BMT in hemophilia A mouse model	Improved clotting, IR8 more effective than hBF8, except for the cremaster model, probably because of delayed onset	(Greene et al., 2010)
human B-domain deleted FVIII	LV	hGPIIb (-889 to +35)	BMT in pre-immunized FVIII^{null} mice	Platelet-specific delivery improves the phenotype	(Kuether et al., 2012)
human/porcine B-domain deleted FVIII hybrid	SIV-LV, LV	CMV, EF1a, yeast PGK	BMT in hemophilia A mouse model	SIV-LV with CMV promoter selected as the best performing vector	(Johnston et al., 2013)
human B-domain deleted FVIII fused to the VWF D domain	LV	hGPIIb (-889 to +35) hGPIIb (-673 to +35)	hemophilia A dog model[2]	Sustained long term correction of the bleeding phenotype, granule targeting	(Du et al., 2013)
human and canine B-domain deleted FVIII	LV	mPf4 (-1120 to +82)	BMT in hemophilia A mouse model	canine more active than human, but also increases apoptosis in MK	(Greene et al., 2014)
human B-domain deleted FVIII-MGMT(P140K)	LV	hGPIIb (-889 to +35)	BMT in hemophilia A mouse model non-myeloablative preconditioning	Enrichment to mean 5% FVIII expression in platelets by BG/BCNU treatment	(Schroeder et al., 2014)
human B-domain deleted FVIII	LV	hGPIIb (-889 to +35)	CD34+ into NSG or NSGF8KO mice	Improved hemostasis in NSGF8KO mice, lo contribution of human platelets	(Shi et al., 2014)
human B-domain deleted FVIII	LV	MND or EF-1α-short, hGPIba	Intraosseal injection, hemophilia A mice	Long term expression, platelet-specific (in the case of hGPIba), phenotype correction	(Wang et al., 2015)
Hemophilia A models expressing FVIIa					
activated FVII, insertion of two RKR motives into the factor X activation–cleavage site	SIV-LV	hGPIba (-58 to +330)	BMT in hemophilia A mouse model	Correction of bleeding phenotype in mice with hemophilia A in the presence of neutralizing antibodies	(Ohmori et al., 2008)

Hemophilia B models expressing Factor IX					
hFIX-PGK.MGMT	LV	Beta globin locus control region, reverse orientation	BMT in hemophilia B mouse model[3]	Erythrocyte-specific expression of FIX, (>500 ng/ml by vector copy number 1-2)	(Chang et al., 2008)
hFIX	LV	hGPIIb (-889 to +35)	BMT in hemophilia B mouse model	release from the platelets, phenotype correction	(Zhang et al., 2010)
hFIX	LV	hGPIIb (-889 to +35)	BMT in hemophilia B mouse model	Sustained phenotype correction in serial transplants, no inhibitors developed	(Chen et al., 2014)
Models expressing cell surfaces receptors					
Human Integrin beta3	GV	hGPIIb (-889 to +35)	CD34+ cells of Glanzmann patients with RSΔ9β3 and EAY115Cβ3 mutations	Ex vivo phenotype correction	(Wilcox et al., 2000)
Human GPIIb-MGMT(P140K)	LV	hGPIIb (-889 to +35)	BMT in the αIIbβ3 deficient Dog model[4]	Improved hemostatic function, enrichment of transduced by srug selection	(Fang et al., 2011)
Human GPIBa, HA tagged	LV	hGPIIb (-889 to +35)	DAMI cells, CD34+ cells	Detection of the GPIBa on the cell surface	(Shi et al., 2004)
Human GPIBA	LV	hGPIIb (-889 to +35)	BMT in mouse model for Bernard-Soulier syndrome[5]	Phenotype correction, 70% of the platelets pos.	(Kanaji et al., 2012)
Murine Mpl	GV	mMpl (-2085 to +42)	BMT in wt mice, Mpl-/- cells into wt recipients	Lineage-specific Mpl expression prevents toxicity of ectopic expression	(Wicke et al., 2010)
Murine Mpl	LV	mMpl (-2085 to +42) hMpl (-770 to+44) hGPIba (-258 to +330)	BMT in the Mpl-deficient mouse model[6]	Correction of aplastic anemia and thrombocytopenia	(Heckl et al., 2011)

Vectors: LV: HIV-based lentiviral vector, SIV-LV: Simian immunodeficiency virus-based lentiviral vector, GV: Gammaretroviral vector, MGMT(P140K): human O(6)-methylguanine-DNA-methyltransferase(P140K)

Promoters: hGPIba: human glycoprotein Ib alpa; hGPIIb: human glycoprotein IIb, also known as integrin alpha 2b and CD41; mPf4: murine platelet factor 4, EF1a: elongation factor 1 alpha; CMV: cytomegalovirus; PGK: phosphoglycerate kinase, MND: modified MoMuLV promoter with myeloproliferative sarcoma virus enhancer; Mpl: myeloproliferative leukemia oncogene, also known as the receptor for thrombopoietin

Mouse models

[1] Bi, L., Lawler, A.M., Antonarakis, S.E., High, K.A., Gearhart, J.D., Kazazian, H.H. Jr. (1995).Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet. 1995 May;10(1):119-21

[2] Lozier, J.N., Dutra, A., Pak, E., Zhou, N., Zheng, Z., Nichols, T.C., Bellinger, D.A., Read, M., Morgan, R.A. (2002). The Chapel Hill hemophilia A dog colony exhibits a factor VIII gene inversion. Proc. Natl Acad. Sci. USA 99, 12991–12996

[3] Wang, L., Zoppè, M., Hackeng, T.M., Griffin, J.H., Lee, K.F., Verma, I.M. (1997). Factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

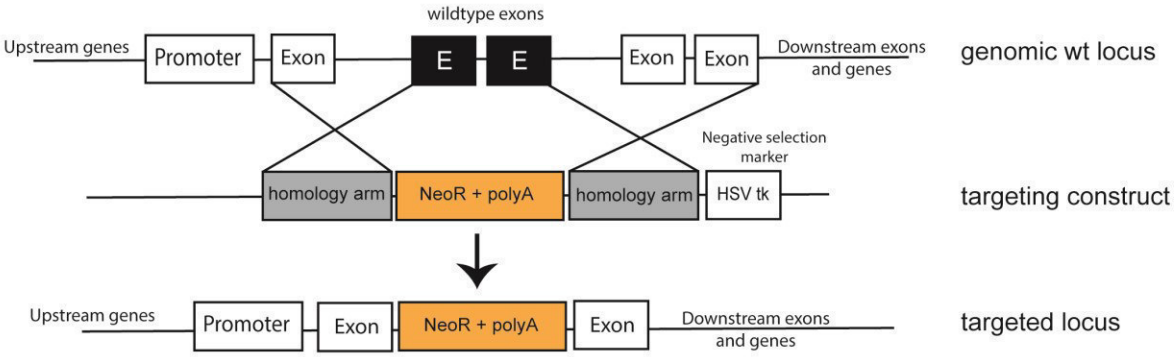
[4] Lipscomb, D.L., Bourne, C., and Boudreaux, M.K. (2000). Two genetic defects in alphaIIb are associated with type I Glanzmann's thrombasthenia in a Great Pyrenees dog: a 14-base insertion in exon 13 and a splicing defect of intron 13. Veterinary pathology *37*, 581-588

[5] Ware, J., Russell, S., and Ruggeri, Z.M. (2000a). Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proceedings of the National Academy of Sciences of the United States of America *97*, 2803-2808

[6] Alexander, W.S., Roberts, A.W., Nicola, N.A., Li, R., Metcalf, D. (1996). Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. Blood 87(6):2162-70.

Figure 1: Homologous recombination

A Consitutive knockout



B Conditional knockout

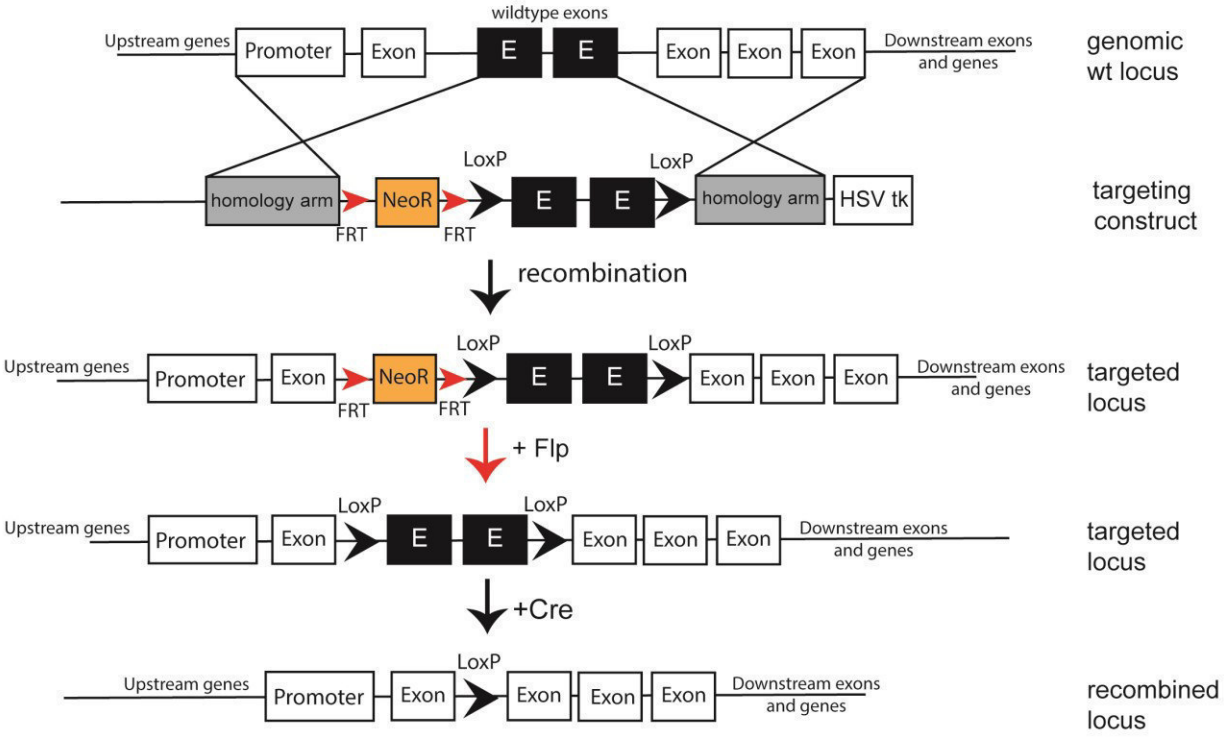


Figure 2: Cre inducible systems

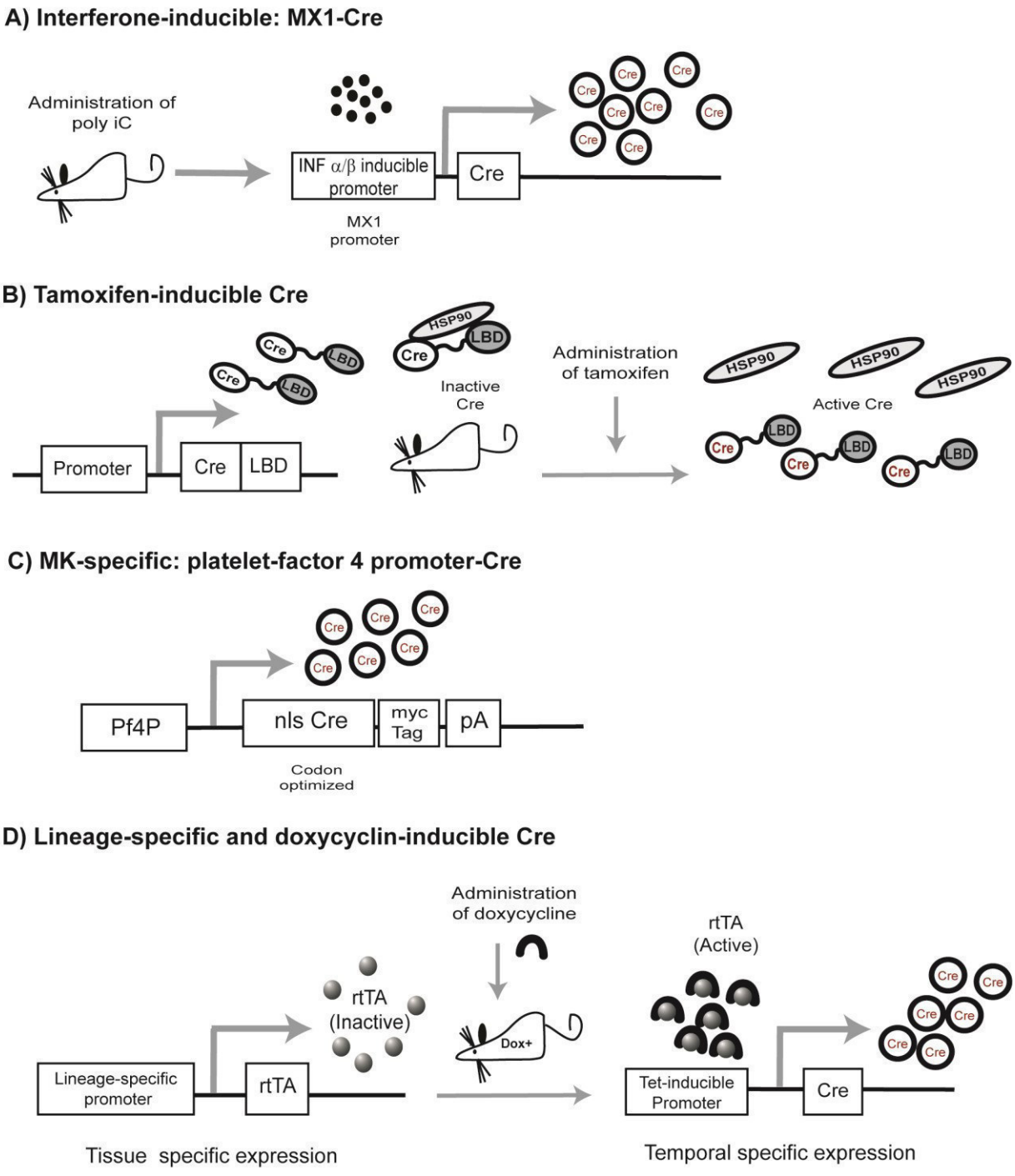
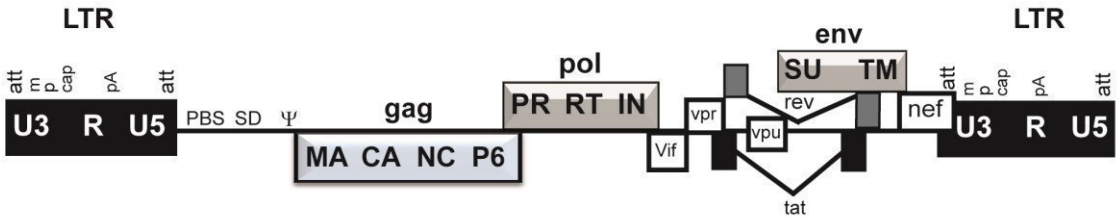
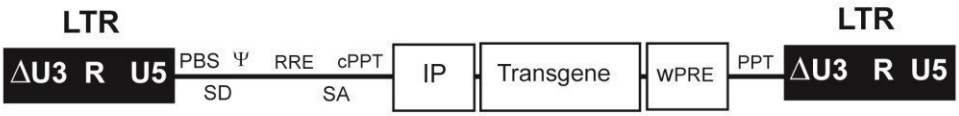


Figure 3: Viral genome editing for viral vector development

A) Proviral genome structure of the human immunodeficiency virus



B) Architecture of a lentiviral vector



c) Helper plasmids for viral production

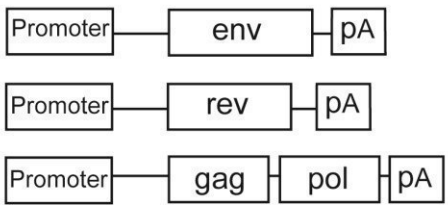
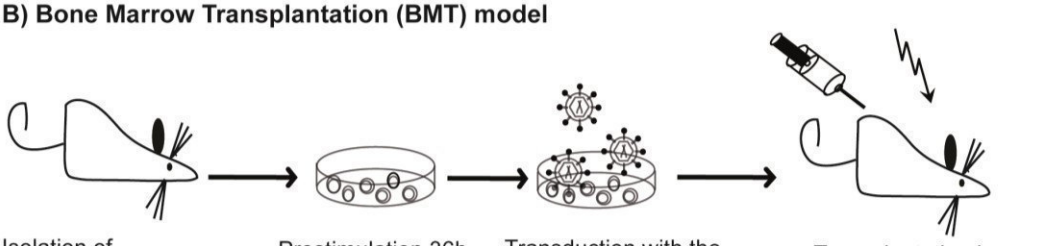


Figure 4: Lineage specific expression using lentiviral vectors

A) Lineage-specific lentiviral vector

B) Bone Marrow Transplantation (BMT) model



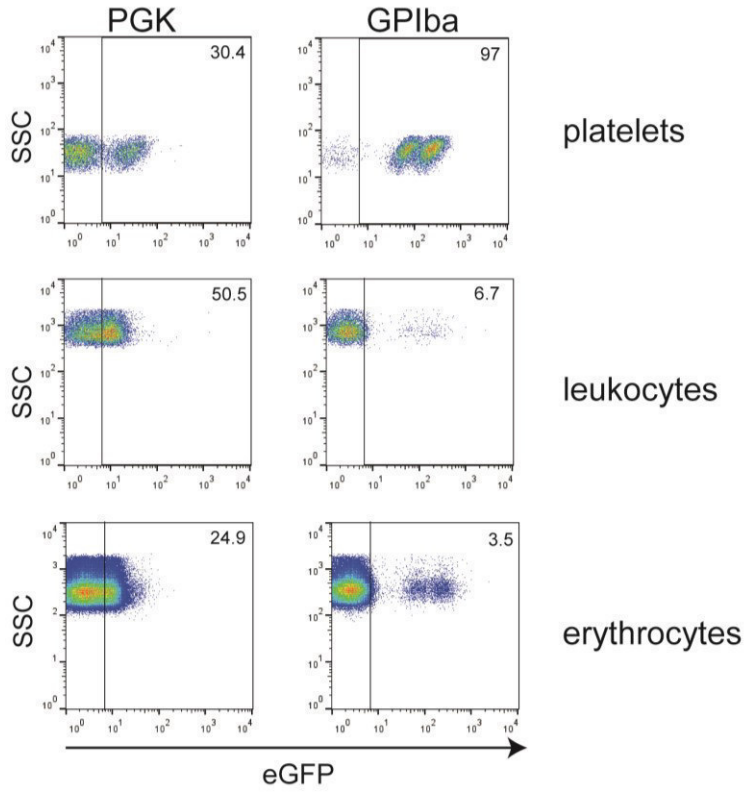
Isolation of lineage-marker negative cells (Lin-) from the bone marrow

Prestimulation 36h with cytokines

Transduction with the VSV-g pseudotype viral particles on Retronectin coated well plates.

Transplantation in lethally irradiated mice

C) Lineage-specific expression of GFP using lentiviral vectors



IP: murine Mpl (2,127 bp), human Mpl (806 bp), human GPIbaP (594 bp) , human PGK (511 bp)

Figure legend

Figure 1: Homologous recombination. A) Constitutive Knockout. The targeting vector contains an internal cassette including the neomycin resistant gene and a poly A sequence (shown in yellow), flanked by two homology arms (grey boxes) which are able to recombine with the targeted wild type locus within the genome. The HSV-tk it is included as a negative selection marker. After recombination, the neoR cassette is inserted in the targeted locus replacing the wild type sequence, and therefore disrupting the coding sequence of the wild type gene. **B) Conditional Knockout.** In a first step, the targeting vector is used to generate an embryonic stem cell which contains, flanked with 2 loxP sites, the coding sequence of the exons to be deleted (shown in black boxes), the promoter-neo resistance gene cassette (NeoR) is flanked by Frt sites (yellow box). The neoR can be removed by the administration of the Flp recombinase. After the generation of the mice with the engineered genomic locus, these mice can be bred to mice expressing the Cre recombinase. These mice can express Cre in all tissues, in a tissue-specific or inducible manner.

Figure 2: Cre inducible systems. A) Interferon inducible: MX1-Cre. The interferon inducible MX1 promoter is used to drive the expression of the Cre. The promoter is induced after the administration of the pI-pC (synthetic double-stranded RNA polyinosinic-polycytidylic acid) which activates the cells' immune response and induced interferon. Mice carrying the floxed targeted gene will only develop the targeted mutation, once the Cre is expressed after the administration of the pI-pC (Kuhn et al., 1995). **B) Tamoxifen inducible Cre**. The Cre recombinase is fused with the estrogen receptor ligand-binding domain (LBD), and to avoid permanent induction by the endogenous steroids, internal mutation are introduced in the LBD domain, to keep the affinity just for synthetic hormones. One modification of this model was done by Feil and colleagues, were by the introduction of the point mutation G521R, the induction of the LBD domain became sensitive to tamoxifen. In a non-induced state, the Cre is expressed but remains inactive by the binding of the LBD to a heat shock protein (HSP90). After administration of the tamoxifen, LBD is not bound anymore, and Cre becomes active to mediate the recombination event (Feil et al., 1997). **C) Megakaryocyte-specific platelet factor 4 Cre.** In this model, the rat platelet factor 4 promoter drives the expression of the Cre, which has been fused to a nuclear localization signal and a myc-tag. The coding sequence is followed by a poly-adenylation signal (poly-A). Because of the use of a promoter fragment of a megakaryocyte- specific gene, Cre is expected to be expressed only in the megakaryocyte lineage (Tiedt et al., 2007). **D) Lineage-specific and**

doxycycline-inducible Cre. This approach allows the spatial and temporal control of the Cre expression by the use of a tissue-specific promoter driving the expression of the reverse tetracycline-controlled transactivator protein (rtTA), which remains inactive until doxycycline is administrated. Once doxycycline is present, it binds to the rtTA, allowing by this the induction of the Tet-inducible promoter and therefore the expression of Cre (Utomo et al., 1999).

Figure 3: Viral vector development. A) Proviral genome structure of the human immunodeficiency virus. The proviral genome is flanked by long terminal repeats (LTR) (in black boxes), that provide the transcriptional control (by enhancer-promoter elements), start and polyadenylation signals. The leader region contains the primer binding site (PBS) for the initiation of the reverse transcription, the splice donor site (SD) as well as the packaging signal Ψ. Downstream the viral coding sequence is differentiated into gag (coding for structural proteins matrix (MA), capsid (CA), nucleocaspid (NC) and P6 protein); the pol (coding for replication enzymes, PR: protease, RT. reverse transcriptase, IN: integrase), and env (envelope protein). The lentiviral genome contains accessory proteins (viral infectivity factor (vif), viral protein r (vpr), viral protein u (vpu), regulator of expression of virion proteins (rev), transactivator of transcription (tat), negative factor (nef)) that are generated by alternative splicing and promote the maintenance of the viral infectivity. **B) Architecture of a lentiviral vector.** For therapeutic purposes, the genome of the virus is modified, in order to generate replicating deficient viral particles. The gag, pol and envelop proteins are removed, and replaced by the coding sequence of the transgene. Elements like PBS, SD, Ψ, splice acceptor (SA) are retained in the vector to guarantee packaging of the viral RNA (Ψ) and the initiation of reverse transcription (PBS). **C) Helper plasmids for viral production**. For the generation of infectious viral particles, proteins like gag, pol, env, and rev are delivered in *trans* (from different expression plasmids). This allows the packaging and assembly of all structural proteins needed, but because the RNA of these plasmids lack the packaging signal, just the RNA coding the transgene is packaged in the viral particles.

Figure 4: Lentiviral vector for lineage specific expression. A) Lineage-specific lentiviral vector. The enhancer/promoter in the U3 region of the LTR is deleted (ΔU3). This modification allows the use of an internal cellular promoter to control the expression of the gene of interest, in this case eGFP. (wPre: woodchuck hepatitis virus posttranscriptional regulatory element, SD: splice donor, SA: splice acceptor, RRE: rev responsive element, cPPT: central polypurine tract). Modified from (Heckl et al., 2011). **B) Bone Marrow Transplantation model (BMT).** For genetic modification in the hematopoietic stem cells (HSC) with lentiviral vectors, first the lineage marker negatives cells were isolated from the bone marrow of donor mice. Cells were cultured for 36 hours for prestimulation with cytokines, then transduced by addition of VSV-G pseudotyped lentiviral particles to the medium on Retronectin coated wells. Transduced cells are then transplanted into a lethally irradiated recipient mice. **C) Lineage-specific GFP expression in blood cell.** Six weeks after transplantation blood cells were analyzed by flow cytometry for eGFP expression. With the use of GP1b alpha promoter as internal promoter in the lentiviral vector, lineage specific expression of eGFP was achieved: 97% of the platelets expressed eGFP compare to the leukocytes (6.7%) and erythrocytes (3.5%). Using the ubiquitously expressing PGK (*phosphoglycerate kinase)* promoter as control, eGFP expression was observed in all blood cells without lineage-specificity (30.4% 50.5%, 24.9% in platelets, leukocytes, erythrocytes respectively). Data from D. Heckl, U. Modlich et al..