Role of Rho GTPases in Migration of Stem and Progenitor Cells

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ABBREVIATIONS

AAV	Adeno-Associated Virus
APC	Allophycocyanin
Asn	Asparagine
ATCC	American Type Culture Collection
attR	recombination sites
BD	Becton Dickinson
bp	Base pairs
BSA	Bovine Serum Albumin
ccdB	Cytotoxic ccdB suicide gene
CD	Cluster of Differentiation
cDNA	Coding DNA
CMV	Cytomegalovirus (immediate early promoter)
cPPT/CTS	central polypurine tract/central termination sequence
Da	Dalton
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
EBM-2	Endothelial cell medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
eEPC	Embryonic Endothelial Progenitor Cell
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope (HIV)
EPC	Endothelial progenitor cells
$F(ab')_2$	Fragment antigen binding
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
Flt-3L	Fetal liver tyrosine kinase-3 ligand
Fn	Fibronectin
Gag	Group-specific antigen (HIV)
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
HBSS	Hanks' Balanced Salt Solution
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HRP	Horse radish peroxidase
HS	Horse serum
HSA	Human serum albumin
HPC	Hematopoietic Progenitor Cells
HUVEC	Human umbilical vein endothelial cells
IgG	Immunoglobulin G
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium

IPTG	Isopropyl-α-D-1-thiogalactopyranosid
IRES	Internal ribosome entry site
IU	International Unit(s)
kb	Kilo basepairs
kDa	Kilo Dalton
LB	Luria Bertonie
Ln	Laminin
LTR	Long terminal repeat
M-CSF	Macrophage Colony Stimulating Factor
MACS	Magnetic Activated Cell Separation
MCS	Multiple cloning site
MOI	Multiplicity of infection
mRNA	messenger RNA
MSC	Mesenchymal Stem Cell
Nef	Negative regulation factor (HIV)
NLS	Nuclear localisation signal
NOD	Non-obese diabetic
pA	Polyadenylation sequence
PAGE	Polyacrylamide gel electrophoresis
PBS	Dulbecco's Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PIGF	Placental growth factor
PLL	Poly-L-lysine
PMSF	Phenyl methylsulfonyl fluoride
Pol	Polymerase (HIV)
polyA	Polyadenylation Signal
PVDF	Polyvinyldifluoride
PVP	Polyvinylpyrrolidine
Rev	Regulator of viral expression (HIV)
rh	recombinant human
Rho	Ras homologous protein
rm	recombinant murine
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRE	Rev-responsive element
RSV	Rous-Sarcoma Virus
RT	Room temperature
S1P	Sphingosine-1-phosphate
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
SCID	Severe Combined Immunodeficient
SDF-1α	Stromal Derived Factor-1alpha
SDS	Sodium dodecyl sulfate
SFFV	Spleen focus forming virus
SIN	Self Inactivation
SV40	Simian virus type 40
Tat	Transactivator (HIV)
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylene diamine
TNF-1α	Tumor Necrosis factor-1alpha
TPO	Thrombopoietin
Tris-Cl	Tris [hydroxymethyl] aminomethane hydrochloride
Trizma base	Tris [hydroxymethyl] aminomethane

TU	Transducing units
UTR	Untranslated region
VEGF (-R)	Vascular endothelial growth factor (receptor)
Vif	viral infectivity (HIV)
Vn	Vitronectin
Vpr	Viral Protein R (HIV)
Vpu	Viral Protein U (HIV)
VSV-G	G-Glycoprotein from Vesicular Somatitis Virus
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element

SUMMARY

Stem cells capable of self-renewal and differentiation into multiple tissues are important in medicine to reconstitute the hematopoietic system after myelo-ablative chemo- or radiotherapy. In the present situation, adult stem cells such as Mesenchymal stem cells (MSC) and Hematopoietic stem cells (HSC) are used for therapeutic purposes. For tissue regeneration and tissue constitution, engraftment of transplanted stem cells is a necessary feature. However, in many instances, the transplanted stem cells reach the tissues with low efficiency.

Considering the three-step model of leukocyte extravasation by Springer et al, the rolling, adhesion and transmigration form the three major steps for the transplanted stem cells to enter the desired tissues. One of the molecular switches reported to be involved in these mechanisms are the Rho family GTPases.

The present study investigates the role of Rho GTPases in adhesion and migration of stem and progenitor cells. Chemotactic and chemokinetic migration assays, transendothelial migration assays, migration of cells under shear stress, microinjection, retroviral and lentiviral gene transfer methods, oligonucleotide microarray analysis and pull down assays were employed in this study for the elucidation of Rho GTPase involvement in migration and adhesion of stem and progenitor cells.

The transmigration assay used for the migration determination of the adherent cell type, MSC, was optimized for the efficient and effective assessment of the migrating cells. The involvement of Rho was found to be critical for stem and progenitor cell migration where inactivation of Rho by C2I-C3 transferase toxin and/or overexpression of C3 transferase cDNA increased the migration rate of Hematopoietic progenitor cells (HPC) and MSC. Moreover, modulation of Rho caused predictable cytoskeletal and morphological changes in MSC.

Assessment of Rho GTPase involvement in the interacting partner, the endothelial cells during stem cell migration, revealed that active Rho expression induced E-selectin expression. The increased levels of E-selectin were functionally confirmed by the increased adhesion of progenitor cells (HPC) to the Human umbilical vein endothelial cell (HUVEC) layer. Moreover, inhibition of Rac in the migrating endothelial progenitor cells

(eEPC) increased their adhesion to HUVEC correlating with the increased percentage expression of cell surface receptor, CD44 in Rac inactivated eEPC.

In conclusion, this study shows that Rho GTPases control the adhesion and migration of stem and progenitor cells, HPC and MSC. Rho inhibition drives the cells to migrate in the blood vessels. The substantial increase in the level of active Rho in endothelial layer, manifested by the E-selectin surface expression assists the better adhesion of stem and progenitor cells to the endothelial layer. Serum factors and growth factors in the physiological system influence the Rho GTPase expression in both migrating stem cells and the barrier endothelial cells. Thus, specific modulation of Rho GTPases in the transplanted stem and progenitor cells could be an interesting tool to improve the migration and homing processes of stem cells for cellular therapy in future.

ZUSAMMENFASSUNG

Stammzellen zeichnen sich durch die Fähigkeit zu Selbst-Erneuerung und Differenzierbarkeit in unterschiedliche Gewebe aus. Dies macht sie zur Anwendung in der Medizin attraktiv. Gegenwärtig werden adulte Stammzellen wie mesenchymale Stammzellen (MSC) und hämatopoietische Stammzellen (HSC) zu therapeutischen Zwecken transplantiert. Mesenchymale Stammzellen werden seit einigen Jahren als interessantes Mittel zur Organregeneration z.B. des Herzens diskutiert, während hämatopoietische Stammzellen medizinisch zur Rekonstitution des hämatopoietischen Systems nach myeloablativer Therapie eingesetzt werden. Notwendig ist hierbei das Anwachsen der transplantierten Stammzellen im Empfänger; in vielen Fällen aber geschieht dies nur mit sehr geringer Effizienz.

In diesem Zusammenhang interessant ist die Anwendung des dreischrittigen Modells von Springer et al. für die Auswanderung von Leukozyten aus den Blutgefäßen ins Gewebe auf Stammzellen, das Rolling, Adhäsion und Transmigration von Zellen als die entscheidenden Schritte beschreibt. Nach der Transplantation, die in der Regel in den Blutkreislauf erfolgt, wandern Stammzellen selbständig in die Blut bildenden Organe ein, die ihr eigentliches "Zuhause" im Körper sind. Ein wichtiger molekularer Schalter bei diesen "homing"-Mechanismen sind die so genannten Rho-GTPasen (*ras*-homologe Guanosin-Triphosphatasen).

Die Rho-GTPasen sind eine Familie kleiner, monomerer Proteine in einer Größenordnung von ca. 25 kD, die in zwei Formen vorliegen können: Einer aktiven Form, die GTP gebunden hat, und einer inaktiven Form, die GDP gebunden hat. Beide sind durch Umwandlung von GTP in GDP bzw. Austausch von GDP gegen GTP ineinander überführbar. Liegt die Rho-GTPase in ihrer aktiven Form vor, wirkt sie an Signalwegen der G-Protein-gekoppelten Rezeptoren mit, die die Proliferation von Zellen, Differenzierung und Zellbewegungen regulieren. Die aktive, GTP-bindende Form der Rho-GTPasen wird von GTPase-aktivierenden Proteinen (GAPs) durch Hydrolyse des Triphosphats in die inaktive, GDP-bindende Form überführt. Diese wird durch Anlagerung von so genannten Guaninnukleotid-Dissoziationsinhibitoren (GDIs) stabilisiert, bis das GDP erneut gegen GTP ausgetauscht und die Rho-GTPase aktiviert wird. Dieser Austausch wiederum erfolgt durch so genannte Guaninnucleotid-Austausch-Faktoren (Guanin nucleotide exchange factors – GEFs). Rho-GTPasen spielen in vielen Signalwegen eine wichtige Rolle, unter

anderem bei der Regulation des Aktinzytoskeletts, das für die Morphologie und damit für die Migration von Zellen von entscheidender Bedeutung ist.

Die vorliegende Arbeit untersucht die Bedeutung der Rho-GTPasen für die Adhäsion und Migration von Stamm- und Vorläuferzellen. Im Einzelnen geht es um den Einfluss von Rho-GTPasen auf das Migrationsverhalten von HSC und MSC und um die Regulation der Adhäsion von Stammzellen an endotheliale Zellen. Bisher wurde in Studien zum Migrationsverhalten mesenchymaler Stammzellen das Hauptaugenmerk meist auf die Verteilung der Zellen in Organen nach einer Transplantation gelegt. Im Gegensatz dazu steht hier die Migration nach Stimulation von Adhäsionsmolekülen und Chemokinrezeptoren selbst im Mittelpunkt. Erkenntnisse hierzu könnten helfen, die adhärenten MSC besser mobilisierbar zu machen.

Ein wichtiges Mittel zur Untersuchung von Rho-GTPasen bietet die Natur: Eine Anzahl pathogener Mikroorganismen bildet natürliche Inhibitoren von Rho-GTPasen, auf denen ihre Pathogenität beruht. Zur Manipulation der Rho-GTPasen wurden in dieser Studie Toxin B, C2I-C3-Transferase und LT (lethal toxin) aus verschiedenen *Clostridien*- bzw. *E. coli*-Stämmen verwendet, die eine unterschiedliche Spezifität der Inhibition von Rho-GTPasen aufweisen. Außerdem wurden dominant-negative oder konstitutiv-aktive Varianten von Rho-GTPasen in Zellen eingebracht, um den Effekt der aktiven bzw. inaktiven Variante einer Rho-GTPase auf die Zelle studieren zu können.
In dieser Arbeit wurden drei Mitglieder der Rho-GTPasen untersucht: Rho, Rac und Cdc42. Alle drei sind an der Aktivierung des Zytoskeletts beteiligt: Rho induziert so genannte Stressfasern in Zellen sowie Fokale Adhäsionskomplexe, die bei der Anheftung von Zellen auf ihrem Untergrund von Bedeutung sind; von Rac und Cdc42 konnte gezeigt werden, dass sie die Bildung so genannter "actin ruffles" kontrollieren und Ausstülpungen der Zellmembran regulieren; alle diese Phänomene sind für Migration von Zellen wichtig.

Die Versuchsbedingungen für Migrationsexperimente mit MSC - adhärenten Stammzellen - wurden zunächst optimiert, um das Wanderungsverhalten der Zellen effektiv erfassen und darstellen zu können. Hierbei gelang es, durch Vorbehandlung der für die Migrationsexperimente verwendeten Zellkulturplatten mit verschiedenen Substanzen der extrazellulären Matrix (Vitronektin, Laminin, Fibronektin) eine Versuchsanordnung zu etablieren, mit deren Hilfe chemotaktische, d.h. auf einen chemischen Reiz hin gerichtete, Migration von chemokinetischer Migration unterschieden werden kann, die keine Richtung zeigt.

Frühere Studien zeigten, dass für die Migration von HSC die Interaktion von SDF-1α mit seinem Rezeptor CXCR4 auf den Zellen essentiell ist. In dieser Studie wurde das chemotaktische Verhalten von MSC auf SDF-1α hin untersucht. Es konnte keine klare Migration von MSC auf SDF-1α hin festgestellt werden. Als Alternative zu SDF-1α wurde humanes Blutplasma verwendet, das in verdünnter Form in der Lage war, Migration von MSC zu induzieren. Es gelang allerdings noch nicht, den dafür verantwortlichen Faktor im Plasma zu identifizieren.

Die Bedeutung von Rho für die Migration von Stamm- und Vorläuferzellen zeigte sich, als die Inaktivierung von Rho in den Migrationsexperimenten überraschenderweise die chemokinetische Migration von HPC und MSC signifikant erhöhte. Hierbei wurde eine spezifische Inhibition von Rho durch Verwendung von C2I-C3-Transferase, einem bakteriellen Toxin, erzielt. Eine generelle Inhibiton der Rho-GTPasen durch Toxin B, das Rho, Rac und Cdc42 inaktiviert, führte zu einer niedrigeren Migration von HPC auf SDF-1α bzw. MSC auf Plasma hin, dasselbe wurde nach Verwendung von LT (lethal toxin) beobachtet, das Rac und Cdc42 inaktiviert, nicht aber Rho. Eine generelle Aktivierung der Rho-GTPasen durch LPA führte ebenfalls zu einer niedrigeren Migrationsrate der Zellen.
Neben Plasma konnte auch eine Migration von MSC auf rhPDGF-bb und rhHGF hin gezeigt werden, die allerdings von der Aktivierung von Rho abzuhängen scheint.

Außerdem bewirkte eine Modulation von Rho durch Expression dominant-negativer bzw. konstitutiv-aktiver Varianten deutliche Veränderungen in der Morphologie und im Zytoskelett von MSC. Die Zellen nahmen nach Inhibition von Rho eine gestreckte, flache Konformation an, die Ausformung so genannter Lamellipodien und Pseudopodien wurde nach einigen Stunden beobachtet. Interessanterweise führte die längerfristige Expression dominant-negativen Rhos in MSC zu einem anderen Phänotyp: Nach einigen Tagen wurde eine Häufung der Zellen beobachtet, eine Ablösung der Zellen von ihrem Untergrund und eine generelle Rundung ihrer Gestalt. Überraschend war, dass Ähnliches auch nach Expression der konstitutiv-aktiven Variante von Rho in MSC zu beobachten war, nicht aber nach Expression von aktivem Rac. Dies führt zu der Annahme, dass eine zweiphasische Regulation des Zytoskeletts durch Rho und Rac erfolgt. Es kommen aber auch adaptive Prozesse in den Zellen für eine Erklärung dieser Phänomene in Betracht.

Neben der Migration ist für ein erfolgreiches Einwandern von Zellen ins Gewebe die Expression von Adhäsionsmolekülen, das "rolling" von Zellen auf dem Endothel und die

Anheftung der Zellen an endotheliale Zellen erforderlich, bevor es zum Durchtreten der Zellen durch das Endothel kommen kann.

Um diese Vorgänge möglichst nah am physiologischen Sachverhalt untersuchen zu können, wurde eine "Flow Chamber" verwendet, eine schmale Kammer, in der ein konstanter Flüssigkeitsstrom definierter Geschwindigkeit den Blutstrom in den Adern und dessen Scherkraft simuliert. Diese Scherkraft, "shear stress", hat im physiologischen Sachverhalt großen Einfluß auf das "homing"-Verhalten von Zellen und sollte deshalb bei Untersuchungen hierzu nicht vernachlässigt werden. Am Boden der Kammer befindet sich eine Lage endothelialer Zellen aus Nabelschnurblut (HUVEC), die als Modell für das Endothel der Blutgefäße dienen.

In dieser Arbeit wurde die Veränderung des "homing" von Stamm- und Vorläuferzellen unter verschiedenen Scherkräften und nach Stimulation mit dem Entzündungsmediator TNF-α untersucht, außerdem wurde die Rolle der Rho-GTPasen in diesem Zusammenhang beleuchtet. Dies geschah entweder durch Inhibition der Rho-GTPasen mit Hilfe der Toxine oder durch Überexpression dominant-negativer bzw. konstitutiv-aktiver Varianten der Rho-GTPasen in den beteiligten Zellen, also in den HUVEC der endothelialen Lage oder den HSC bzw. MSC im Flüssigkeitsstrom.

Die Analyse der Rho-GTPasen in Endothel-Zellen als den interagierenden Partnern der Stammzellen während ihrer Migration aus den Blutgefäßen in die Gewebe zeigte, dass eine erhöhte Rho-Expression mit einer erhöhten E-Selektin-Expression korrelierte. Die erhöhte E-Selektin-Expression im Endothel konnte funktionell bestätigt werden durch eine erhöhte Adhäsion von Vorläuferzellen an das Endothel. Nicht zuletzt erhöhte die Inhibition von Rac in migrierenden endothelialen Vorläuferzellen (eEPC) deren Adhäsion an HUVEC, korrelierend mit der erhöhten Expression des E-Selektin-Liganden CD44 in diesen Zellen.

Somit lassen sich Migration und "homing" von Stammzellen als eine Sequenz von Phänomenen analog zur Extravasation von Leukozyten ins Gewebe beschreiben. Rho-GTPasen spielen in den verschiedenen Stadien des "homing", "rolling", der Adhäsion und der Transmigration der Zellen eine wichtige Rolle. Die vorliegende Arbeit zeigt, dass durch Modulation von Rho-GTPasen die Einwanderung von MSC ins Knochenmark oder andere Organe verbessert werden könnte.

Während transplantierte MSC bisher aufgrund ihrer Morphologie oft in der Lunge der Empfänger hängen blieben und somit nur zu geringen Anteilen die Gewebe und Organe erreichten, für die sie bestimmt gewesen waren, könnte eine Inhibiton von Rho diese Zellen beweglicher machen, so dass sie die Lunge passieren und die Gewebe und Organe erreichen können. Auf dieser Grundlage könnten neue Therapieansätze zur Knochenheilung, Wundheilung und zur Regeneration untergegangenen Gewebes enstehen.

Die Daten sprechen für ein Modell der Transmigration, in dem aktives Rac das "rolling" von Stammzellen (MSC) im peripheren Blut und ihre Extravasation durch das Endothel reguliert. Auf der anderen Seite führt an Orten von Entzündung oder Verletzung von TNF-α aktiviertes Rho in endothelialen Zellen zu einer verstärkten Expression von E-Selektin im Endothel, was wiederum zu einer verstärkten Adhäsion von Stamm- und Vorläuferzellen führt.

Wachstumsfaktoren wie PDGF (platelet-derived growth factor), die am Ort der Verletzung präsent sind, aktivieren nun Rac. Dies führt dazu, dass Stammzellen beginnen, durch das Endothel in das betreffende Gewebe einzuwandern. In allen Schritten dieses Modells der Transmigration wird eine reziproke Balance von Rac und Rho postuliert, in der Rac die Migration und Rho die Adhäsion von Stamm- und Vorläuferzellen bewirkt, was letztlich das "homing" der Zellen und ihre Einwanderung in das Gewebe reguliert.

Zusammengefasst zeigt diese Arbeit, dass Rho-GTPasen die Adhäsion und Migration von Stamm- und Vorläuferzellen regulieren. Inhibiton von Rho führt zu erhöhter Mobilisation dieser Zellen in den Blutkreislauf. Die Zunahme der aktiven Form von Rho in den Zellen des Endothels, die sich in erhöhter E-Selektin-Expression auf den Zellen manifestiert, führt zu einer Erhöhung der Adhäsion von Stamm- und Vorläuferzellen an das Endothel. Wachstumsfaktoren und Faktoren im Serum beeinflussen die Expression von Rho-GTPasen sowohl in zirkulierenden Stammzellen als auch im Endothel.

Deshalb könnte eine spezifische Regulation der Rho-GTPasen in transplantierten Stamm- und Vorläuferzellen ein wichtiges Mittel zur Verbesserung der Transplantationseffizienz und damit der Stammzelltherapie insgesamt sein.

1.0 SCIENTIFIC BACKGROUND

1.1 Stem Cells

Stem cells are considered as “building blocks” of multicellular organisms. They have the potential of self-renewal and can differentiate into multiple lineages depending on specific signals. Stem cells could be obtained either from embryonic tissue (embryonic stem cells) or from adult tissues (adult stem cells). Embryonic stem cells (ES cells) are pluripotent cells derived from the inner mass of the blastocyst that can be cultured *in vitro* indefinitely in an undifferentiated state (Tuan*, et al* 2003). Adult stem cells could be found in many tissues of the body including hematopoietic, hepatic, mesenchymal, and epidermal tissue. Although adult stem cells are not pluripotent like ES cells, some stem cells have been termed multipotent, i.e. they are capable of differentiation *in vitro* and to contribute to multiple lineages other than their tissues of origin.

Embryonic stem cells (ES cells)

In medicine, stem cells have gained increasing attention due to the need for stem cells for the transplantation and regeneration of organs. For many reasons, ES cells isolated from human however have not entered into therapy. The first and the foremost reason are ethical considerations. The ES cells used to treat human diseases would have to be derived from human embryos. Although embryos used to produce ES cells were never destined to be re-implanted in the uterus, in most areas of the world it is an ethically unacceptable situation to destroy human embryos even for therapeutic purposes. Secondly, there are technical obstacles in the use of ES cells. Although several human ES cell lines have been established, they are not immunologically compatible with most of the patients. So a necessity occurs where separate ES cell lines would have to be made available for each patient by therapeutic cloning (Weissman 2002). However, cloning a patient’s DNA into existing stem cells is laborious and ethically contentious (Pearson 2002). A third reason for not using undifferentiated ES cells is that they have been found to form teratoma (Orkin and Morrison 2002). Also, the differentiated cells derived from cultured ES cells have been seen to function inefficiently in *in vitro* studies (Lumelsky*, et al* 2001). Recent studies report that a neo-antigen, sialic acid Neu5Gc, gets incorporated into the cultured ES cells, which may lead to the elimination of cultured ES cells by the immune system when used in the transplantation setting. Many humans were found to have circulating antibodies against Neu5Gc (Martin*, et al* 2005).

Multipotent Adult Progenitor Cells (MAPC) and Mesenchymal Stem Cells (MSC)

The identification of MAPC through the years has allowed researchers to side step the disadvantages of ES cells (Jiang*, et al* 2002). MAPC were isolated from bone marrow of mice and rats and could be cultured in an undifferentiated state for up to 100 population doublings (Reyes*, et al* 2001) or 20 to 30 cell doublings in case of human cells (Prockop 1997). Apart from bone marrow, multipotent stem-like cells were identified in brain (Taupin and Gage 2002), muscle (Majka*, et al* 2003), fat (Zuk*, et al* 2001) and liver (Campagnoli*, et al* 2001). Polymerase Chain Reaction (PCR), immunofluorescence staining, and retroviral marking methods have identified a single cell giving rise to continuous growing cultures and differentiated progeny. When transplanted into non-obese diabetic/severe-combined immunodeficient (NOD/SCID) mice, these cells were found to engraft and differentiate into cells expressing different markers specific for various tissues e.g., bone, muscle, etc. (Jiang*, et al* 2002). In addition, blastocysts injected with these cells gave rise to chimeric progeny. No tumour or teratoma formation was seen in the immunodeficient mice that received the MAPC unlike in mice transplanted with embryonic stem cells (Jiang*, et al* 2002). Moreover, adult stem cells were found to maintain their differentiation potential throughout the life of the organism. Lineage or tissue type commitment of cells of mesenchymal origin is reversible (Cancedda*, et al* 2003). In addition, the adult stem cells do not create any substantial ethical considerations.

Though adult stem cells were found to be therapeutically more feasible than ES cells, certain drawbacks occur in any cases. One disadvantage of MAPC so far is that only few groups have been capable of reproducibly isolating them. However, a similar and in many respects very comparable cell population, which is also of a fibroblastoid phenotype and which can be isolated from bone marrow are Mesenchymal Stem Cells (MSC) (Caplan and Bruder 2001, Pittenger*, et al* 1999). MSC are capable of differentiating into a number of different cell types, including neuronal progenitors (Bacigalupo 2004, Woodbury*, et al* 2000) osteoblasts, chondrocytes, adipocytes or myocytes, fat or bone cells and in some cases to muscle cells (Saltiel 2003), as shown in Fig. 1.1. Systemically infused MSC were found to home to non-hematopoietic organs and might possess the capacity to proliferate within these tissues (Devine*, et al* 2003).

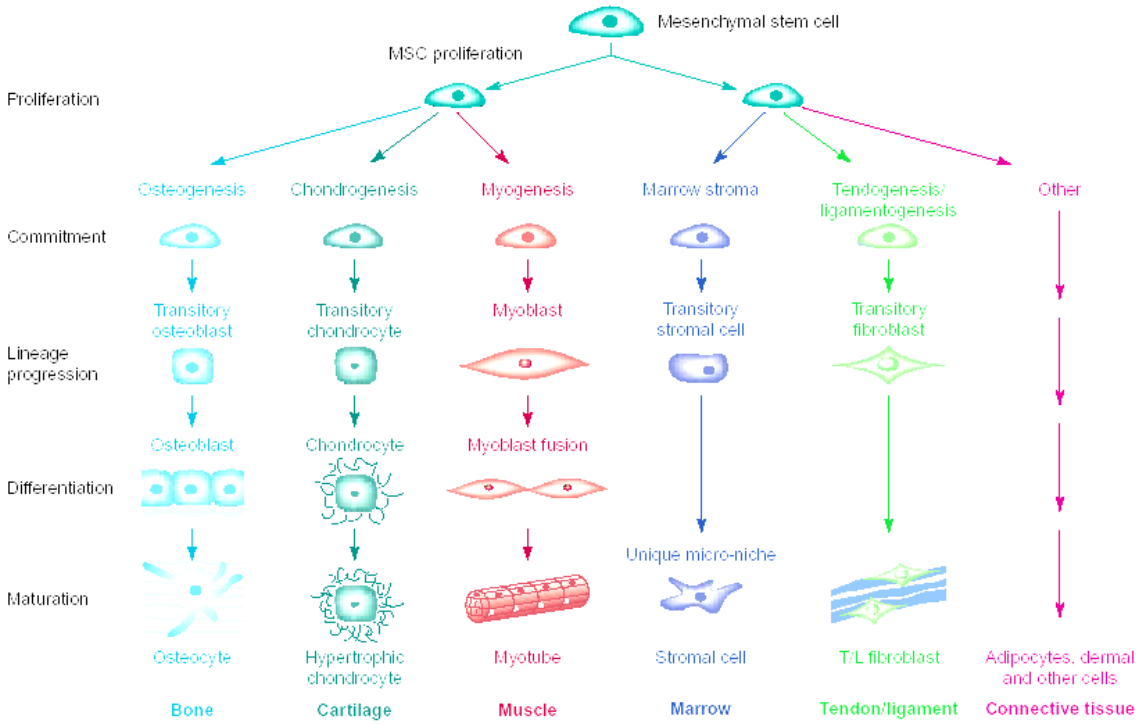


Fig.1.1 *Schematic representation of hierarchy of MSC according to Caplan (Caplan and Bruder 2001).*

Hematopoietic stem cells (HSC)

Apart from MSC, a group of blood forming stem cells, was successfully isolated from human and murine tissues by various groups (Baum*, et al* 1992, Morrison and Weissman 1994, Osawa*, et al* 1996, Spangrude*, et al* 1988). These are hematopoietic stem cells (HSC), defined by the combination of cell surface markers and by their ability to reconstitute the immune system of lethally irradiated mice.

Using *in vitro* culture techniques and *in vivo* HSC transplantation and bone marrow repopulation assays, a hierarchy of hematopoietic stem and progenitor cells has been defined. At the top of this hierarchy, a very small population of long-term bone marrow reconstituting HSC (LT-HSC) has been identified, which have the capability to reconstitute all hematopoietic lineages in an entire organism over a period of 3 months or longer (Uchida 1994, Uchida 1992) (see Fig. 1.2). Short-term repopulating HSC (ST-HSC) do the same, yet over a shorter time point. Cells with a high proliferation capacity, which are however not capable of self-renewal over extended periods or the lifetime of an animal, are termed committed hematopoietic progenitor cells (HPC). In addition to the above mentioned HSC types, multipotent (MPP), common lymphoid (CLP) or common myeloid

progenitors (CMP), as well as bi- or unilineage-determined progenitor cell types have been defined by the use unique of cell surface marker combinations (Fig. 1.2) (Passegue, *et al* 2003).

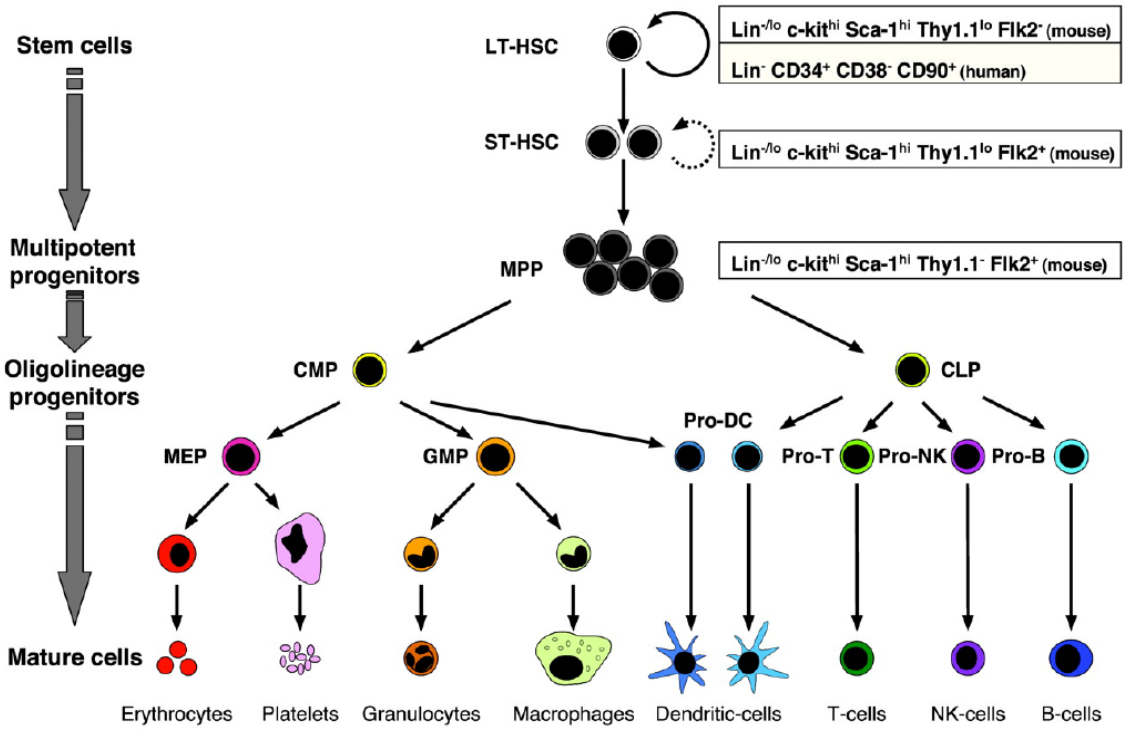


Fig 1.2 Scheme of the hematopoietic hierarchy according to Passegue (Passegue, et al 2003) showing HSC and HPC types in the course of hematopoietic lineage differentiation. *LT-HSC: Long-term hematopoietic stem cells, ST-HSC: Short term hematopoietic stem cells, MPP: Multipotent progenitor cells, CMP: Common myeloid progenitor, CLP: Common lymphoid progenitor, MEP: Megakaryocyte Erythroid progenitor, GMP: Granulocyte macrophage progenitor.*

Furthermore, using *in vitro* clonal culture systems in semisolid media (hematopoietic colony forming assays), it has been possible to enrich and purify hematopoietic growth factors. This includes, colony-stimulating factors (CSFs; factors which when given alone stimulate the growth of colonies from HPC) including Granulocyte (G-), Macrophage (M-), GM- or Interleukin (IL)-3 which activates multipotent progenitors, and on the other side a number of synergistic factors such as Stem Cell Factor (SCF), FLT3 Ligand (FL), IL-1 and IL-6 (Metcalf 1984, Metcalf 1989). Whereas clonal semisolid culture systems have been shown to primarily indicate the presence of lineage-committed HPC, long-term culture systems which are supported by a stromal cell layer have been used to analyse the content of transplantable progenitor and stem cell populations of very primitive precursor cells, termed long-term bone marrow culture initiating cells (LTC-IC) (Dexter 1977, Eaves 1988).

The role of stem cells in tissue replenishment and repair

In recent years, the ability of stem cells and progenitor cells to circulate in the blood has been recognized to be of crucial importance for a number of physiological processes such as tissue repair, regulation of the size of the hematopoietic stem cell compartment, tumour metastasis formation, and vasculogenesis. Hematopoietic stem cell transplantation has evolved as a routine therapeutic procedure for a number of patients with human malignancies including leukaemia and lymphoma, if a suitable allogeneic donor is available (Thomas 1999a, Thomas 1975). Experimental animal studies using monkeys or mice have demonstrated that the best and clinically safe way for the application of transplanted stem and progenitor cells is the intravenous route (Thomas 1970, van Bekkum 1978) where the transplanted stem cells extravasate from the blood stream to certain tissues. HSC have been shown to home to the sites of hematopoiesis within the first hours to days after transplantation. However, only a proportion of the transplanted HSC or HPC could be retrieved from bone marrow and spleen in several different models (Oostendorp*, et al* 2000, Quesenberry 1999, Szilvassy 1989). Similar results have been reported after transplantation of human HSC (van Hennik 1999) or murine MSC in immunodeficient mice (Anjos-Afonso*, et al* 2004). In patients with ischemic disease, such as myocardial infarction or stroke, support by stem or progenitor cells is sought for improved re-endothelialization and in consequence more rapid reformation of infracted vessels and the surrounding tissue. More recently, it has been demonstrated that some of the stem cells homing to the bone marrow are capable of differentiation into more cell types than hematopoietic, including epithelial and mesenchymal differentiation (Goodell 1996, Goodell 2001, Krause 2001). Also, the adult stem cells were reported to seek out the damaged tissue and repair them (LaBarge and Blau 2002, Pereira*, et al* 1998). However, in an application for bone repair, intravenously administered progenitor cells have been found to engraft with very low efficiency, indicating a potential defect in the homing of the transplanted cells (Cancedda*, et al* 2003).

1.2 Adhesion molecule(s) and mechanisms regulating the extravasation of circulating cells through endothelium

Hematopoietic stem cells (HSC) have been established to rescue the hematopoietic system after transplantation into patients with hematopoietic malignancies undergoing myelo-ablative chemotherapy or irradiation (Thomas 1999b). They were found to home to

the bone marrow and to initiate a new hematopoietic system, but the mechanisms which regulate their homing behaviour in many respects remained unclear. However, it can be inferred from mechanisms which regulate leukocyte extravasation to inflamed tissues that the engraftment step involves transplanted cells crossing the bone marrow endothelium to make their way into the bone marrow to its niche (microenvironment) using specific types of adhesion molecules. In this model, homing is an active migration process, and it involves 3 steps where the transplanted cells:

1) roll on the endothelium mediated by selectins,
2) adhere firmly to the endothelial layer through integrins and
3) transmigrate across the endothelium into the bone marrow in co-operation with chemokines (Springer 1994).

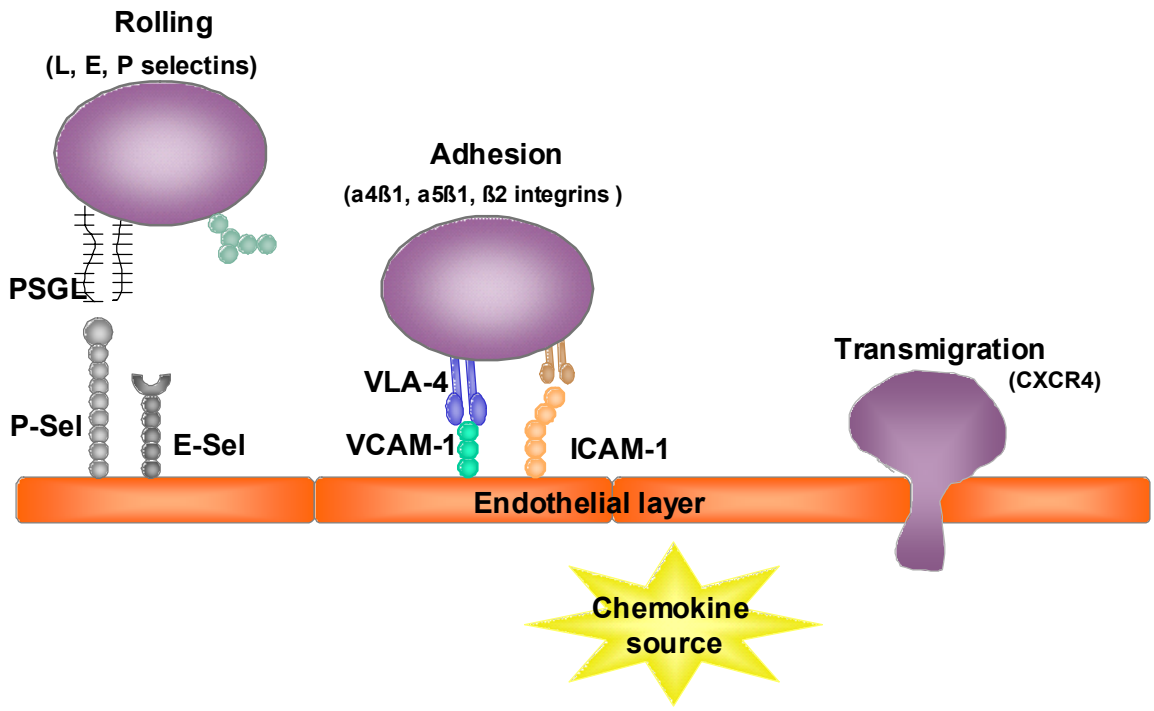


Fig. 1.3 Representation of various steps (Rolling, Adhesion and Transmigration) involved in the homing of transplanted cells. *Model according to Springer (Springer 1994).*

As shown in Fig. 1.3, the transplanted cells in *in vivo* tether and roll on the endothelial layer of the blood vessels. At some instance, the cells stop and adhere firmly to the

endothelial cell layer until they transmigrate through the endothelium. Several classes of adhesion receptors have been found to be involved in this process. These include lectin ligands named selectins (Frenette*, et al* 1998), integrins and other calcium-dependent adhesion molecules (CAMs) (Texeido 1992). Endothelial cells can express E-selectin and P-selectin (Weibel Palade bodies), leukocytes express L-selectin, and P-selectin was found in intracellular granules of platelets (α-granules) (Bevilacqua and Nelson 1993). The interaction of the migrating cells with the endothelial P-selectin was found to be mediated by P-Selectin Glycoprotein Ligand (PSGL) (Pouyani and Seed 1995). These selectins were found to mediate rolling of the cells, whereas integrins mediate adhesion and transmigration in co-operation with chemokines. Studies by several groups showed that very late antigen-4 (VLA-4) is an important homing integrin in hematopoietic stem cells (Quesenberry and Becker 1998) and other integrins involved in the process were α5β1, and β2 integrins (Verfaillie*, et al* 1994, Williams 1991). Some of the adhesion molecules so far have been found to be of relevance for the interaction of HPC with endothelial cells during the homing process as represented in Fig. 1.3. Peled et al demonstrated that SDF-1α is involved in the transmigration of HPC (Peled*, et al* 1999). Thus understanding the endothelial adhesion molecules and their interaction with the stem cells will shed light on the mechanisms of stem cell homing.

The work of a number of groups, pioneered by the studies of T. Springer and co-workers (Springer 1994), has revealed that transmigration is an important and complex process where a network of signal transduction pathways are triggered in the endothelium as well as in the migrating cells in co-operation with external chemokines which attract the cells. In addition to adhesion molecules, Stromal cell Derived Factor-1alpha (SDF-1α) has been identified as a powerful chemoattractant for bone marrow derived $CD34^+$ cells (HSC) which signals through CXCR4 receptor (Aiuti*, et al* 1997). Moreover, HSC have been suggested to migrate from the circulation into the bone marrow across the endothelium by passing through the intercellular junctions of endothelial cells (van Buul*, et al* 2002). In co-operation with selectins, integrins and chemokine mediated transmigration; many other signalling pathways are triggered in both the migrating cells and the endothelium and the surrounding cells during transmigration and homing. G protein coupled receptors were found to be an important player in this process. Recently identified small G proteins, the Rho GTPases, were reported to regulate tightly chemotaxis, actin polymerisation, and cell migration in neutrophils (Gardiner*, et al* 2002, Weiner*, et al* 1999).

1.3 Rho family small GTPases

Signalling through G proteins

G proteins are a family of small cytoplasmic signalling molecules, which have been implicated in the control of a number of crucial cellular functions, such as cell growth, differentiation, gene expression, and cell movement. G-protein coupled receptors (GPCR) transmit signals to intracellular targets through G-proteins. GPCR as cell surface receptors are characterized by seven-transmembrane α-helices. These receptors undergo conformational change due to external ligand binding which allows G-protein to bind to the cytosolic domain of the receptor and thus become activated. Thus, G proteins act as physiological switches that regulate the activity of intracellular targets in response to extracellular signals (Cooper 2000).

The role of Rho GTPase signalling in the biology of stem cells

Rho GTPases have been described in the early 1990s as members of Ras super family of small guanosine triphosphatases (GTPases) (Hall 1998). Rho stands for Ras homologous proteins. They are monomeric GTPases with a molecular weight in the range of 25kD. These proteins cycle between an active GTP-bound form and an inactive GDP-bound form (Fig. 1.4). The active state is terminated by GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis. The switch between the GTP and the GDP-bound form is catalysed by guanine nucleotide exchange factors (GEFs) (Kjoller and Hall 1999); (Rossman*, et al* 2005). The guanine nucleotide exchange inhibitors (GDIs) stabilize the inactive GDP-bound form, and thus prevent the interaction of Rho GTPases with the plasma membrane but not with the downstream targets. GEFs have multiple or single specific targets (e.g., Tiam was shown to be specific for Rac) (Kjoller and Hall 1999). These molecules significantly regulate Rho GTPases spatially and temporally (Fukata*, et al* 2003).

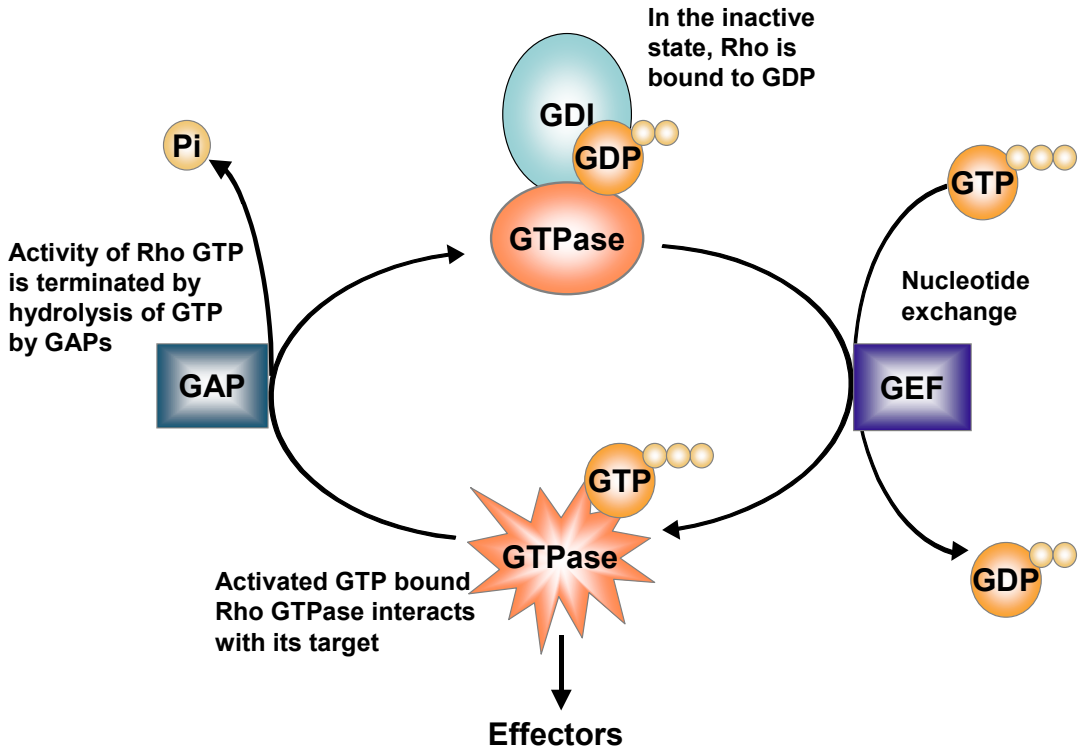


Fig. 1.4 *Schema of regulation of Small GTPase activation showing that small GTPases alternate between inactive GDP-bound and active GTP-bound states. Adapted from (Mackay and Hall 1998).*

Rho proteins act as a molecular switch controlling a variety of signal transduction processes and are also involved in the regulation of actin cytoskeleton (Raftopoulou and Hall 2004). These small Rho GTPases are involved in the control of cell-cell contact, integrin signalling (Hotchin and Hall 1996), endocytosis, proliferation (Westwick*, et al* 1998), apoptosis (Malliri and Collard 2003), transcriptional activation, endothelial cell permeability (Wojciak-Stothard*, et al* 2001) and cell transformation (Bar-Sagi and Hall 2000, Cleverley*, et al* 2000). The signalling processes contributed by Rho proteins are found to be important in host-pathogen interactions, signal transduction of cells in the immune system and host defence mechanisms. Rho proteins have also been found to be important for the invasiveness of bacteria in the gastrointestinal tract. Specific bacterial toxins have been shown to target Rho proteins in this context and thus mediate bacterial entry into mucosal cells (Aktories 1997).

The three important members of the Rho family are Rho, Rac and Cdc42. The roles of individual Rho family members, Rho, Rac and Cdc42 in cell migration are interpreted to depend primarily on the regulation of actin dynamics and organization (Hall 1998).

Migration begins when a cell polarizes in response to a chemotactic or chemokinetic signal. Rounded or amoeboid modes of cell migration have been shown to require signalling through RhoA, whereas elongated mode of cell movement is driven by Rac. The migratory behaviour was characterized by a polarized interaction at the leading edge between collagen fibres with integrin β1 and matrix metalloproteinases co-clusters at these sites (Webb and Horwitz 2003).

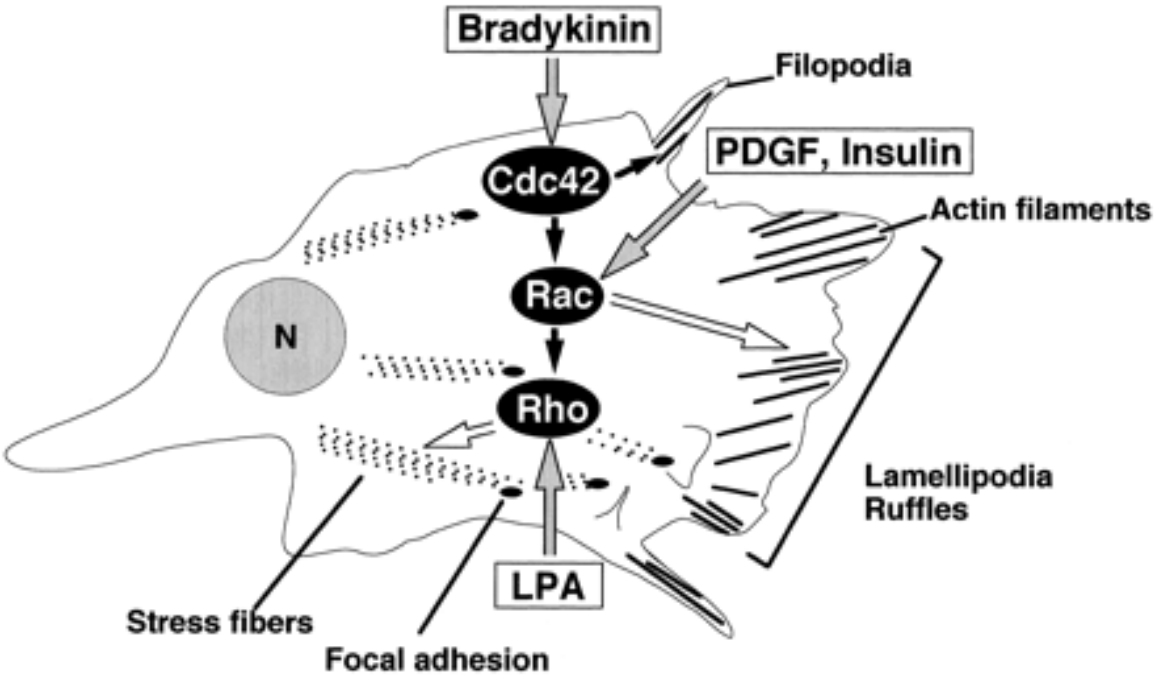


Fig. 1.5 *Small G proteins in control of actin cytoskeleton according to Matozaki (Matozaki, et al 2000). LPA: Lysophosphatidic acid, PDGF: Platelet derived growth factor*

Rho GTPases shuttle between the cytosol and specific membrane sites upon extracellular stimulation, such as growth factors and cell-adhesion molecules (Fukata*, et al* 2003). A post-translational modification, e.g. prenylation of the Rho proteins has been found to regulate their localization to different membrane compartments (Michaelson*, et al* 2001). The first molecule identified to activate Rho proteins was lysophosphatidic acid (Ridley and Hall 1992). LPA is a natural occurring water-soluble compound that was originally identified as a key molecule in de novo lipid biosynthesis (Moolenaar 1999, Moolenaar 2000). It was also regarded as an important extracellular mediator regulating a broad range of biological functions. LPA was found to be stored inside the cells at concentrations up to 60 µM and could be released into the extracellular space (Goetzl and Lynch 2000). Rho, Rac and Cdc42 have been shown to be the master regulators of actin cytoskeletal activation (Ridley and Hall 1992). Rho activation has been shown to result in the formation

of focal adhesions and stress fibres (Ridley, *et al* 1992), whereas Rac participates in the control of lamellipodia (Ridley and Hall 1992) and Cdc42 in the formation of filopodia (Nobes, *et al* 1995). This is schematically represented in figure 1.5. Upon activation Rho becomes translocated to the plasma membrane and interacts with its effectors in the GTP-bound form, whereas in the GDP-bound form where Rho is complexed with the GDI, it is restricted to the cytosol.

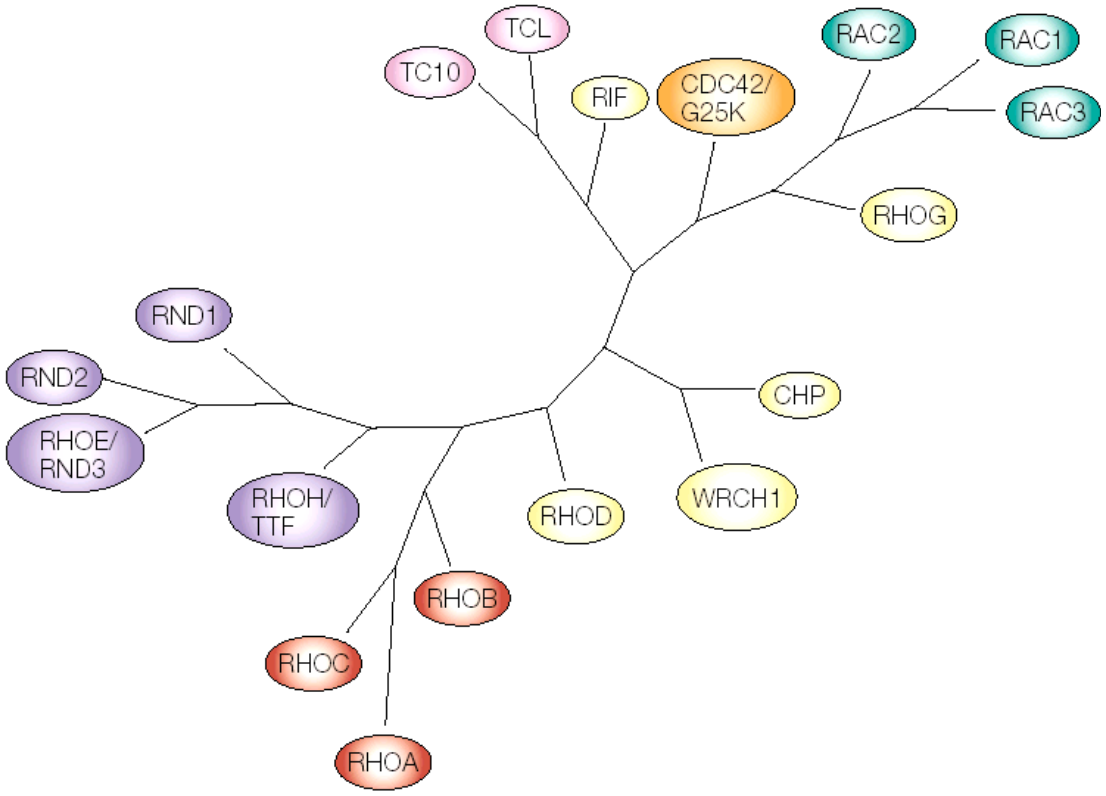


Fig. 1.6 *Rho protein family tree based on functional and biochemical relationships according to Sahai (Sahai and Marshall 2002) .*

A cross-talk between members of the Rho family GTPases has been described in primary cells (Bar-Sagi and Hall 2000). An increase in Rac level was found to down regulate the Rho activity (Rottner, *et al* 1999, Sander, *et al* 1999). Rac mediates the production of Reactive Oxygen Species (ROS) which inhibits the protein tyrosine phosphatase leading to the phosphorylation and thereby to the activation of p190RhoGAP which down regulates the Rho activity (Nimnual, *et al* 2003). Functional and biochemical relation between the members of the Rho family is represented in figure 1.6.

The possibility to study the signalling pathways and morphological changes mediated by Rho GTPases has relied on the ability to interfere with activation of individual Rho

GTPases. The various approaches include:

1. Use of bacterial toxins that specifically target Rho GTPases (Aktories 1997)
2. Constitutively active and dominant negative isoforms of the Rho GTPases (Feig 1999)
3. Introduction of GTPase binding domains that inhibit Rho GTPases (Osada*, et al* 1997).
4. Small molecule chemical inhibitors that interfere with molecules in Rho GTPase pathway

Bacterial toxins that influence Rho proteins. The toxins which have been used to influence the activity of Rho GTPases and their main characteristics are shown here in table 1.1.

Toxin	Source	Target	Mode of Action	Effect	Reference
ADP-ribosyl transferase C3	*Clostridium botulinum* type C and D strains	Rho	ADP ribosylation at asparagine 41	Inactivation	(Genth*, et al* 1999) & (Genth*, et al* 2003)
Toxin B	*Clostridium difficile*	Rho, Rac and Cdc42	Monoglucosylation of Rho proteins at threonine-37 for Rho and threonine 35 for Rac and Cdc42	Inactivation	(Aktories 1997)
Lethal toxin (LT)	*Clostridium sordellii*	Rac and Cdc42	Monoglucosylation using UDP-glucose as co-substrate	Inactivation	(Aktories 1997)
Cytotoxic necrotizing factor 1 (CNF1)	*Escherichia col*i (UPEC)	Rho, Rac and Cdc42	Deamination of glutamine 63 of Rho GTPase or glutamine 61 in Rac and Cdc42	Constitutive activation	(Waksman 2002)

Table 1.1 *The bacterial toxins that target Rho GTPases.*

Clostridium botulinum type C and D strains produce ADP-ribosyltransferase C3 that targets the Rho proteins (Aktories and Hall 1989), (Genth*, et al* 1999). Several isoforms sharing 30-70% identity of C3 exist with a molecular mass of ~ 25 kD. The enzyme lacks cell binding and membrane translocation unit and usually enters the cells by diffusion. This enzyme modifies Rho (Rho A, Rho B, and Rho C) but not Rac or Cdc42. The modification occurs at asparagine 41 in the effector region of the GTPase, resulting in the inactivation of Rho leading to rounding up of cells, and inhibition of interaction of Rho with its effector

proteins. The toxin causes the redistribution of the actin cytoskeleton, thus making it a potent cytotoxin which is widely used as a selective inhibitor of Rho GTPase. A cell permeable form of the toxin C2I-C3 was used for the present study, because C3 was found inefficient in penetrating the cells. *Clostridium difficile* toxins B also target Rho proteins (Aktories 1997) which is designated as cytotoxin. Motifs of repetitive amino acid sequences are located at the C-terminus of Toxin B which is required for its binding to the carbohydrates of the eukaryotic cell surface. The middle part of the molecule comprises a short hydrophobic region probably involved in membrane translocation. The biological activity of toxin B has been found to be located at the N-terminus. Toxin B monoglucosylates Rho proteins using UDP-glucose as co-substrate. Monoglucosylation occurs at threonine 37 for Rho and threonine 35 for Rac and Cdc42, thereby blocking its interaction with the effector proteins. *C. difficile* toxins affect the actin cytoskeleton by inactivating the Rho proteins. *C. sordellii* produces lethal toxin (LT) that shares 90% similarity with *C. difficile* Toxin B and uses UDP-glucose as co-substrate. LT has been shown not to affect Rho, whereas it glucosylates Rac and thus inactivates it. Its ability to modify Cdc42 varies between toxins from various strains (Aktories 1997).

Constitutively active and dominant negative mutants of Rho GTPases have been constructed analogous to the mutations identified in Ras proteins (Feig and Cooper 1988). Constitutively active mutants were created by mutagenesis of Gly→Val at codon 12 for Rac V12 (Xu*, et al* 1994) and Ala→Val at codon 14 for RhoA V14 (Qiu*, et al* 1995). These amino acids are conserved among the GTP binding proteins (Freeman*, et al* 1996) and mutations in these GTP binding domains or 'effector regions' were found to inhibit GTP hydrolysis (Xu*, et al* 1994). Dominant negative mutants were created by mutagenesis of Thr→Asn at codon 17 for Rac N17 (Bishop and Hall 2000) or Thr→Asn at codon 19 for Rho N19 (Qiu*, et al* 1995). Dominant negative proteins appear to function by sequestering GEFs (Kiyono*, et al* 1999) competing with the corresponding endogenous GTPase, to form a complex which could not generate a downstream response (Feig 1999). Expression of these mutant GTPases were found to modulate actin organization and cell polarity in epithelial cells (Jou and Nelson 1998).

So far, only few studies have been carried out to investigate the role of Rho GTPases in stem cell homing. All the three members of the Rho GTPases (RhoA, Rac1, Cdc42) have been reported to be ubiquitously expressed whereas Rac2 expression was found to be hematopoietic specific (Yang*, et al* 2001). On the other hand, Rac1 but not Rac2 was found to be important for the engraftment of HSC (Gu*, et al* 2003). Mice lacking Rac1 and

Rac2 showed increased mobilization of HSC from the bone marrow (Cancelas*, et al* 2005). Moreover, expression of dominant negative RhoA in the bone marrow cells led to increased stem cell engraftment (Gu*, et al* 2003).

Thus, the members of Rho family GTPases are candidates for the regulation of migration of stem cells populations. They might prove useful to understand the molecular mechanisms governing the migration and enhance the homing and engraftment of stem cells after bone marrow transplantation and may have a great impact on stem cell therapy.

2.0 AIMS

Little is known so far about the role of small GTPases in the migration and homing behaviour of stem cells. In cellular therapy, the homing behaviour of HSC has been described after *ex vivo* manipulation, especially after culture steps. Moreover, since 1999 MSC have become an additional attractive stem cell population for transplantation. However, homing and migration studies with MSC, have so far focused on the analysis of MSC distribution in the organs, and the response of MSC towards adhesion molecule and chemokine receptor stimulation is largely unknown. In addition, MSC represent a tightly adherent cell population, and it has been difficult to mobilize them to migrate in the chemotaxis system. Finally, the roles which endothelial cells play during the homing process await further elucidation.

This study concentrates on the impact of Rho family small GTPases in stem and progenitor cell populations, as well as model type endothelial cells. It wishes to investigate and clarify

1) the impact of Rho GTPase involvement in the chemotactic and chemokinetic migration of HSC,
2) the possibility to mobilize adherent MSC to better migrate and response to physiological stimuli of migration,
3) the regulation of adhesion functions mediated by Rho GTPases in endothelial cells

To interfere with Rho GTPase activation, the study is meant to use purified natural inhibitors of Rho GTPase as well as transfection and subsequent overexpression of dominant negative or constitutively active GTPase isoforms. The study aims to provide further insight into methods which will allow the manipulation and engineered targeting of potential therapeutic stem and progenitor cell populations for application in transplantations.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

2-Propanol	Roth (# T910.1), Karlsruhe, DE
Acetic acid, Glacial Hygroscopic	Sigma (# A-6283), Steinheim, DE
Acetone	Roth (# 9372.4), Karlsruhe, DE
Acrylamide solution	Roth (# 3030), Karlsruhe, DE
Agarose Type VII:	
-low gelling temperature	Sigma (# A-9045), Steinheim, DE
Albumin, Fraction V	Sigma (# A-3059), Steinheim, DE
Ammonium persulfate	Sigma (# A-3678), Steinheim, DE
Ammonium Chloride	Sigma (# A-5666), Steinheim, DE
Ampicillin	Sigma (# A-9518), Steinheim, DE
β-mercaptoethanol	Fluka (#63689), Buchs, CH
Bombesin	Sigma (# B-4272), Steinheim, DE
Bromophenol blue	Sigma (# B-5525), Steinheim, DE
Calcium chloride (dihydrate)	Sigma (# C-3306), Steinheim, DE
Chloroform	Sigma (# C-2432), Steinheim, DE
Dry milk (non-fat)	Bio-Rad Laboratories (# 170-6404), Munich, DE
Ethanol	Roth (# 5054.1), Karlsruhe, DE
EDTA, disodium, dihydrate	Sigma (# E-9884), Steinheim, DE
EZ-Detect Rac1 activation kit	Perbio Science (# 89856Y), Bonn, DE
EZ-Detect Rho activation kit	Perbio Science (# 89854Y), Bonn, DE
Gelatin Type A: from porcine skin	Sigma (#G-2500), Steinheim, DE
Giemsa's solution	Merck (# 00109204), Darmstadt, DE
D-(+)-Glucose (anhydrous)	Sigma (# G-8270), Steinheim, DE
Glycerol	Sigma (# G-5116), Steinheim, DE
Glycine	Sigma (# G-8898), Steinheim, DE
HEPES	Sigma (# H-7523), Steinheim, DE
Hexamethrin bromide (polybrene)	Sigma (# H-9268), Steinheim, DE
Hydrochloric acid	Roth (# 9277.2), Karlsruhe, DE
Laemmli sample buffer	Bio-Rad Laboratories (# 161-0737), Munich, DE
Lauryl Sulphate (SDS)	Sigma (# L-3771), Steinheim, DE
Lysophosphatidic Acid (LPA)	Sigma (# L-7260), Steinheim, DE
Magnesium chloride, Hexahydrate	Sigma (# M-2670), Steinheim, DE
May Grünwald solution	Merck (# 1.01424.2500), Darmstadt, DE
Methanol	Merck (# 1.6009.1011), Darmstadt, DE
Mobi Glow Mounting medium	Mo Bi Tec (# MGM01), Goettingen, DE
Nonidet P-40	Sigma (# N-7320), Steinheim, DE
Parafilm M	Roth (# 20021151), Karlsruhe, DE
Paraformaldehyde	Sigma (# P-6148), Taufkirchen, DE
Phalloidin	
-FITC from *Amanita phalloides*	Sigma (# P-5282), Steinheim, DE
-TRITC from *Amanita phalloides*	Sigma (# P-1951), Steinheim, DE
Plasmid Maxi preparation kit	Genomed (# 220020), Löhne, DE
PKH26 Red fluorescence kit	Sigma (# PKH26GL), Steinheim, DE

PKH67 Green fluorescence kit	Sigma (# MINI67), Steinheim, DE
Ponceau S	Sigma (# P-7170), Steinheim, DE
Potassium carbonate	Sigma (# P-4379), Steinheim, DE
Potassium chloride	Sigma (# P-9333), Steinheim, DE
Pre-stained SDS-PAGE standards,	
-Low molecular weight range	Bio-Rad Laboratories (# 161-0305), Munich, DE
- Broad range	Bio-Rad Laboratories (# 161-0373), Munich, DE
Protein detection kit	Bio-Rad Laboratories (# 500-0006), Munich, DE
Propidium iodide	Sigma (# P-4864), Steinheim, DE
Sodium acetate, anhydrous	Sigma (# S-2889), Steinheim, DE
Sodium azide	Fluka (# 71289), Buchs, CH
Sodium bicarbonate	Sigma (# S-6279), Steinheim, DE
Sodium chloride	Roth (# P029.2), Karlsruhe, DE
Sodium hydroxide	Merck (# 6327139), Darmstadt, DE
Sodium phosphate	Sigma (# S-7907), Steinheim, DE
Shingosine-1-phosphate	Sigma (# S-9666), Steinheim, DE
TEMED	Sigma (# T-7024), Steinheim, DE
Triton X-100	Sigma (# T-6878), Steinheim, DE
Trizma base	Sigma (# T-6066), Steinheim, DE
Trizma Hydrochloride	Sigma (# T-5491), Steinheim, DE
TRIzol reagent	Invitrogen Life Technologies (# 15596-018), Karlsruhe, DE
Tweeņ 20	Fluka (# 93773), Buchs, CH
Western blot detection kit	Upstate Biotechnology (# 17-318), Hamburg, DE
Western blot detection kit (ECL plus)	Amersham BioSciences (# RPN2133), Freiburg, DE
Y27632 in solution	Calbiochem (# 688001), Schwalbach/Ts, DE

3.1.2 Toxins

Cytochalasin D	Sigma (# C-8273), Steinheim, DE

The Rho GTPase inhibiting toxins, C2I-C3, a cell permeable form of C3 transferase from *Clostridium botulinum*, Lethal Toxin (LT) from *Clostridium sordellii* Toxin B from *Clostridium difficile* were a kind gift from Dr. Klaus Aktories, Institute of Pharmacology, University of Freiburg, Germany.

3.1.3 Protease inhibitors

Aprotinin from bovine lung	Sigma (# A-1153), Steinheim, DE
Dithiothreitol	Sigma (# D-5545), Steinheim, DE
Leupeptin Trifluoroacetate salt	Sigma (# L-2023), Steinheim, DE
N-Ethylmaleimide	Sigma (# E-1271), Steinheim, DE
Pepstatin A	Sigma (# P-4265), Steinheim, DE
PMSF	Sigma (# P-7626), Steinheim, DE

3.1.4 Cell culture media and reagents

BSA, V solution	Sigma (# A-1595), Steinheim, DE
Chloroquine	Sigma (# C-6628), Steinheim, DE
Dextran -FITC, 3000MW, anionic	Molecular Probes, USA
DMSO	Sigma (# D-2650), Steinheim, DE
DMEM	
-High glucose (4.5g/l)	PAA Laboratories (# E15-810), Cölbe, DE
-Low glucose (1g/l)	PAA Laboratories (# E15-806), Cölbe, DE
EBM-1	Clonetics (# CC-3156), Oldendorf, DE
FBS	PAN Biotech (# 3302-P222303), Aidenbach, DE
Fibronectin from human plasma	Sigma (# F-2006), Steinheim, DE

Stock solutions of 1 mg/ml prepared in PBS and stored in small aliquots at -80°C.

Ficoll separating solution	Biochrom (# L-6115), Berlin, DE
Gelatin (From Porcine Skin)	Sigma (G-1890), Steinheim, DE
HBSS	Gibco (# 24020-091), Karlsruhe, DE
HEPES solution	Gibco (# 15630-056), Karlsruhe, DE
Horse serum	Gibco (# 16050-122), Karlsruhe, DE
HSA, 20% solution	DRK-Blutspendedienst Niedersachsen, Springe, DE
IMDM	Gibco (# 21980-032), Karlsruhe, DE
Laminin from human placenta	Sigma (# L-6274), Steinheim, DE

Stock solutions of 1mg/ml prepared in PBS and stored in small aliquots at -80°C.

LB Agar (Lennox L agar)	Invitrogen life Technologies (# 22700-025), Karlsruhe, DE
LB broth base	Invitrogen life Technologies (# 12780-052), Karlsruhe, DE
Non-essential amino acid solution	Gibco (# 11140-035), Karlsruhe, DE
Penicillin-Streptomycin solution	Gibco (# 15240-062), Karlsruhe, DE
PBS	Gibco (# 14190-094), Karlsruhe, DE
PLL	Sigma (# P-4707), Munich, DE
Protamin sulfate	Sigma (# P-4020), Steinheim, DE
Red blood cell lysis buffer	Sigma (# R-7757), Taufkirchen, DE
Retronectin	Takara, Shiga, Japan
RNase I	
RPMI 1640 supplemented with L-Glutamine	Gibco (# 21875-034), Karlsruhe, DE
S. O. C. Medium	Invitrogen life Technologies (# 15544-034), Karlsruhe, DE
Trypan blue stain (0.4%)	Gibco (# 15250-061), Karlsruhe, DE
Trypsin EDTA, 0.25% solution	Gibco (# 35400-010), Karlsruhe, DE
Vitronectin from human serum	Sigma (# V-8379), Steinheim, DE

Stock solutions of 1 mg/ml prepared in PBS and stored in small aliquots at -80°C.

Water, endotoxin-free	Sigma (# W-3500), Steinheim, DE

3.1.5 Antibodies

3.1.5.1 Antibodies used for Western blotting and Immunofluorescence

Anti-mouse IgG-HRP	Sigma (# A-9917), Steinheim, DE
Donkey anti-goat IgG-HRP	Santa Cruz Biotechnology (# sc-2020),
Anti-mouse IgG-FITC	Sigma (# F-5262), Steinheim, DE
Anti-Paxillin	BD Transduction Laboratories (Clone 349), Heidelberg, DE
Anti-mVEGF R1 (Flt-1)	R & D Systems, Wiesbaden, DE
Goat anti-human E-selectin	R & D Systems (# BBA18), Wiesbaden, DE
Monoclonal anti-ß-Actin	Sigma (Clone AC-74), Steinheim, DE
Monoclonal anti-Cdc42	Perbio Science, Bonn, DE
Monoclonal anti-Rac1	Perbio Science, Bonn, DE
Monoclonal anti-Rho	Perbio Science, Bonn, DE
Monoclonal mouse anti-RhoA	Santa Cruz Biotechnology (Clone 26C4), DE
Monoclonal mouse anti-Rho Clone 55	BD Transduction laboratories, Heidelberg, DE
Polyclonal anti-human E-selectin	R & D Systems (# BBA18), Wiesbaden, DE
Polyclonal Rabbit anti-Goat IgG/FITC	Dako Cytomation, Hamburg, DE

3.1.5.2 Antibodies used for flow cytometric analysis

The following antibodies from BD Pharmingen were used with the indicated chromophores:

Antibody	Chromophore	Clone/Isotype	Species	Cat. No
CD3	PE	HIT3a/Mouse IgG_{2a},k	Mouse anti-human	555340
CD11b/Mac-1	PE	M1/70/Rat (DA) IgG_{2b}, k	Rat anti-mouse	553311
CD117/c-Kit R	PE	2B8/Rat IgG_{2b}, k	Rat anti-mouse	553869
CD31/PECAM-1	-	MEC13.3/Rat $IgG2_a$,k	Rat anti-mouse	01951D
CD44	FITC	IM7/Rat IgG_{2b}, k	Rat anti-mouse	01224D
CD45/LCA	Cy-chrome	30-F11/Rat IgG_{2b}, k	Rat anti-mouse	011184
CD45R/B220	PE	RA3-6B2/Rat IgG_{2a},k	Rat anti-mouse	553089
CD49d/Int α4	FITC	R1-2/Rat IgG_{2b}, k	Rat anti-mouse	01274D
CD62L/L-sel	PE	MEL-14/Rat IgG_{2a},k	Rat anti-mouse	01265B
CD54/ICAM-1	PE	3E2/Rat IgG, group 1, k*	Hamster anti-mouse	01545B
CD73	PE	AD2/Mouse IgG_1,k	Mouse anti-human	550257
CD105	-	266/Mouse IgG_1,k	Mouse anti-human	555690
CD135/Flk-2	PE	A2F10.1/Rat (WI) $IgG2_a$,k	Rat anti-mouse	553842
Gr-1	PE	RB6-8C5/Rat IgG_{2b}, k	Rat anti-mouse	553128
IgG_{2a}, k	FITC	MOPC-21	Mouse	11024C
IgG_{2b}, k	PE	A95-1	Rat	11185A
Sca-1	FITC	E13-161.7/Rat IgG_{2a},k	Rat anti-mouse	553335
TER-119	PE	TER-119/Rat IgG_{2b}, k	Rat anti-mouse	553673
VEGFR2/Flk-1	PE	Avas12α1/ Rat IgG_{2a},k	Mouse	555308

3.1.6 Magnetically labelled microbeads for cell separation

Anti-mouse B220	Miltenyi Biotech (# 120-000-299), Bergisch Gladbach, DE
Anti-mouse CD11b/Mac-1	Miltenyi Biotech (# 120-000-300), Bergisch Gladbach, DE
Anti-mouse TER-119	Miltenyi Biotech (# 120-000-303), Bergisch Gladbach, DE
Anti-mouse Sca-1	Miltenyi Biotech (# 120-001-503), Bergisch Gladbach, DE

3.1.7 Chemokines and cytokines

basic FGF	TeBU-bio (# 10019-B), Offenbach, DE
PDGF-BB	TeBU-bio (# 10014-B), Offenbach, DE
rhFLT3-L	TeBU-bio (# 300-19), Offenbach, DE
rhTPO	TeBU-bio (# 300-18), Offenbach, DE
rmGM-CSF	R & D Systems (# 016315-03), Wiesbaden, DE
rmIL-3 (E.coli)	R & D Systems (# 14190-094), Wiesbaden, DE
rmIL-6	TeBU-bio (# 216-16), Offenbach, DE
rmSCF	Peprotech (# 250-53), Offenbach, DE
rmSDF-1α	R&D Systems, Wiesbaden, DE
rhTNF-1α	R&D Systems (# 410-MT), Wiesbaden, DE

3.1.8 Buffers

2 X SDS Gel-loading buffer

Tris-Cl pH 6.8	100 mM
SDS (electrophoresis grade)	4% (w/v)
Bromophenol blue	0.2% (w/v)
Glycerol	20% (v/v)
Dithiothreitol	
(or) β-mercaptoethanol	200 mM

Components were dissolved in dH_2O
Dithiothreitol or β-mercaptoethanol was added to the buffer just before use.

GST-Fish buffer

Tris-HCl pH 7.4	50mM
NaCl	150mM
$MgCl_2$	4mM
Glycerol	10% (v/v)
Nonident P-40	1% (v/v)

Components were dissolved in dH_2O

Isolation buffer for lineage depleted bone marrow cells:
PBS + 0.5% BSA + 2 mM EDTA

Microinjection buffer

KCl pH 7.2	137 mM
NaCl	2.7 mM
Na2HPO4	8.1 mM
KH2PO4	1.5 mM
MgCl2	4 mM

Components were dissolved in dH_2O

0.5M EDTA pH 8.0

Dissolved in dH_2O

Tris Buffer Saline (TBS) pH 7.4

NaCl	150 mM
Tris-Cl	20 mM

Tris-Glycine electrophoresis buffer

Tris	25 mM
Glycine	250 mM
SDS	1% (w/v)

Components were dissolved in dH_2O

Tris-Cl pH 6.8 **1.0 M**

Dissolved in dH_2O

Tris-Cl pH 8.8 **1.5 M**

Dissolved in dH_2O

Tris-EDTA buffer (TE) pH 7.5

Tris-Cl	100 mM
EDTA	10 mM

Components were dissolved in dH_2O

TE/dH_20 buffer

0.1 X TE	2 parts
dH_20	1 part

Salt solution

KCl	1 M
Glucose	20%
Na_2HPO_4	141 mM

Components were added to dH_2O and volume made up to 50 ml.

2X HBS solution

HEPES	42 mM
NaCl	171 mM
Salt solution	1 ml

The volume was made to 85 ml with dH_2O, pH of the solution was adjusted to 7.0, 7.05, 7.10 respectively with NaOH, and the volume made up to 100 ml with dH_2O. The solution was sterile filtered with 0.22 µm filter and stored at -20°C in aliquots until use.

2.5 M $CaCl_2$ solution

$CaCl_2$ 2.5 M

The component was dissolved in dH_2O and sterile filtered with 0.22 µm filter and stored at -20°C in aliquots until use.

3.1.9 Instruments and apparatus

2-well chamber slides	Nunc (# 177437), Rochester, NY
4-well chamber slides	Nunc (# 177429), Rochester, NY
48 well chemotaxis chamber	NeuroProbe, Gaithersburg, MD, USA
Autoclave	Integra Biosciences, Baar, CH
Bacterial incubator	Heraeus, Hanau, DE
Balance	Sartorius, Göttingen, DE
Cell culture flasks	Greiner, Frickenhausen, DE
Cell culture incubator	Heraeus, Hanau, DE
Cell culture dishes	Greiner, Frickenhausen; Becton Dickinson, DE
CELLocate glass cover slips with grids,	
- Grid size 55 µm	Eppendorf (# 5245952.004), Hamburg, DE
Cell scraper	Corning, Wiesbaden, DE
Cell sieve (40 µm)	Becton Dickinson, Heidelberg, DE
Centrifuge	Eppendorf, Hamburg, DE
Cryotubes	Nalgene, Rochester, NY
Electrophoresis apparatus	
- For agarose gels	Life Technologies, Karlsruhe, DE
- For SDS-PAGE	Bio-Rad Laboratories, Munich, DE
FACS tubes	Fisher Scientific (# 352063), Schwerte, DE
Femtotips II	Eppendorf (# 5242 957.000), Hamburg, DE
Femtojet	Eppendorf, Hamburg, DE
Flow chamber kit	
- Circular parallel plate	GlycoTech, Maryland, U.S.A
Flow-cytometer Coulter Epics II	Beckman Coulter, DE
Hyperfilm ECL	Amersham Biosciences (# RPN3103K), Freiburg, DE
Injectman NI 2	Eppendorf, Hamburg, DE
Light microscope	Zeiss, Göttingen; Leica, Wetzlar, DE
Nitrocellulose membrane (0.45 µm)	Schleicher & Schuell (# 10401196), Dassel, DE
MACS separation column	Miltenyi Biotech (# 130-041-306), Bergisch-Gladbach, DE
MACS Pre-separation Filter	Miltenyi Biotec, Bergisch-Gladbach, DE
Microscope Olympus CK-2;	
Zeiss Axiovert 135	Zeiss, Oberkochen, DE
Microloader	Eppendorf (# 5242 956.003), Hamburg, DE
Microtubes	Eppendorf, Hamburg; Sarstedt, Nümbrecht, DE
Micropipette tips	
Micropipettes	
Mini MACS	Miltenyi Biotec, Bergisch-Gladbach, DE
Perfusion pump	(Perfusor secura FT) Braun, Heidelberg, DE
Perfusion syringe	Braun, Melsungen, DE
Petridishes	
pH-Meter	
Polyallomer Centrifuge tubes	Beckman, Krefeld, DE
PVP free, 8 µm pore size filters	NeuroProbe, Gaithersburg, MD, USA

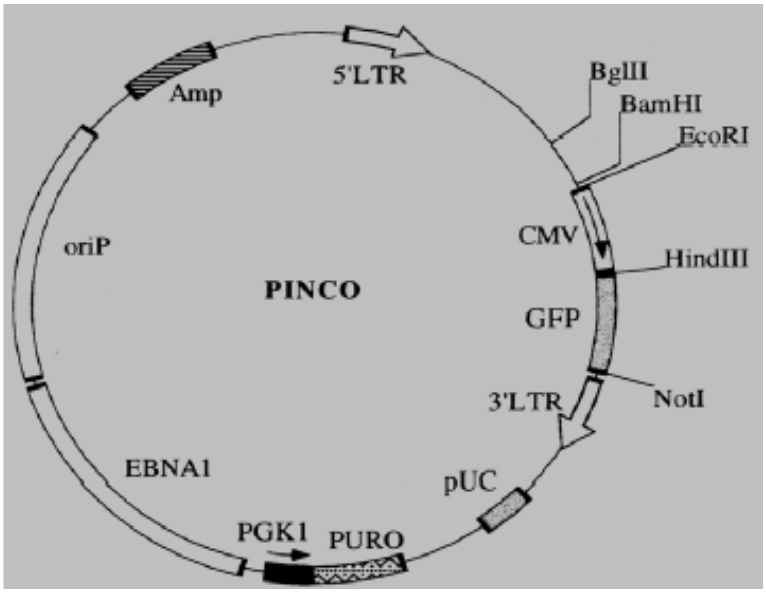
PVDF membrane (0.2 µm)	Schleicher & Schuell, Dassel, DE
Spectrophotometer	
Stericup-Filter	Millipore (# SCGPU05RE), Eschborn, DE
Sterile filter	Millipore, Eschborn, DE
-0.22 µm	Millipore, Eschborn, DE
-0.45 µm	Millipore, Eschborn, DE
Table top centrifuge	Eppendorf, Hamburg, DE
Thermomixer 5436	Eppendorf, Hamburg, DE
Ultra-centrifuge	Beckman, DE
Ultrospec II	Biochrom, Cambridge, GB
Vario MACS	Miltenyi Biotec, Bergisch-Gladbach, DE
Vortex	Eppendorf, Hamburg, DE
Water bath	Julabo Labortechnik, Seelbach, DE
Whatman-Paper	Schleicher & Schuell, Dassel, DE

3.1.10 cDNAs and plasmid vectors

3.1.10.1 Plasmids used for production of Moloney type retroviral vectors

Pinco

Retroviral expression vector derived from Moloney murine Leukemia virus, length = 3506 bp.



Pinco Rho wild type

Homo sapiens Ras homolog gene family member A (RHOA), length = 1926 bp, cloned into Pinco 5' of an ATG and Myc-Tag sequence, was obtained from Dr. Martin Ruthardt, Hematology, J. W. Goethe-University, Frankfurt.

Rho14V25N in Pinco

Mutated Homo sapiens RhoA cDNA, length = 1926 bp, with G14V and F25N transitions which had been cloned into Pinco 5' of an ATG and Myc-Tag sequence.

Pinco RacV12

Homo sapiens Ras-related C3 botulinum toxin substrate 1, Rho family, small GTP binding protein Rac1, length = 2355 bp, with G12V transitions which had been cloned into Pinco 5' of an ATG and Myc-Tag sequence.

Pinco RacN17

Homo sapiens Ras-related C3 botulinum toxin substrate 1, Rho family, small GTP binding protein Rac1, length = 2355 bp, with T17N transitions which had been cloned into Pinco 5' of an ATG and Myc-Tag sequence.

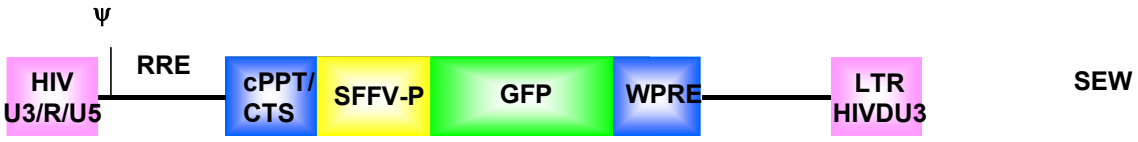
Pinco C3 (C3 transferase d.n. RhoA)

C. limosum C3 gene, Length = 1022 bp, which had been cloned into Pinco 5' of an ATG.

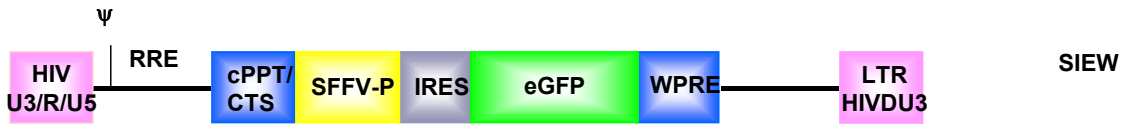
All Moloney type retroviral vectors were kindly provided by Dr. Martin Ruthardt, Hematology, J. W. Goethe-University, Frankfurt.

3.1.10.2 Plasmids used for production of lentiviral vectors

SEW, obtained from Dr. Manuel Grez, Georg Speyer Haus, Biomedical Research Institute, Frankfurt.



SIEW, obtained from Dr. Manuel Grez, Georg Speyer Haus, Biomedical Research Institute, Frankfurt.



SIEW-RFA
SIEW vector with the Gateway recombination sites inserted.

SIEW-RFA-Rho V14

Mutated Homo sapiens RhoA cDNA, length = 1926 bp, with G14V and F25N transitions which had been cloned into SIEW-RFA 5' of an ATG.

SIEW-RFA-C3

C. limosum C3 gene, Length = 1022 bp, which had been cloned into SIEW-RFA 5' of an ATG.

SIEW-RFA-Rac V12

Homo sapiens Ras-related C3 botulinum toxin substrate 1, Rho family, small GTP binding protein Rac1, length = 2355 bp, with G12V transitions which had been cloned into SIEW-RFA 5' of an ATG.

SIEW-RFA-Rac N17

Homo sapiens Ras-related C3 botulinum toxin substrate 1, Rho family, small GTP binding protein Rac1, length = 2355 bp, with T17N transitions which had been cloned into SIEW-RFA 5' of an ATG.

All SIEW-RFA constructs were provided by Dr. B. Rüster, Stem Cell Biology, DRK-Blutspendedienst, Frankfurt, Germany.

3.1.10.3 Construction of SIEW-RFA

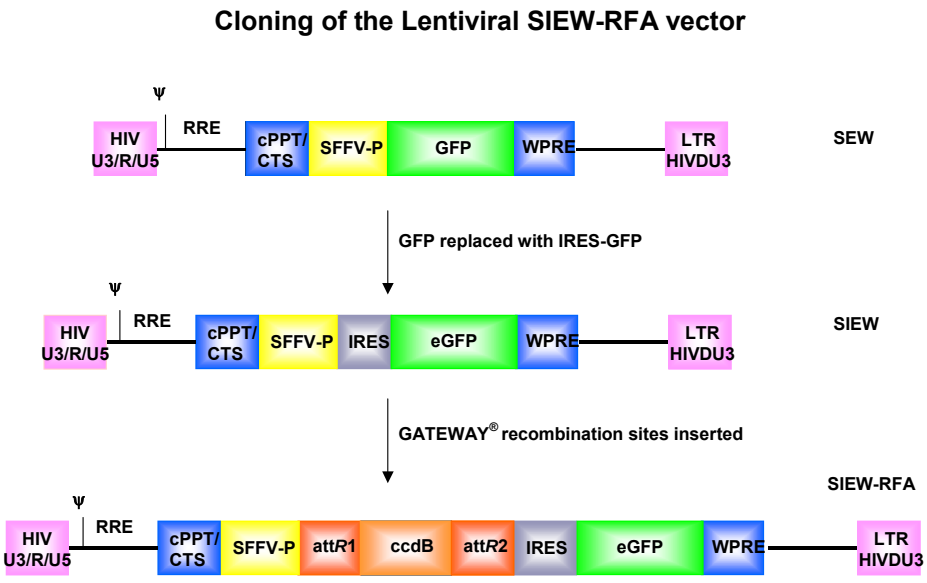


Fig.3.1 Construction of SIEW-RFA*. Within the SEW vector, the GFP sequence was replaced by an IRES-GFP cassette. Subsequently, attR1 and attR2 recombination sites flanking a ccdB suicide sequence were inserted 5´of IRES-GFP.*

Recombination of a mutant Rac gene into SIEW-RFA

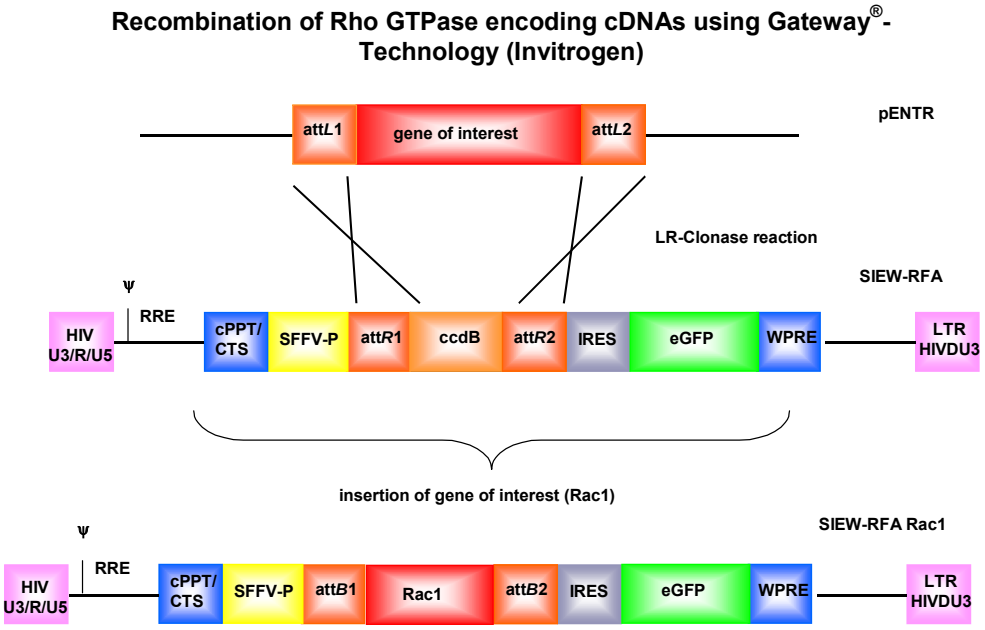


Fig.3.2 Recombination of a mutant Rac gene into SIEW-RFA*. A cDNA encoding a mutated Rho GTPase, subcloned into the pENTR "Entry" vector is incubated with SIEW-RFA in a clonase reaction, yielding SIEW-RFA, e.g. RacV12.*

3.1.10.4 Vectors used for microinjection

Pinco

Cloning vector pCNS, complete sequence, length = 3506 bp.

Pinco Rho14V25N

Mutated Homo sapiens RhoA cDNA, length = 1926 bp, with G14V and F25N transitions which had been cloned into Pinco 5' of an ATG and Myc-Tag sequence.

Pinco RacV12

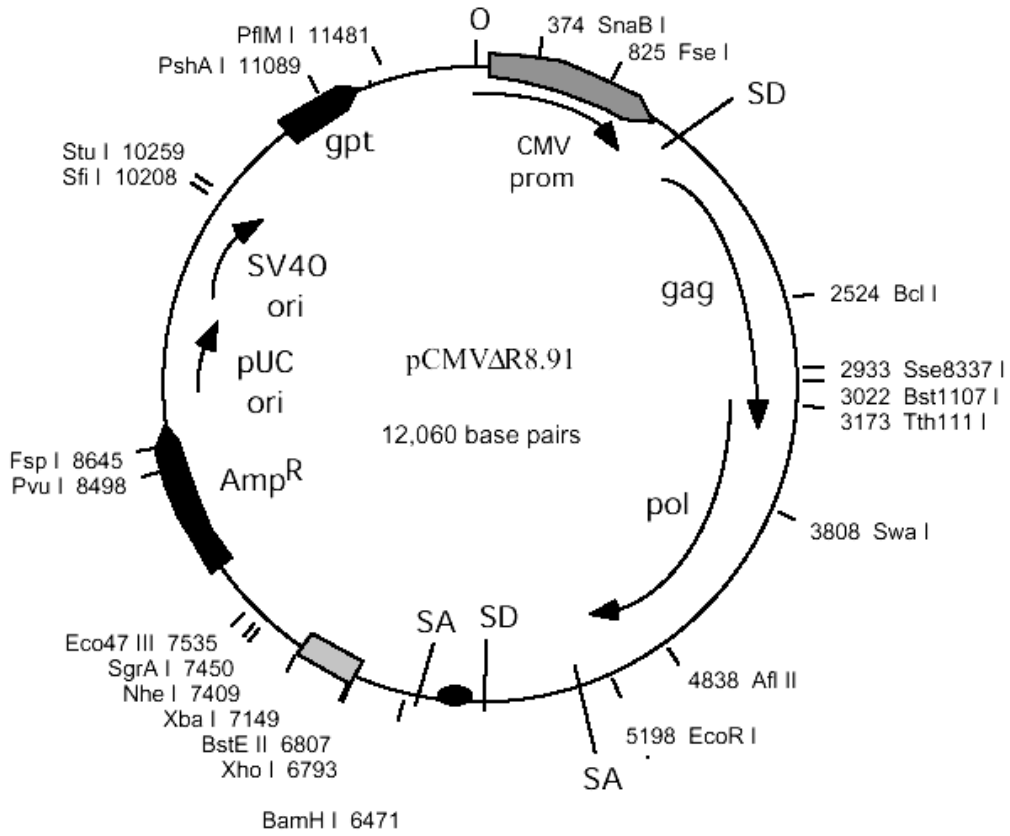
Homo sapiens Ras-related C3 botulinum toxin substrate 1, Rho family, small GTP binding protein Rac1, length = 2355 bp, with G12V transitions which had been cloned into Pinco 5' of an ATG and Myc-Tag sequence.

Pinco C3 (C3 transferase d.n. RhoA)

C. limosum C3 gene, Length = 1022 bp, which had been cloned into Pinco 5' of an ATG.

3.1.10.5 Packaging vectors for lentivirus production

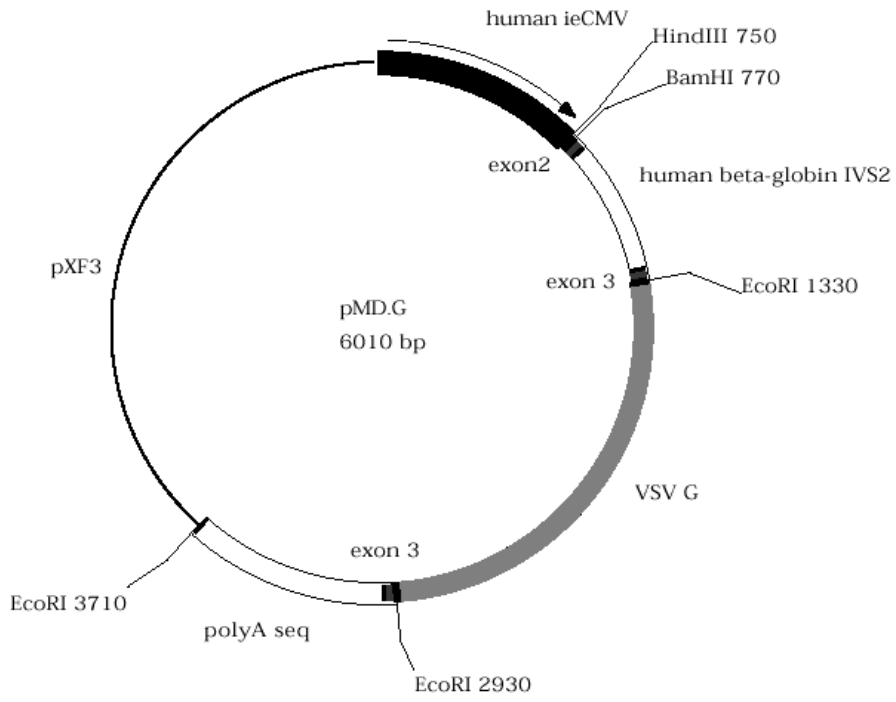
pCMVΔR8.91



pCMVΔR8.91

Lentiviral packaging vector expressing lentiviral gag-pol under the control of CMV promoter was kindly provided by Dr. Manuel Grez, Georg Speyer Haus, Biomedical Research Institute, Frankfurt.

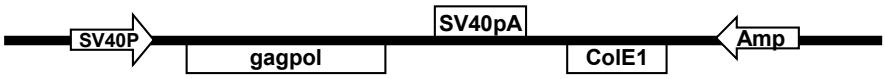
pMD.2.VSVG



The lentiviral packaging vector expressing pseudotyped envelope was kindly provided by Dr. Manuel Grez, Georg Speyer Haus, Biomedical Research Institute, Frankfurt.

3.1.10.6 Packaging vectors for retrovirus production

M57



M57 was kindly provided by Dr. Manuel Grez, Georg Speyer Haus, Biomedical Research Institute, Frankfurt. M57, a retroviral packaging vector expressing retroviral gag-pol under the control of SV40 promoter was used to complement the gag-pol expressed by retroviral packaging cell line Phoenix (Amphotropic or Ecotropic) during lentiviral production.

Abbreviations (for 3.1.10)

attR recombination sites
ccdB cytotoxic ccdB suicide gene CMV Immediate early promoter of Cytomegalovirus
cPPT/CTS central polypurine tract/central termination sequence (from HIV-1)
EGFP Enhanced green fluorescent protein (Sequence from pIRES2-EGFP from Clontech; Wild type-Gene from *Aequorea victoria*:

	Prasher et al., 1992)
Gag	Group-specific antigen (from HIV-1)
IRES	Internal ribosome entry site (Sequence from pIRES2-EGFP from Clontech; IRES from Encephalomyocarditis virus [ECMV])
LTR HIV.U3	Long terminal repeat from HIV-1 with Deletion in the U3-Sequence
MCS	Multiple cloning site
pA	polyadenylation signal
Pol	Polymerase (from HIV-1)
Rev	Regulator of viral expression (from HIV-1)
RRE	Rev-responsive element (from HIV-1)
RSV	LTR of Rous-Sarcoma virus
R/U5	R- and U5-Sequences from HIV-1-LTR
SA	Splice acceptor site
SD	Splice donor site
SFFV	3'-LTR of Spleen Focus Forming Virus
SV40	Simian virus type 40
Tat	Transactivator (from HIV-1)
VSV-G	G-Glycoprotein from Vesicular Somatitis Virus
WPRE	Woodchuck hepatitis virus post transcriptional regulatory element
ψ	Packaging signal (from HIV-1)

3.1.11 Cells

3.1.11.1 FDCP mix cells clone A4

The murine multipotent factor-dependent cells FDCP (Factor Dependent Cell-Paterson) mix cells have previously been established from long-term bone marrow cultures of BDF1 mice; (Spooncer*, et al* 1984), (Spooncer*, et al* 1986); These cells were stably transfected with enhanced Green Fluorescent Protein (eGFP) cDNA driven by the EF-1α promoter using a neomycin resistance gene. The CMV promoter of the plasmid pEGFP-N1 (Clontech Laboratories, Palo Alto, CA, USA) was deleted and replaced with the EF-1α promoter from the plasmid pEF-BOS (Goldman*, et al* 1996). FDCP mix cells were transfected by electroporation and selected with 0.7 mg/ml G418 (Gibco, Paisley, UK). These cells are dependent on IL-3; FDCP mix cells were maintained in a primitive state by culturing them in IMDM supplemented with 20% horse serum and 20 ng/ml IL-3. The cells were cultured at a cell density higher than $2x10^5$ cells /ml.

3.1.11.2 Murine primary lineage-depleted (lin^-) and Stem Cell Antigen-1 (Sca-1) enriched (lin^- $Sca\text{-}1^+$) bone marrow cells

The mouse lin^-Sca1^+ cells were freshly isolated from the femurs of 6-12 week old female Black 6 (C57BL6) mice. Murine bone marrow cells were negatively selected for lineage markers (TER-119, CD11b, B-220, Gr-1), lin^-, and positively selected for stem cell antigen ($Sca\text{-}1^+$) by magnetic activated cell sorting. The isolation of lin^- $Sca\text{-}1^+$ cells is described in detail in 3.2.5.1. The cells were cultured at a density of $5x10^5$ cells/ml in DMEM low-glucose (1 g/l) supplemented with 20% (v/v) Fetal Bovine Serum (FBS) containing rmSCF (100 ng/ml), rmIL-3 (20 ng/ml), rmIL-6 (20 ng/ml), rmFlt-3 ligand (20 ng/ml) and rhTPO (20 ng/ml) for up to 24 hours.

3.1.11.3 Mesenchymal Stem Cells (MSC)

MSC were isolated from bone marrow samples of patients undergoing hip surgery. The cells were separated by density gradient centrifugation and adherence selection. The detailed selection procedure is described in 3.2.5.2. The cells were flow cytometrically analysed for the expression of mesenchymal markers CD73 and CD105 and for absence of CD45. MSC were cultured in DMEM low-glucose (1 g/l) supplemented with 20% (v/v)

FBS, basic Fibroblast Growth factor (bFGF, 10 ng/ml) and were passaged 1:3 every 3 to 4 days by trypsinization (described in 3.2.1).

3.1.11.4 Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC cells were procured from Cell Systems (Endothelial umbilical pool 500K). The deep frozen vial was thawed in a 37°C water bath rapidly and 1ml of pre-warmed EBM-2 medium was added drop-wise every minute until the volume reached 10 ml. Cells were seeded in a tissue culture flask which was pre-coated for 10 minutes at room temperature with 0.1% gelatin. The ells were grown in EBM-2 medium. After reaching 90% confluence cells were passaged approximately every 3 days at 1:6 by trypsinization (described in 3.2.1). The cells were used until passage 7 for the experiments.

3.1.11.5 Murine Embryonic Endothelial Progenitor Cells (eEPC)

Murine embryonic endothelial progenitor cells were a kind gift from Prof. Jens Gille, Dermatology, J. W. Goethe-University, Frankfurt. They have originally been isolated from mouse embryos at E7.5 to E7.8 of development (Hatzopoulos*, et al* 1998). The cells were grown on 0.1% gelatin coated tissue cultures flasks and passaged every 3-4 days by trypsinization (described in 3.2.1). The culture medium used was DMEM high-glucose (4.5g/l), supplemented with 10% (v/v) FBS, 1% (w/v) L-glutamine, non-essential amino acids and β-mercaptoethanol.

3.1.11.6 Human 293T cells

293T cells, originally called 293tsA1609ne (DuBridge*, et al* 1987) and derived from 293 cells, a continuous human embryonic kidney cell line transformed by sheared Type 5 Adenovirus E1a and carrying a temperature sensitive T antigen co-selected with neomycin DNA (ATCC Number CRL-1573), (Graham*, et al* 1977). The cells were grown in DMEM containing glucose (4.5 g/l) supplemented with 10% (v/v) FBS and 1% Penicillin/Streptomycin. The cells were passaged 1:10 every 3 to 4 days by trypsinization (described in 3.2.1).

3.1.11.7 Phoenix cells

Phoenix cells are second-generation retrovirus producer lines for the generation of helper free ecotropic and amphotropic retroviruses. The lines are based on the 293T cell line. The cell line was created by placing into 293T cells constructs capable of producing gag-pol, and envelope protein for ecotropic and amphotropic retroviruses. IRES-CD8 surface marker cDNA is situated downstream of the reading frame of the gag-pol construct. Thus, CD8 expression is a direct reflection of intracellular gag-pol expression and the stability of the producer cell population's ability to produce gag-pol can be readily monitored by flow cytometry. For both the gag-pol and envelope constructs, Non-Moloney virus promoters were used to minimize recombination potential. Gag-pol was introduced with hygromycin as the co-selectable marker and the envelope proteins were introduced with diphtheria resistance as the co-selectable marker (Nolan) The cells were grown in DMEM high-glucose (4.5 g/l) supplemented with 10% (v/v) FBS and passaged 1:10 every 3 to 4 days by trypsinization (described in 3.2.1).

3.2 Methods

3.2.1 Trypsinization of adherent cells

The culture medium was removed by aspiration and the cell sheet was washed with PBS. Two ml of 0.025% trypsin solution was added to the adherent cells. After incubating for 5-10 minutes at 37°C, cells were observed under the microscope. The cell culture flask was tapped slightly on the sides to detach the cells. When the cells were detached, 5 ml of FBS was added to inhibit the trypsin action. The cells were washed by centrifugation at 300 x g for 5 minutes and resuspended in the desired cell concentration for various assays or seeded at a reduced factor.

3.2.2 Determination of protein concentration

Protein concentrations were determined by Bradford-assay with Bio-Rad protein detection kit according to the manufacturer's instruction and quantified with a spectrophotometer at 595 nm. The standards for protein concentration were prepared with different concentrations of BSA (0.1 to 1 µg/ml).

3.2.3 Fluorescent labelling of MSC

MSC were labelled with the fluorescent dye PKH-26 for migration assays. The cell sheet was washed and trypsinized as described in 3.2.1. The cells were pelleted by centrifugation at 300 x g for 5 minutes. The supernatant was carefully removed and the cells were resuspended with 1 ml of Diluent C at a concentration of 10^7 cells/ml. 1 ml of PKH-26 stain (8 µl of PKH-26 + 1 ml Diluent C) was added to the cell suspension. The mix was incubated in the dark at room temperature for 5 minutes with gentle agitation. 2 ml of FBS was added and incubated for 1 minute at room temperature with gentle agitation. Equal volume of serum medium containing was added to the mix and the cells were pelleted at 300 x g for 5 minutes. The cells were washed twice with the complete medium (DMEM low-glucose (1 g/l) supplemented with 20% (v/v) FBS) by centrifugation. The cell pellet was resuspended at a concentration of $5x10^5$ cells/ml.

3.2.4 Labelling of cells with fluorochrome conjugated antibodies for flow cytometric analysis

The adherent or suspension cells were pelleted by centrifugation at 300 x g for 5 minutes. The cells were washed with PBS by centrifugation at 300 x g for 5 minutes. The resulting

cell pellet was resuspended at a density of $1x10^7$ to $1x10^8$ cells/ml in ice-cold PBS supplemented with 0.5% BSA. 10 µl of fluorochrome conjugated antibodies (FITC, PE) were added to the cell suspension and incubated for 30 minutes on ice. The cells were washed by centrifugation and analysed in a flow cytometer.

3.2.5 Cell Isolation methods

3.2.5.1 Isolation of lin$^-$ Sca-1$^+$ cells from mouse bone marrow

The murine lin$^-$Sca1$^+$ cells were isolated from the femurs of 6-12 week old female Black 6 (C12BL6) mice. The femurs and tibiae of the mice were collected and bone marrow was flushed with isolation buffer using a syringe with a 25 G needle. The resulting cell suspension was passed through a 40 µm nylon filter which had been pre-wetted with PBS to disaggregate any cell clumps. The cells were washed by addition of PBS and subsequent centrifugation at 300 x g for 10 minutes. The supernatant was removed and the cells were resuspended at a density of 10^7 cells per 90 µl in the isolation buffer (described under 3.1.8). The cell suspension was incubated with 10 µl each of magnetic labelled microbeads coupled to antibodies against TER-119, CD11b, B-220, and Gr-1 for 10^7 cells for 30 minutes in the dark at 4°C. A 10-fold volume of isolation buffer was added to the cell suspension and cells were washed twice by centrifugation at 300 x g for 10 minutes. The cells were resuspended with 1 ml of ice-cold isolation buffer. The lineage depletion column (LD column) was placed in the magnetic field (Mini MACS, Miltenyi Biotech) and prepared by rinsing twice with the isolation buffer. The cell suspension was added onto the column. The unlabelled cells that passed through the column were collected as lineage depleted cells and the column was washed twice with the isolation buffer. The lineage depleted cells were washed by centrifugation at 300 x g for 10 minutes and counted. Aliquots of cells were taken for flow cytometric analysis. If positive selection for Sca1$^+$ cells was performed, the remaining cells were resuspended in the isolation buffer at a density of 10^7 cells per 80 µl. 20 µl of magnetic labelled microbeads coupled to antibody against Sca-1 were added to the cells and incubated for 15 minutes at 4°C in dark. The positive selection column was prepared by placing in the magnetic field and rinsing twice with isolation buffer. The cell suspension was loaded onto the column and the column was washed twice with the buffer. The column was removed from the magnetic field and placed over a 5 ml polypropylene collection tube. Isolation buffer was added to the column and the magnetically labelled cells were flushed out immediately by application of pressure with the plunger provided with the column to enrich for Sca-1$^+$

cells. The cells were counted using a Neubauer counting chamber and an aliquot was taken for flow cytometric analysis. Before further processing, the cells were washed by centrifugation and resuspended in the culture medium at a density of $5x10^5$ cells/ml DMEM low-glucose (1 g/l) supplemented with 20% (v/v) Fetal Bovine Serum (FBS) containing rmSCF (100 ng/ml), rmIL-3 (20 ng/ml), rmIL-6 (20 ng/ml), rmFlt-3 ligand (20 ng/ml) and rhTPO (20 ng/ml) for up to 24 hours.

The efficiency of separation was determined by staining aliquots of cells taken before and after separation by labelling with fluorochrome-conjugated antibodies (FITC or PE) against TER-119, CD11b, B-220, and Sca-1 for 30 minutes on ice in dark. The cells were washed by centrifugation at 300 x g for 10 minutes. The pellet was resuspended in PBS and analysed by flow cytometry.

3.2.5.2 Isolation of human bone marrow MSC

MSC were isolated from bone marrow samples of patients undergoing hip surgery after informed consent by a procedure approved by the local ethics committee. The bone marrow cells were suspended in IMDM supplemented with 500 U/ml Heparin. Within 24 hours after the operation, cells were passed through a 100 µm nylon filter and washed with PBS by centrifugation at 400 x g for 5 minutes. Cells were subsequently separated by density gradient centrifugation at 300 x g for 20 minutes without brake by layering them on ficoll solution (ρ, 1.709 g/cm^3). The mononuclear interphase cells were collected carefully using a sterile pipette and washed twice with PBS by centrifugation at 300 x g for 5 minutes. The pellet was resuspended in DMEM low-glucose (1 g/l) supplemented with 20% (v/v) FBS and seeded at a density of $5x10^6$ cells/ml into tissue culture containing bFGF (10 ng/ml). Cells were incubated at 37°C and 5% CO_2. The medium was changed every 2-3 days. After three weeks, a layer of spindle-shaped cells had formed (MSC). These cells stained negative for CD45, and positive for the mesenchymal cell markers CD105 and CD73 using flow cytometric analysis. The cells were passaged by trypsinization and split regularly 1:3 before the cells reached confluence. Their pluripotency was confirmed by successful induction of osteoblastic, chondrogenic, and adipogenic differentiation upon addition of the respective media. MSC populations could be passaged 10 to 12 times, without the loss of their multipotentiality before their growth slowed or stopped. MSC were cultured in DMEM low-glucose (1 g/l) supplemented with 20% (v/v) FBS, basic Fibroblast Growth factor (bFGF, 10 ng/ml).

3.2.6 Cell Migration Methods

3.2.6.1 Boyden chamber type migration assay

A Boyden chamber type migration assay was performed to asses the transmigration potential of the cells. The system consists of two chambers, separated by a polycarbonate, polyvinylpyrrolidine-free membrane of a definite pore size through which the cells migrate. A membrane with 8 µm pore size was used to asses the migration of MSC whereas a 5 µm pore-sized membrane was used for HSC. The membrane was pre-coated with fibronectin (1 µg/ml), laminin (100 ng/ml), vitronectin (100 ng/ml), or poly-L-lysine (10 µg/ml) in sterile PBS for 2 hours at 37°C. The membranes were air-dried for few minutes before mounting in the chamber. The adherent cell types (e.g., MSC) were trypsinized, washed once with PBS, and resuspended at a density of $5x10^6$ cells/ml in DMEM containing 0.5% (w/v) Bovine Serum Albumin (BSA), and if necessary the test substances were added to the cells suspensions. The cell suspension was incubated at 37°C, 5% CO_2 for 1 h in a 10 ml polypropylene tube in an upright position. The test substances were added to the lower wells of the chamber in 32 µl volume in pre-warmed medium. The pre-coated membrane was placed in-between the two chambers. The upper chamber was assembled and the system was screwed tight until the resistance to unscrew surmounted the effect of slightly pressured clockwise turning. The cells were seeded into the upper wells in a pre-warmed medium of DMEM containing 0.5% BSA containing 25,000 cells per well.

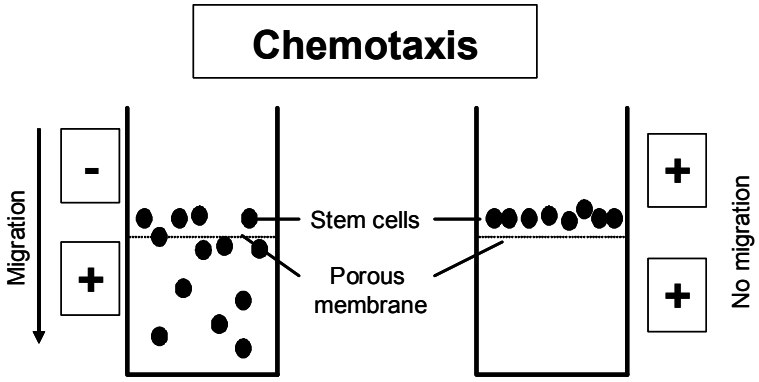


Fig 3.1 Pictorial representation of chemotaxis chamber *(Modified Boyden migration chamber). + represents the presence of chemoattractant, - represents the absence of chemoattractant.*

Cells were allowed to migrate through the pores, across the thickness of the membrane, and towards the source of chemoattractant in the lower chamber (Fig 3.1). Cells that migrated across the membrane and attached to the underside were stained (adherent cells) and microscopically counted or analysed by flow cytometer. If non-adherent cells were used, they were collected from the lower chamber into tubes for flow cytometry. To the cells collected in tubes, a fixed number of unlabelled cells were added. Flow cytometric analysis was performed to determine the ratio between unlabelled and labelled cells in the migrated fraction. By comparison of this ratio to that of the input control, the number of migrated cells was quantified.

Pre-coating of chemotaxis membrane

The porous membrane (8 µm) used for migrated experiments in Boyden chamber type migration assay were pre-coated with extracellular matrix (ECM) substances. ECM substances were diluted in PBS and the membranes were coated on both the sides unless otherwise specified with ECM substances by passive adsorption for 2 hours at 37°C or 4°C overnight. The membranes were washed once with PBS and air-dried before mounting in the chemotaxis chamber.

May-Grünwald Giemsa staining of migrated cells on chemotaxis membrane

The migrated cells on the chemotaxis membrane were visualized by May-Grünwald Giemsa's staining. The migrated cells (MSC) after migration were found attached to the lower surface of the chemotaxis membrane. The migrated cells on the membrane were fixed with ice-cold methanol (100%) for 15 minutes, stained with May-Grünwald solution for 5 minutes. After a brief wash in the distilled water, the cells were stained with Giemsa's solution (1:10 dilution of the stock) for 15 minutes, rinsed with distilled water briefly, visualized under the light microscope and documented.

3.2.6.2 Chemokinesis Assay

The non-directional random migration was determined by chemokinesis assay (Strobel*, et al* 1997). Adherent and non-adherent cells were used for chemokinesis assays. Ninety-six well plates for non-adherent cells and 8-chamber glass slides for adherent cells were used. The wells or the chambers were pre-coated with human fibronectin (10 µg/ml),

gelatin (0.1%), laminin (100 ng/ml), vitronectin (100 ng/ml), or poly-L-lysine (10 µg/ml) for 2 hours at 37°C or at 4°C overnight. The coating medium was removed and the wells or chambers were washed briefly with PBS twice. 10,000 non-adherent cells or 25,000 adherent cells were seeded per well/chamber in a 96-well plate or a 8-chamber slide. The plate or the chamber slide was positioned at an angle of elevation of 80° immediately after seeding at 37°C, 5% CO_2 for 24 hours so that the cells could accumulate at the lower end of the well or the chamber. After adding the test substances, the plate or the slide was re-positioned at an angle of 15°C for 24 hours. The distance to which the cells have migrated was documented by a colour photograph. Video microscopic imaging was performed of the cells kept at 15° inclined position each for 20 minutes, 1 hour or overnight at 200 x or 400 x magnification. The test substances were added for certain experiments 2 hours before the video microscopic imaging. The microscopic table was pre-warmed to 37°C throughout the assay period. A box ventilated with an atmosphere with 5% CO_2 in air was constructed to keep the pH constant during the observation periods. In some experiments, medium buffered with HEPES was also used.

3.2.6.3 Transendothelial migration assay

Transendothelial migration assay was performed to investigate the migration behaviour of progenitor cells through a layer of endothelial cells. The assay was performed with transwells of pore size 5 µm to asses HPC migration and 8 µm pore size membranes for MSC migration. HUVEC were seeded on gelatine (0.1%) coated transwells at concentration of 30,000 cells per well. The non-adherent cells were removed 24 hours after seeding the cells by medium flushing. The confluence and the membrane integrity of the endothelial cells were checked by measuring the permeability for fluorescein isothiocyanate (FITC)-dextran 3000. FITC-dextran 3000 was added to the upper chamber of the transwells, incubated at 37°C, 5% CO_2 for 2 h. Aliquots of the medium were taken from the upper and the lower chamber of the transwells and the fluorescence is measured with a Enzyme Linked Immunosorbent Assay (ELISA) reader at a wavelength of 521 nm. The endothelial cells were used stimulated or unstimulated with rhTNF-1α for 2 hours at 37°C. For some experiments, the endothelial cells were treated with toxins, inhibitors, or inducers before adding primary cells. Endothelial cells were washed twice with the assay medium before adding the primary cells in the upper compartment. Twenty-five thousand (25,000) cells per well in case of MSC (labelled with PKH-26) or $5x10^4$ cells per well in case of HSC (GFP positive or fluorescent labelled) were added to the upper compartment

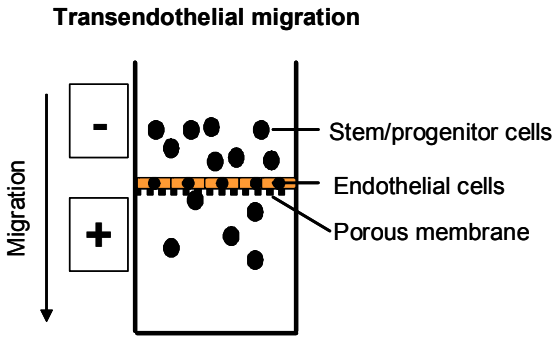


Fig 3.2 Pictorial representation of transendothelial migration of stem cells*. + represents the presence of chemoattractant, – represents the absence of chemoattractant.*

with 0.3 ml assay medium (DMEM containing 0.1% (w/v) BSA) with or without the test substances. 1 ml of the assay medium with or without the test substances was added to the lower compartment. The plate was incubated at 37°C, 5% CO_2 for 6 hours. The cells that migrated to the lower compartment or the underside of the membrane (Fig 3.2) were collected in tubes or fixed, stained and counted. To the cells collected in tubes for flow-cytometric analysis, a fixed number of unlabelled cells were added. Flow-cytometric analysis was used to determine the ratio between unlabelled and labelled cells in the migrated fraction. By comparison of this ratio to that of the input control, the number of migrated cells was quantified.

3.2.6.4 Migration of cells under shear stress

The migration of cells is an important step in homing of stem cells into the stem cell niche. The assays described so far were migration of cells in static conditions. But in *in vivo* conditions, the migration occurs in a condition of shear stress where the migrating or homing cells interact with the underlying endothelium and extravasate through the endothelial cell layer as described by Springer (Springer 1994). Flow chamber migrating assay was employed for this purpose.

The apparatus (Fig 3.3) consists of a circular flow chamber with an inlet port through which the stem or progenitor cells were injected, an outlet port, where the flow out medium and cells pass through and a vacuum port which creates a vacuum. This apparatus was mounted on a glass slide coated with endothelial layer (HUVEC). The entire setup was placed on an inverted microscope connected to a video system to document the motility of

the cells. The tubing system was connected to the flow chamber ports by luer locks. The stem or progenitor cells were flushed into the chamber with a constant shear stress (0.1 dyn/cm^2 to 4 dyn/cm^2) through a tubing system connected to an injection syringe which in turn is operated by a perfusion pump whose velocity could be modified manually (2.5 ml/hr to 99 ml/hr). The system creates a laminar flow of the liquid over the endothelial layer. The shear stress formed in the system was calculated using the formula:

Shear stress = $6Q\mu/bh^2$
Where,
Q = flow rate (cm^3/s)
μ = fluid viscosity (cp or dyn s/cm^2)
h = channel height (254 μm for gasket used)
b = channel width (5 mm for 0.010 mm gasket)

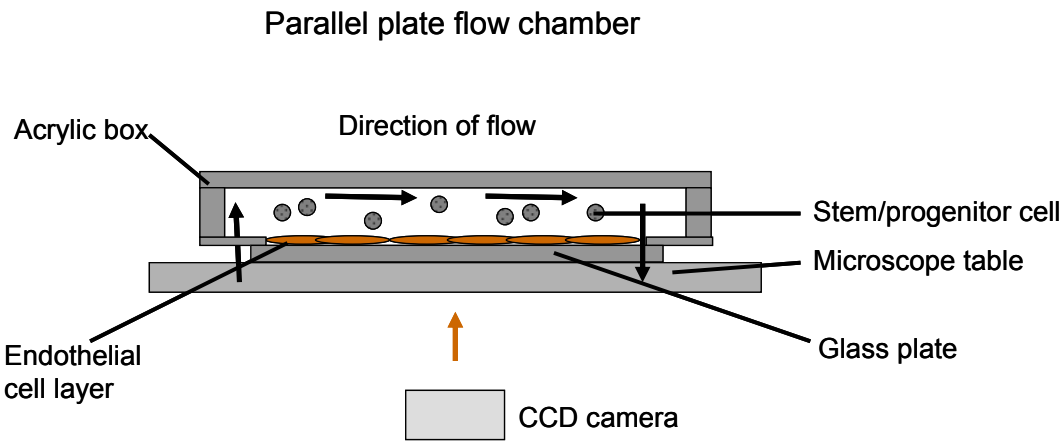


Fig 3.3 Pictorial representation of parallel plate flow chamber.

The rolling and adhesion of the stem or progenitor cells over the endothelial layer was studied using this system. Initially, the stem or progenitor cells were flushed at a constant shear stress of 0.1 dyn/cm^2 (2.5 ml/h) at a rate of ~ 4x10^4 cells/min for 10 minutes over the endothelial layer. Representative pictures of three different fields were taken to asses the adhesion of cells in lower shear stress. A real time movie was made at this shear rate for 10-20 seconds to asses the rolling of cells over the endothelium. The shear stress was increased to a rate of 2 dyn/cm^2 for 10 minutes where most of the progenitor cells bound lightly to the endothelium was flushed away and tightly bound cells remain.

3.2.7 Microinjection of Rho GTPase cDNA

Microinjection is the physical introduction of DNA into the living cells. The Rho GTPase cDNAs RacV12 (activated Rac), RhoV14 (activated Rho), C3 transferase (C3 transferase from *C. botulinum*) were subcloned into Pinco retroviral expression vector were microinjected into the nucleus of the cells using an Eppendorf InjectMan NI 2 and FemtoJet mounted to a Zeiss microscope Axiovert 135. As instrumental platform, an inverted microscope was fitted with a needle holder which can be moved at definite positions in x-, y- and z coordinates within the microscopic field is provided on a movable tray that takes up the culture vessel with the cells to be injected (Fig 3.4). CELLocate glass cover slips with microgrids grids for relocating the cells with grid size of 55 µm were coated by addition of human laminin (100 ng/ml) for 2 hours at 37°C or 4°C overnight incubation. The coating solution was removed; the cover slips were washed twice with PBS. MSC were seeded on the cover slips which were placed in a cell culture dish at a constant cell density so that the cells remained without contact to neighbouring cells. The cells were allowed to attach at 37°C, 5% CO_2 for 24 hours.

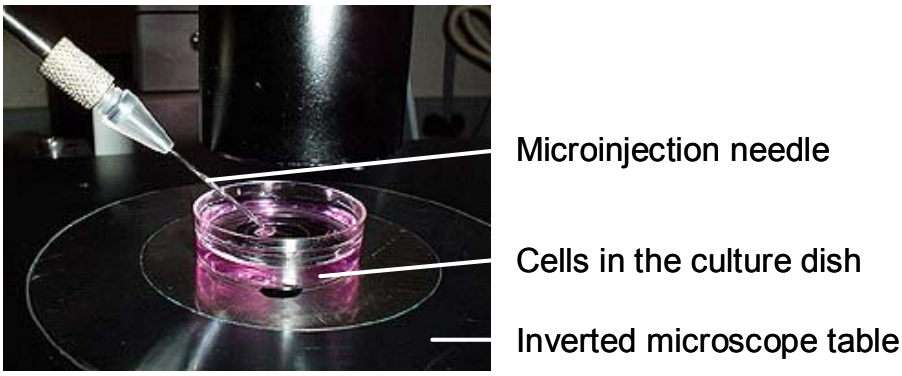


Fig 3.4 Microinjection of adherent cells. *Adherent cells were seeded in a culture dish on the cover slips. Microinjection was performed using an inverted microscope.*

The cells were cultured in a buffered medium (DMEM containing glucose (1 g/l) supplemented with 20% (v/v) FBS containing 25 mm HEPES, pH 7.2) to maintain the pH during the prolonged exposure to ambient conditions during the microinjection process. The vectors to be microinjected were prepared by centrifuging the sample for 15 minutes at 2000 rpm to sediment the debris/aggregates.

Cells to be microinjected were brought into focus of the microscope. 2-10 µl of the sample to be microinjected were loaded into the microinjection needles (Femtotips). The needle

was mounted into the needle holder and the tip of the needle was brought immediately into the medium to avoid drying and crystal formation at the tip of the needle. The instrument pressure was set to low. The Z limit was set for the cells, the needle was moved to the search position, and injection of cells was started. Z limit was set where the tip of the microinjection needle was focused on the centre of the cell and it made an invagination of 1 µm without piercing the cell. Once the Z limit was set, the needle was moved upwards manually above the cells. This plane, from which the various cells were injected, was the search plane. 40X magnification were used for microinjection of adherent cells. The microscopic table was pre-warmed if the microinjection procedure took more than 30 minutes (Fig 3.2). The cells were left in the incubator at 37°C between 30 minutes to 24h after microinjection and were immunohistochemically analysed.

3.2.8 Viral gene transfer methods

3.2.8.1 Production of Lentiviral particles

Plating of packaging cells

293T cells were used for packaging of lentiviral particles. The cells were trypsinized, washed by centrifugation at 1600 rpm (450 x g) for 5 minutes at room temperature and plated 24 hours before transfection in cell culture dishes at a density of $5x10^6$ cells/10 cm^2 in DMEM containing glucose (4.5 g/l) supplemented with 10% (v/v) FBS, Penicillin (25 U/ml), Streptomycin (25 U/ml).

Transfection of packaging cells

The cells were transfected by the calcium-phosphate mediated method. Fresh medium was given to the cells 2 hours before transfection. Chloroquine was added at a concentration of 100 mM to the cells 5 minutes before adding the transfection reagent mix. The transfection reagent mix was prepared by adding 3.5 µg ENV plasmid (pMD2-VSV-G), 6.5 µg Packaging plasmid (CMVΔR8.91), and 10 µg of plasmid containing the gene to be expressed (described in 3.1.10.2) together per 10 cm plate. The volume was made to 450 µl with TE/dH_2O buffer. 50 µl of 2.5 M $CaCl_2$ was added finally. The precipitate was formed by the drop-wise addition of 2X HBS to the DNA-$CaCl_2$ mix while bubbling. The mixture was let at room temperature for 3-5 minutes before adding to the cells. The precipitate was removed from the cells after 16 hours and fresh medium (RPMI supplemented with 10% (v/v) FBS) was added to the cells for viral collection.

Viral collection

The virus was collected 24 hours and 48 hours after the medium change after the transfection. The medium with the virus (viral supernatant, VS) was filtered through a 0.22 µm filter pre-wetted with PBS. The supernatant was stored in 1 ml aliquots at –80°C. After the viral collection, the packaging cells were trypsinized and flow cytometrically analysed for the transfection efficiency.

Concentration of Lentiviral particles

The viral particles (293T supernatant) were collected from the packaging cells 24 and 48 hours after the medium change. The viral supernatant was centrifuged at 500 x g for 5 minutes and filtered through a 0.22 µm pore filter (pre-wetted with PBS). The viral particles were concentrated by ultracentrifugation at 50,000 x g for 140 minutes at 4°C. The resulting supernatant was discarded and the pellet was resuspended in 200 µl of PBS containing 1% HSA or BSA. The pellet of concentrated virus from the first collection was stored at 4°C overnight and pooled with the concentrated virus on the following day. The pellets were resuspended in a small tube on a rotating wheel for 1h. The resulting concentrated virus was aliquoted, stored in -80°C, and titered after freezing. The concentrated virus was used for transducing the target cells.

Viral titre determination

293T cells were used for viral titre determination. $2x10^5$ cells were seeded per well in a 24 well plate. After 24 hours, the medium was removed and 500 µl of fresh medium containing 4 µg/ml of polybrene with various dilutions of the virus (1:10, 1:100, and 1:1000) was added to the cells. The cells were incubated for 72 hours at 37°C, 5% CO_2. Subsequently, the medium was removed, the cells washed with PBS and trypsinized as described in 3.2.1. The cells were collected into the tubes and washed by centrifugation at 300 x g for 5 minutes. The supernatant was aspirated and the cells were analysed by flow cytometry for the number of GFP positive cells. The viral titre was determined using the formula:

$$\text{No. of cells seeded} \times 2 \times \frac{\text{GFP positive cells \%}}{100} \times \text{dilution factor}$$

The viral titre was expressed as transducing units per ml (TU/ml)

Transduction of the target cells

a) Transduction of adherent cells

The cells were plated in a 6 or 24 well plate 24 hours before transduction, $2x10^5$ cells/well in 6 well plate or $5x10^4$ cells/well in 24 well plate. The medium was removed and 1 ml of the fresh complete medium having 4 µg/ml polybrene and 1 ml of the viral supernatant or MOI ~ 2.0 -3.0 was added to the cells. The cells were centrifuged at 2500 rpm (1260 x g) at 32°C for 90 minutes. The plates were incubated at 37°C, 5% CO_2 for 18 hours after centrifugation. The medium was removed, the cells were trypsinized, an aliquot was analysed by flow cytometry for the expression of reporter gene (eGFP) to quantify the transduction efficiency, and the rest of the cells were split and cultured.

b) Transduction of non-adherent cells

For the transduction of non-adherent cells, a non-tissue culture treated 24-well plate pre-coated with human retronectin (50 µg/ml) in PBS for 1h at room temperature or 4°C overnight was used. The retronectin was removed and the wells were washed twice with PBS. The cells were seeded into the wells ($2x10^5$ cells/well) and the viral supernatant was added to the cells to be transduced at an MOI of ~ 1.0-2.0. For transduction of mouse bone marrow primary cells (lin^- $Sca\text{-}1^+$), cytokines (rmSCF, 50 ng/ml; rmIL-3, 20ng/ml; rmIL-6, 20 ng/ml) were added to the viral supernatant. Protamin sulfate was added to the cells during transduction at a final concentration of 4 µg/ml. The cells with the viral supernatant were centrifuged at 1260 x g for 90 minutes at 32°C. The plate was removed from the centrifuge and incubated overnight at 37°C, 5% CO_2. The medium was removed and an aliquot of the cells was analysed by flow cytometry to quantify the transduction efficiency and the rest of the cells were split at a cell density of $2x10^5$ cells /ml (for mouse bone marrow lin^- $Sca\text{-}1^+$ cells).

3.2.8.2 Production of Moloney type retroviral particles

The following viral packaging cell lines were used. Phoenix A for amphotropic packaging to infect human cells and Phoenix E for Ecotropic packaging to infect murine cells were kindly provided by Dr. Grez, Biomedical Research Institute, Goerg Speyer Haus, Frankfurt. Packaging cells were cultured in DMEM containing glucose (4.5 g/l) supplemented with 10% (v/v) FBS and purity checked for the expression of CD8 surface antigen for the expression of gag-pol and selected with hygromycin and clostridium toxin for 2 weeks every 6 months.

Production of amphotropic retroviral particles

Phoenix A cells were used for the production of amphotropic viral particles. Cells were trypsinized and washed by centrifugation at 1600 rpm (450 x g), 5 minutes at room temperature and plated at a density of $1.5x10^6$ cells per 10 cm^2 cell culture dishes, 24 hours before transfection in DMEM with glucose (4.5 g/l) supplemented with 10% (v/v) FBS, Penicillin (25U/ml) and Streptomycin (25 U/ml).

Transfection of packaging cells

The cells were tansfected by calcium-phosphate mediated method. Fresh medium was added to the cells 2 hours before transfection. Chloroquine (100 mM) was given to the cells 5 minutes before adding the transfection reagent. The transfection reagent mix was prepared by adding 3 µg of M57 plasmid (gag-pol expression plasmid) and 8 µg of Gene transfer plasmid (3.1.10.1) together per 10 cm plate. The volume was made to 450 µl with TE/dH_2O buffer. 50 µl of 2.5 M $CaCl_2$ was added finally. The precipitate was formed by the drop-wise addition of 2X HBS to the DNA-$CaCl_2$ mix while bubbling. The mixture was left at room temperature for 5-10 minutes before adding to the cells. The precipitate was removed form the cells after 16 hours and fresh medium (RPMI supplemented with 10% FBS) was added to the cells for viral collection.

Viral collection

The virus was collected 24 hours and 48 hours after the last medium change. The medium with the virus (viral supernatant, VS) was filtered through a 0.22 µm filter pre-wetted with PBS before filtration. The supernatant was used immediately or stored in 1ml aliquots in -80°C. After the viral collection, the packaging cells were washed with PBS and trypsinized. The cells were washed by centrifugation at 1000 rpm for 5 minutes, resuspended in PBS and flow cytometrically analysed for transfection efficiency for the reporter gene expression (eGFP).

Transduction of the target cells

a) Transduction of adherent cells:

The target cells were plated in 6 or 24 well plate 24 hours before transduction at $5x10^4$ cells/well in 24 well plates or $2x10^5$ cells/well in 6 well plates. The medium was removed, 1-2 ml of the fresh complete medium (EBM-2 for HUVEC; DMEM containing glucose (4.5 g/l) supplemented with 10% (v/v) FBS for eEPC) having 4 µg/ml polybrene and 1-2 ml of the viral supernatant was added to the cells. The cells were centrifuged at 2500 rpm (1260

x g) at 32°C for 90 minutes. The plates were incubated at 37°C, 5% CO_2 for 18 hours after centrifugation. The medium was removed, fresh viral supernatant added and the transduction was repeated like the first day and cells were incubated for 24h at 37°C, 5% CO_2. The cells were trypsinized and an aliquot was analysed by flow cytometry for the expression of reporter gene (eGFP) to quantify the transduction efficiency and the rest of the cells were split and cultured.

b) Transduction of non-adherent cells:

For non-adherent cells, the cells were transduced in a non-tissue culture treated plate pre-coated with human retronectin. For this, 24 well plates which consisted of non-tissue culture plate pre-coated with retronectin (50 µg/ml) in PBS for 1 hour at room temperature or 4°C overnight. The retronectin was removed and the wells were washed twice with PBS. The cells were seeded in to the wells ($2x10^5$ cells/well) and the viral supernatant was added to the cells. For transduction of mouse bone marrow primary cells (lin^- $Sca\text{-}1^+$), cytokines (rmSCF, 50 ng/ml; rmIL-3, 20ng/ml; rmIL-6, 20 ng/ml) were added to the viral supernatant. Protamin sulfate was added to the cells before transduction at a concentration of 4 µg/ml. The cells with the viral supernatant were centrifuged at 2500 rpm (1260 x g) for 90 minutes at 32°C. The plate was removed from the centrifuge and incubated overnight at 37°C, 5% CO_2. A new non-tissue culture plastic type plate was pre-coated with retronectin (as mentioned above). The cells which had undergone the first round of transduction were transferred to new retronectin coated plates and the transduction was repeated with the new viral supernatant like on the day 1. The cells were incubated for 24 hours at 37°C, 5% CO_2. The medium was removed and an aliquot of the cells was analysed by flow cytometry to quantify the transduction efficiency and the rest were split at a cell density of $2x10^5$ cells /ml (for mouse bone marrow lin^- $Sca\text{-}1^+$ cells). When the transduction efficiency was found to be less than 50%, the transduced cells were flow cytometrically sorted for reporter gene expression (eGFP) to yield a homogenous population of infected cells.

3.2.9 RNA extraction for Microarray analysis

The medium was aspirated from the cells and the cell sheet was washed twice with ice-cold PBS. 1ml of TRIzol reagent (Invitrogen) was added to the cells and the cells were scrapped into a clean nuclease free microcentrifuge tube, and incubated for 5 minutes at room temperature. 250 µl of chloroform was added; the mixture was vortexed for 15 seconds and incubated at room temperature for 2-3 minutes. The cells were then

centrifuged at 11, 400 rpm (9000 x g) for 15 minutes at 4°C. The upper aqueous phase was transferred to a new microcentrifuge tube, mixed with an equal volume of isopropanol, and incubated overnight at –20°C or 10 minutes at room temperature. The mix was centrifuged at 9000 x g, 4°C for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol by centrifugation at 9000 x g for 5 minutes at 4°C. The supernatant was discarded; pellet air-dried and resuspended in 20 µl RNase free DEPC-water. The pellet was incubated at 55°C for 10 minutes with gentle agitation to enhance the dissolving of the pellet. The RNA was analysed on 1% agarose gel or the sample was frozen at –80°C until use.

3.2.10 Oligonucleotide Microarray

Oligonucleotide microarray analysis to determine the modulation of gene expression in Rho GTPase modified cells were carried out in collaboration with Dr. Elena Puccetti, Hematology, Johann Wolfgang Goethe University, Frankfurt, Germany.

RNA (8 µg) from the transduced cells were reverse transcribed to generate biotinylated complementary RNA (cRNA). The fragmented cRNA were hybridised to a murine Genome microarray (Affymetrix). The fluorescence intensity was normalized for the entire microarray. Data analysis was performed using Genespring Software (Silicon Genetics, San Carlos, CA). Genes which were either up or down regulated at least 2-fold in each case were selected.

3.2.11 Pull down assays for Rho GTPases

Pull down assays for Rho GTPases was performed using a kit obtained from Pierce Biotechnology. Adherent cells were cultured to confluence in a 10 cm cell culture dish or $1x10^7$ non-adherent cells were put on fibronectin (1 µg/ml) coated plates for 2 hours before the assay. The cells were treated with or without Rho GTPase inhibitor toxins or inducers for 2 hours at 37°C, 5% CO_2 before the assay. Cells transduced with constitutively active Rac (RacV12), constitutively active Rho (RhoV14), dominant negative Rac (RacN17) or dominant negative Rho (RhoN17) were also used for the pull down assay.

For adherent cells:
The cell sheet was washed with ice-cold TBS. 0.5-1 ml of the lysis or wash buffer (Pierce) with the protease inhibitors was added to the cells and the cells were scraped off into a

clean microcentrifuge tube. The cells were vortexed briefly and incubated on ice for 5 minutes. The cells were centrifuged at 16,000 x g for 15 minutes at 4°C. The supernatant was transferred to another clean microcentrifuge tube.

For non-adherent cells:
The cells were centrifuged at 100 x g for 5 minutes. The medium was removed and the cells were washed with 10ml of ice-cold TBS by centrifugation at 100 x g for minutes. The TBS was removed and the cells were resuspended with 0.5-1 ml of the lysis/wash buffer and vortexed briefly and incubated on ice for 5 minutes. The cells were centrifuged at 16,000 x g for 15 minutes at 4°C. The supernatant was transferred to another clean microcentrifuge tube.

***In vitro* GTPγS or GDP treatment**

The GTPγS or GDP treatment was done to ensure the proper working of the assay. 10 µl of 0.5 M EDTA, pH 8.0 (for a final concentration of 10 mM) was added to 500 µl of cell lysate. For a positive control, 5 µl of 10 mM GTPγS (for a final concentration of 0.1 mM) and for negative control 5 µl of 100 mM GDP (for a final concentration of 1 mM) were added. The mixture was incubated at 30°C for 30 minutes with constant agitation. The reaction was terminated by placing the sample on ice and adding 32 µl of 1 M $MgCl_2$ (for a final concentration of 60 mM).

Affinity precipitation of activated Rho GTPases
One Swell Gel Immobilized Glutathione Disc was placed into a spin cup with a collection tube. 20 µg of GST-Pak1-PBD (for Rac1 and Cdc42) or 400 µg of GST-Rhotekin-RBD (for Rho A) was added to the spin column. 700 µl of the lysate was immediately transferred to the spin column. The cap of the collection tube was sealed with Parafilm to prevent leakage due to the high concentration of the detergent. The reaction mixture was incubated at 4°C for 1 hour with gentle rocking. The spin column was centrifuged with collection tube at 7,200 x g for 2 minutes. 400 µl of Lysis/Binding/Wash Buffer was added to the resin and centrifuged at 7,200 x g for 2 minutes and the procedure was repeated twice. 50 µl of 2X SDS sample buffer containing 1 part β-mercaptoethanol to 20 parts of the sample buffer was added to the resin. The samples were boiled at 95-100°C for 5 minutes. The tubes were centrifuged at 7,200 x g for 2 minutes. Samples were electrophoresed on a 12% polyacrylamide gel or stored at -20°C for future use.

3.2.12 Western Blot analysis

The proteins were resolved by 12%-15% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked by incubating in TBS containing 3% (w/v) BSA at room temperature for 2 hours with constant agitation. The membrane was rinsed with TBST (TBS containing 0.05% Tween-20). Transferred samples were incubated in TBS + 3% Non fat dry milk + 1% sodium-azide containing anti-Rac1 antibody (1 µg/ml) or anti-Rho antibody (1 µg/ml) solution at 4°C overnight. The membrane was washed five times for 5 minutes per wash with TBST. The membrane was incubated in the secondary antibody (anti-mouse IgG-HRP-conjugate) 1:5000 dilution at room temperature for 30 minutes to 1 hour. The membrane was washed five times for 5 minutes per wash with TBST. The immunoblotted proteins were visualized with the enhanced chemiluminescent reagents (LumiGLO chemiluminescent substrate, Upstate, or ECL western blot detection reagents, Amersham Biosciences). The bands were developed on X-ray films for various time points.

3.2.13 Immunofluorescent staining

Chamber slides were coated with 0.1% gelatin (w/v) or poly-L-lysine (10 µg/ml) or human fibronectin (1 µg/ml) for 2 hours at 37°C. The coating solution was removed and the chambers were washed twice with PBS. Cells were grown on the coated slides or cover slips at 37°C, 5% CO_2. The cells were pre-treated with or without the inhibitors and inducers at 37°C for various time points. The medium was removed and the cells sheet was washed thrice briefly with PBS. The cells were fixed at room temperature with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes, and incubated with blocking solution (3% (w/v) non-fat dry milk in PBS) containing 0.1% (v/v) Triton X-100 for 1 hour at room temperature. The cells were immunostained with the primary antibody in PBS containing 3% (w/v) non-fat dry milk for 1 hour at room temperature, washed thrice for 5 minutes per wash. The cells were incubated with phalloidin-FITC labeled and or fluorescent-labeled secondary antibody for 1 hour at room temperature. The cells were washed 5 times for 5 minutes per wash with PBS. The chambers of the chamber slide were removed, were mounted with fluorescence protective mounting medium, and observed under the microscope.

3.2.14 Molecular Biology methods

3.2.14.1 Bacterial transformation

One vial of 0.5M β-mercaptoethanol (ME), a frozen vial of top 10 competent cells was thawed on ice. The S. O. C medium was brought to room temperature. 2 µl of 0.5 M β-ME was pipetted into the vial of the competent cells and mixed by stirring gently with the pipette tip. Pipetting up and down was avoided. 10 µl of the vector was pipetted directly into the competent cells and mixed by stirring with the pipette tip and not by pipetting up and down. The vial was incubated on ice for 30 minutes. Water-bath or a thermo shaker was pre-warmed to 42°C. Heat shock was given for exactly 50 seconds at 42°C. The vial was removed from 42°C without shaking or mixing and placed on ice for 2 minutes. 250 ml of the S. O. C medium (at room temperature) was added to the tube. The vial with the transformed cells was shaken horizontally at 37°C for 1 hour at 225 rpm in a rotary shaker incubator. The vial was placed on ice. 2.5 µl of ampicillin (50 mg/ml stock) was given to the cells. 50 µl of the transformed cells was spread on a LB agar ampicillin plate. The plate was incubated for 8 hours at 37°C. Individual colonies were picked and inoculated in 5 ml LB broth. The tubes were incubated overnight at 37°C, 225 rpm in a shaker (starter culture). The starter culture was inoculated into the 200 ml maxi culture (LB broth with 50 µg/ml ampicillin) and incubated at 37°C, 225 rpm overnight.

3.2.14.2 Maxi preparation

Maxi preparation of the plasmids was performed with Jet Star Plasmid Maxi preparation kit (Genomed) according to the manufacture's instructions. The protocol for plasmid extraction includes that the expanded transformed cells were lysed by alkaline lysis and purified in an anion exchange resin columns (Genomed).

3.2.15 Data Analysis

Results were expressed as Means $\pm$SD. Statistical analysis was carried out with Student's t-test using Microsoft Excel (Microsoft Corporation, Redmond, WA).

4.0 RESULTS

4.1 Role of Rho GTPase activation in migration of HPC

4.1.1 Influence of SDF-1α on chemotactic migration of HPC

Migration is an important step in homing of HPC into the stem cell niche after transplantation and is mediated by adhesion molecules and chemokines (Verfaillie*, et al* 1994, Yang*, et al* 2001). SDF-1α has been descried as the most potent inducer of chemotaxis in HPC and also many other cell types circulating in the blood (Aiuti, et al 1997). As HPC, either the murine multipotent progenitor cells FDCP mix or freshly isolated primary bone marrow cells enriched for primitive progenitors were used. Both chemotactic and chemokinetic migration were investigated. The optimal concentration of SDF-1α for the transmigration of FDCP mix HPC under experimental conditions was identified by dose response studies. The highest percentage of HPC migration was observed at concentrations from 30 to 100 ng/ml and at higher concentration reduced migration was observed (300 ng/ml; Fig. 4.1 A).

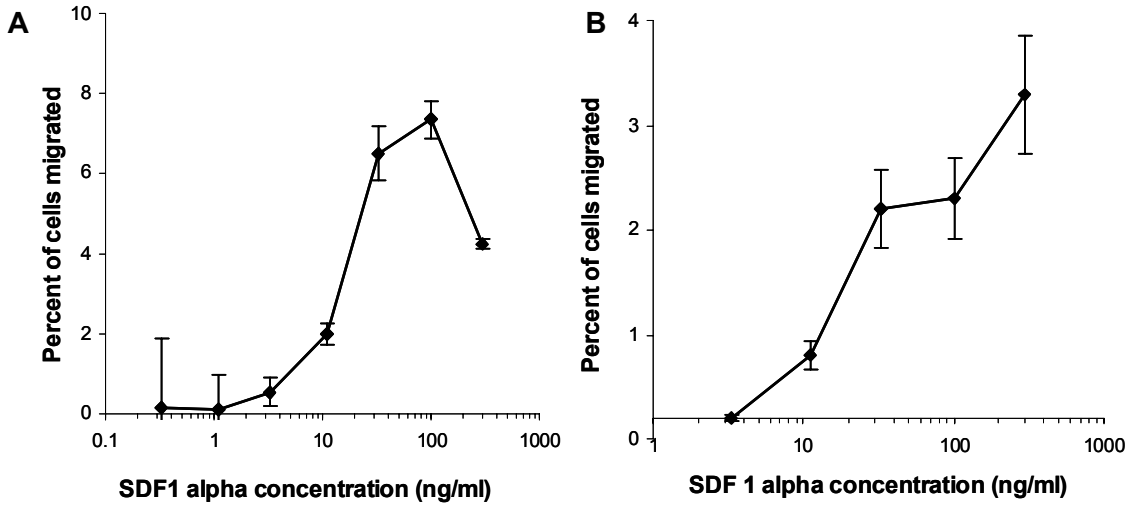


Fig. 4.1 Dose response curve of SDF-1α induced migration of FDCP mix cells. *(A) Transmigration of FDCP mix cells across 5 µm pore membranes in a 48 well Boyden chamber type assay towards various concentrations of SDF-1α* (0.3 to 300 ng/ml) *was determined after 4 hours. Results are expressed as percentage of migrated cells (Means ± SD, n= 3-4). (B) Transmigration of FDCP mix cells across endothelial cell layers (HUVEC) seeded on 5 µm pore membrane in the transwell system towards various concentrations of SDF-1α* (3.3 to 300 ng/ml) *was determined after 4 hours. Results are expressed as percentage of migrated cells (means ± SD, n= 3).*

The transmigration response of HPC to SDF-1α was similar when they were allowed to migrate across an endothelial cell layer (HUVEC). The migration response was dependent on the SDF-1α dose, and the percentage of migrated cells increased over the entire dose range investigated (Fig. 4.1 B). Since the dose response curve of SDF-1α induced migration of FDCP mix cells were similar to that of published results with primary bone marrow progenitor cells (lin^-Sca1^+ cells, see Fig. 4.3), FDCP mix cells were used as an alternate and analogous to HPC in experimental settings.

4.1.2 Rho GTPase modulating bacterial toxins and phospholipids regulate chemotactic migration of HPC

Since Rho GTPases were reported to regulate the migration of neutrophils (Gardiner*, et al* 2002, Roberts*, et al* 1999), it was of interest to examine their involvement in chemotactic migration of HPC towards SDF-1α. HPC were treated with phospholipids and bacterial toxins that target Rho GTPases, and were allowed to migrate in a Boyden chamber type assay.

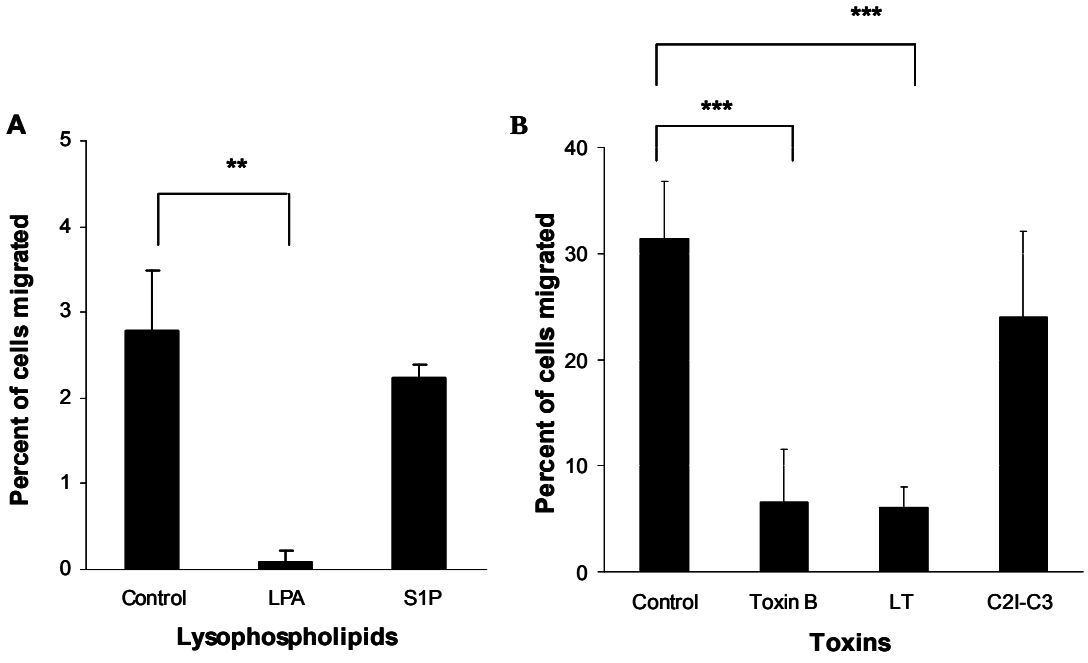


Fig 4.2 Influence of lysophospholipids (A) or pre-treatment with bacterial toxins (B) on SDF-1α induced migration of FDCP mix HPC. *Cells were pre-treated with LPA (25 µM), S1P (10 µM), Toxin B (100 ng/ml), LT (100 ng/ml), C2I-C3 (1000 ng/ml) for 2 hours at 37°C, 5% CO_2. The phospholipids treatment (LPA, S1P in A) and the toxins treatment (Toxin B, LT, C2I-C3 in B) were performed in independent experiments. Transmigration of FDCP mix HPC across 5 µm pore membrane in a 48 well Boyden chamber type assay towards SDF-1α (100 ng/ml) was determined after 6 hours. Results are expressed as percentage of migrated cells (Means ± SD, n= 4). Statistical significance between the groups are represented by asterisks ** $p \leq 0.0002$ *** $p<0.0001$.*

Cells were pre-treated with the experimental substances Lysophosphatidic acid (LPA), Sphingosine-1-phosphate (S1P), Lethal Toxin (LT), Toxin B and C2I-C3 toxin. Pre-treatment with LPA significantly inhibited SDF-1α induced transmigration whereas S1P pre-treatment did not affect the migration rate (Fig. 4.2 A).Toxin B, an inhibitor of Rho, Rac and Cdc42 and LT which inhibits Rac and Cdc42 were found to inhibit HPC migration whereas C2I-C3, a cell-permeable fusion toxin which specifically inactivates Rho but not Rac or Cdc42 did not affect HPC migration (Fig. 4.2 B).

4.1.3 Influence of Rho GTPase modulating bacterial toxins on chemokinetic migration of HPC

In order to analyse the role of Rho GTPases in chemokinetic (random) migration of HPC, $lin^{-}Sca1^{+}$ HPC enriched from mouse bone marrow were treated with Rho GTPase inhibiting toxins and their ability to migrate was assessed by a chemokinetic migration assay on fibronectin. The cells were seeded on a cell culture plate and treated with Rho inhibiting toxins, either C2I-C3, or Toxin B or LT. The migration of the cells was random rather than directional. The migration of the cells was observed under a microscope and recorded using time-lapse video microscopy. Both the migration speed and distance of migration were calculated. Interestingly, the cells treated with C2I-C3 were found to increase both the maximum number of migrating cells and the migrated distance compared to the control cells. However, pre-treatment with Toxin B and LT inhibited the majority of random migration (Fig. 4.3 A and B).

To investigate in more detail the increase in the chemotactic migration of HPC after treatment with Rho inhibiting substances, time course experiments were performed. The effect of C2I-C3 was found to be time dependent, with the peak effect after ~2-3 hours, and declines to basal levels after 14-24 hours (Fig. 4.3 C and D). Taken together, the three different bacterial toxins which target the Rho GTPases showed distinct modulatory effects on the migration capacity of HPC. This implies that Rho GTPases control the chemokinetic migration behaviour in a similar way as the chemotactic migration of HPC towards SDF-1α.

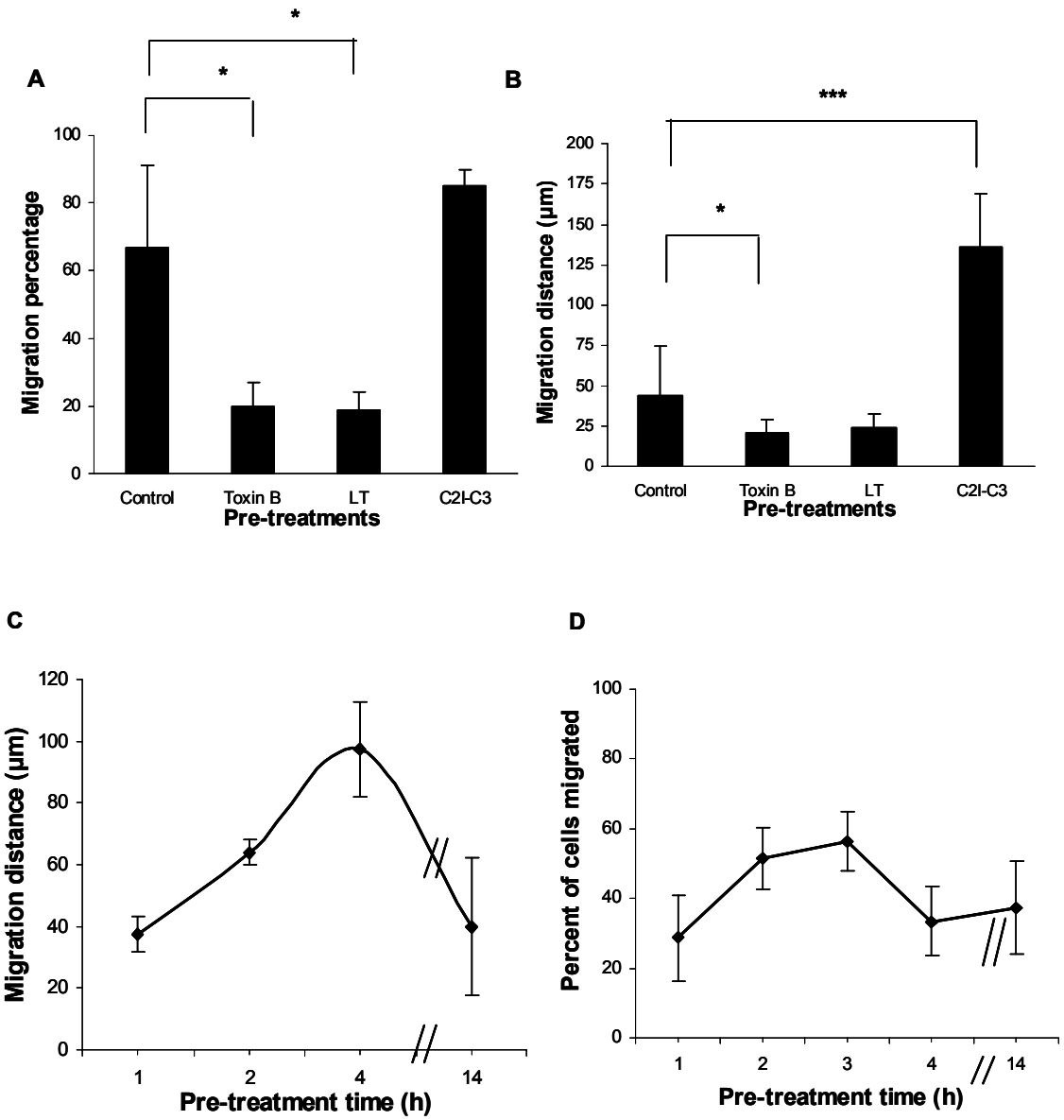
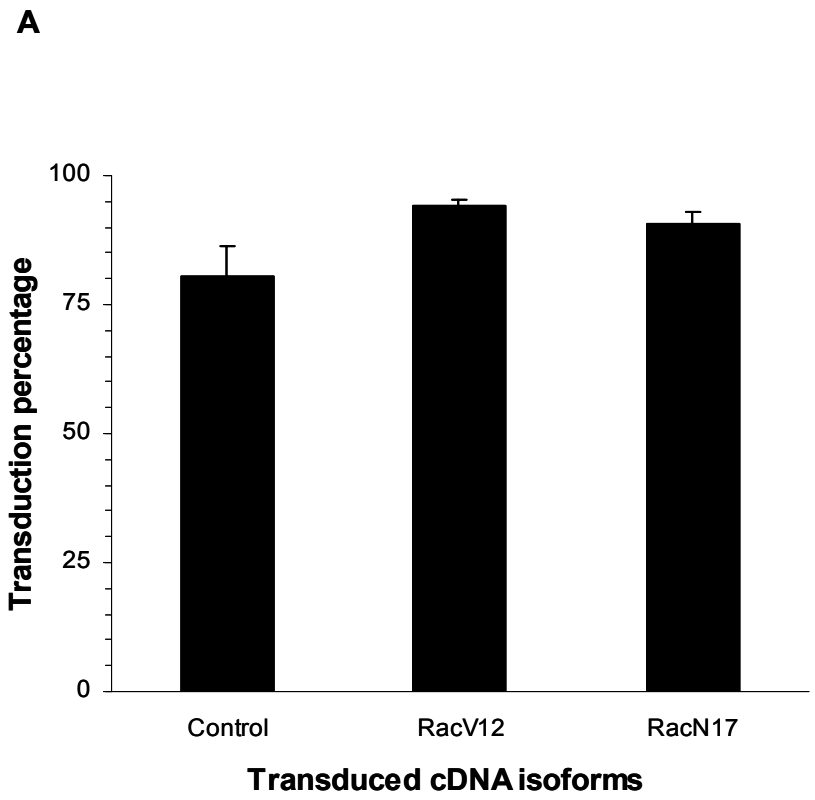


Fig. 4.3 Effect of Rho GTPase modulating toxins on HPC migration. *Chemokinetic migration of HPC treated with C2I-C3 (1 µg/ml), Toxin B (100 ng/ml) and LT (100 ng/ml) was performed on fibronectin (10 µg/ml) pre-coated cell culture plates. The cells were pre-treated with toxins for 2 hours at 37°C, 5%CO_2. Results are expressed as percentage of migrated cells (Means ± SD, n= 7-18). (A) Percentage of cells migrated in the 20 min observation period. Results are expressed as percentage of migrated cells (B) Distance covered by pre-treated migrating cells in 20 min observation period. The results are represented as means ± SD, n= 7-18. (C) Migration distance covered by HPC pre-treated with C2I-C3 (1 µg/ml) for various time points. (D) Percentage of migration of HPC pre-treated with C2I-C3 (1 µg/ml) for various time points. Results are expressed as percentage of migrated cells (Means ± SD, n=6-22). (E) Representative picture showing the chemokinetic migration assay. The cells were tracked for the migration from the beginning until the end of the observation period (20 minutes) and the migration distance was calculated. Statistical significance between the groups are represented by asterisks * $p \leq 0.03$, *** $p<0.0001$.*

4.2 Role of Rho GTPase activation in migration of embryonic endothelial progenitor cells (eEPC)

4.2.1 Transduction of eEPC

Embryonic endothelial progenitors (eEPC) have been reported to be a sub population of bone marrow derived progenitor cells, which take part in vascularization process. eEPC grow as an adherent cell population and are accessible to retroviral gene transduction. To study the role of Rho GTPases in eEPC, the endothelial progenitor cell population was stably transduced with Rac cDNA isoforms using a pseudotyped Moloney-type Retrovirus. The cells were subjected to two sequential transductions 24 hour apart as described in 3.2.8.2 and the transduction efficiency was determined by flow cytometry for GFP expression. The eEPC were found to be highly transducible with retrovirus resulting in transduction efficiency from 80 to 95% 48 hours after the second round of transduction (Fig. 4.4 A and B).



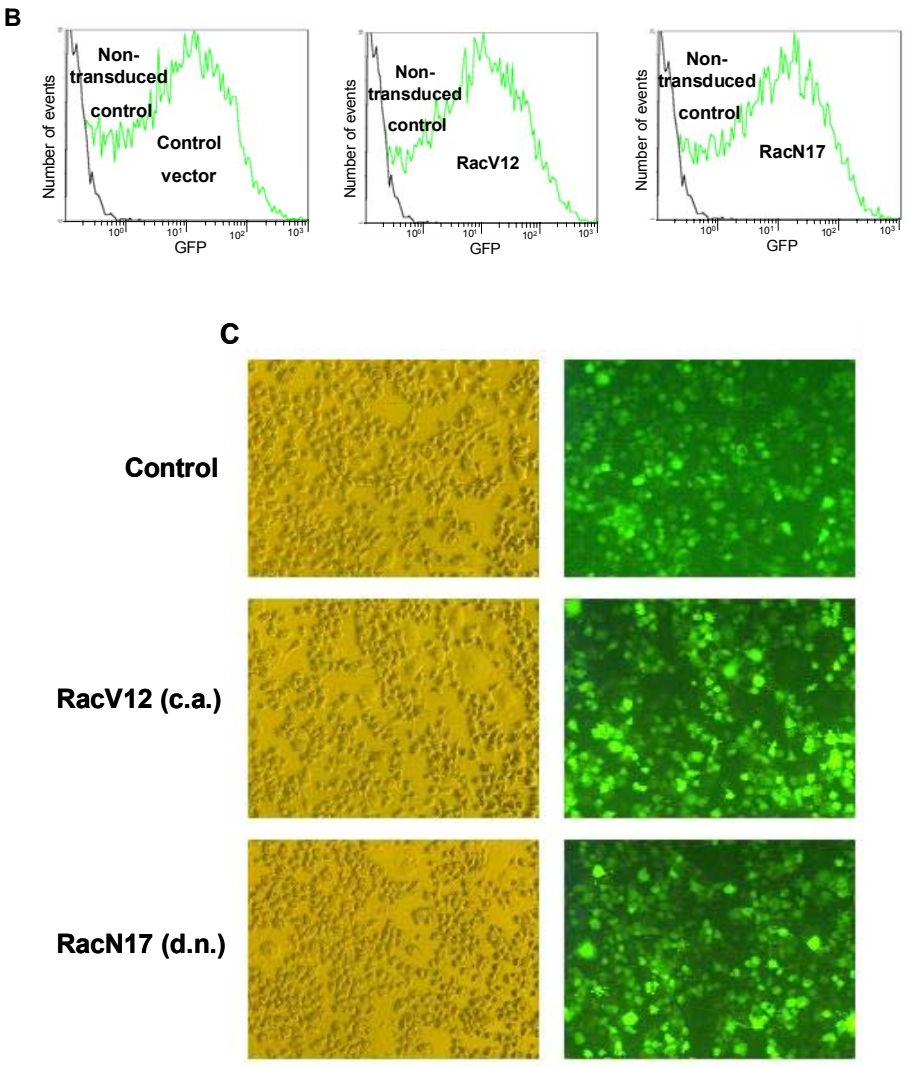


Fig 4.4 Transduction of eEPC with retroviral expression vectors. *(A and B) eEPC transduced with the indicated Rac cDNA isoforms or control vector were analysed for their transduction efficiency by detection of green fluorescence using flow cytometry. Values in A are means ± SD, n=3. (C) Microscopic pictures of transduced eEPC expressing mutant Rac tagged with GFP expression. Left panel in C represents Phase contrast pictures and right panel represents fluorescent microscopic pictures of transduced eEPC expressing mutant Rac with the reporter gene eGFP. Magnification 10 x.*

4.2.2 Morphology of transduced eEPC

The morphological changes brought about by the expression of Rho GTPases in the eEPC were determined by retroviral transduction of eEPC with isoforms of Rho GTPase cDNAs. eEPC were subjected to two sequential transductions 24 hours apart with pseudotyped oncoretrovirus. The control vector transduced cells showed the normal angular morphology (Fig. 4.5 A). The expression of constitutively active Rac (RacV12) led to the formation of numerous lamellipodia like structures on the cell surfaces (Fig. 4.5 B) in

eEPC. In contrast, inhibition of Rac resulted in cells having an elongated morphology (Fig. 4.5 C).

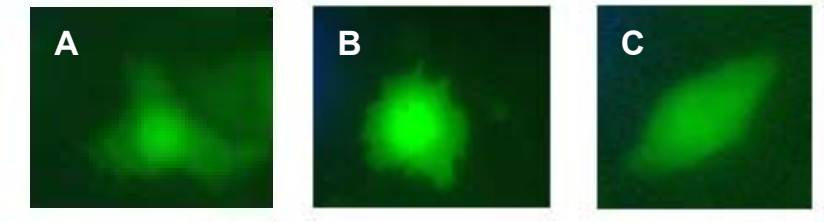


Fig. 4.5 Morphology of transduced eEPC *Retrovirally transduced eEPC expressing the mutant Rac (c.a. and d.n.) upstream of reporter gene eGFP. Fluorescent microscopic pictures showing (A) control vector transduced (B) constitutively active Rac (RacV12) transduced (C) dominant negative Rac (RacN17) eEPC. Magnification 40 x.*

4.2.3 Flow cytometric characterization of Rac GTPase modified eEPC

It was of interest to study the change in the surface receptor expression in eEPC after transduction with the mutant Rac cDNAs, RacV12 (c.a.) and RacN17 (d.n.) which induced a significant change in morphology of the cells. The transduced cells were analysed for the expression of adhesion molecules involved in homing processes, leukocyte specific marker (CD45), and endothelial cell markers Tie-2 and Flk-1. Flow cytometric analysis of transduced eEPC showed an increase in CD44 expression when dominant negative Rac is overexpressed or in Rac inactivated cells. There was also a slight rise in the surface expression of Tie-2 in Rac inactivated eEPC (Table. 4.1). Other receptors tested did not show any change in expression under activated or inactivated Rac conditions in eEPC.

	Control	**RacV12**	**RacN17**
CD44	25	43	52
CD49e	0.7	0.4	0.6
Tie-2	74	76	90
CD11b	0.3	0.3	0.3
CD49d α4	0.2	0.3	0.8
E-selectin	0.7	0.3	0.2
L-selectin	0.2	0.3	0.4
Flk-1	0.2	0.1	0.2
CD45	0.2	0.1	0.1
ICAM-1	0.7	0.3	0.3

Table 4.1 Surface molecule expression of Rac modulated eEPC. *The table shows the percentage of cells stained positive for cell surface molecules in eEPC after retroviral transduction with constitutively active Rac (RacV12) or dominant negative Rac (RacN17) and analysed by flow cytometry. Result of a representative experiment is shown here.*

4.2.4 Adhesion of Rac modified eEPC to HUVEC under shear stress conditions

The morphological changes and the change in cells surface molecule expression in eEPC observed after Rac modulation indicates that it might alter the adhesion behaviour of the cells on the endothelial layer. Moreover, the static migration assay conditions do not always reflect the physiological situation with flow, as it occurs in *in vivo*. In order to determine the function of Rac in shear stress conditions, eEPC were checked for their adhesion efficiency in an *in vitro* model simulating the flow conditions in a flow chamber assay on HUVEC. The adherent eEPC were trypsinized and were incubated for one hour at 37°C for recovery and stabilization of the surface markers. Cells were flushed with a shear rate of 0.1 dyn or 2 dyn/cm^2 for a definite time points over an endothelial cell monolayer (HUVEC). The difference in the adherence of the Rac modulated eEPC over the HUVEC layer was analysed. Under Rac inactivated conditions, eEPC adhered better both with, without TNF-α stimulation on HUVEC compared to respective controls, and regardless of the shear stress applied (Fig. 4.6).

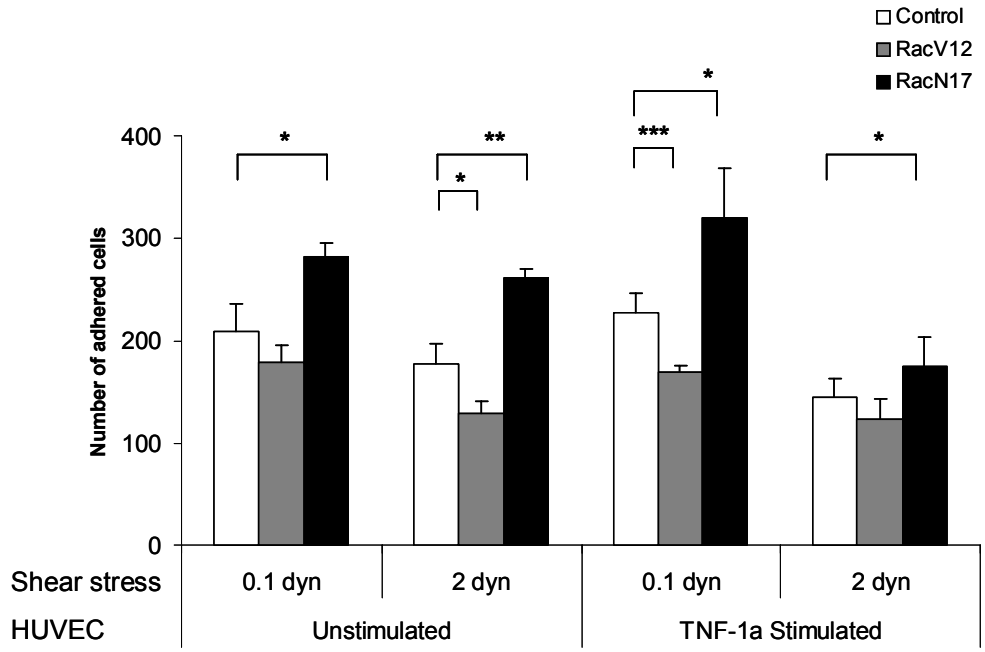


Fig. 4.6 Adhesion of Rac modified eEPC under shear stress conditions. *eEPC transduced with Rac isoforms (RacV12, c.a., RacN17, d.n.) were analysed for their adhesion on HUVEC under shear stress conditions in a flow chamber assay. HUVEC were used stimulated without or with TNF-α for 4 hours. The shear stress applied during the assay was 0.1 and 2 dyn/cm^2. The adhesion was measured after 10 minutes of flushing the eEPC over HUVEC at the mentioned shear stress. Values are means ± SD, n=3-6. Statistical significance between the groups are represented by asterisks * p≤0.05, ** p≤0.002, *** p≤0.0001.*

4.3 Role of Rho GTPase activation in migration of MSC

4.3.1 Migration induction by plasma and SDF-1α in HPC and MSC

MSC have been reported to be important candidates for allogeneic bone marrow transplantation because of their potential to induce immunological tolerance. The basic mechanism involved in stem cell engraftment is the migration of the cells towards the chemotactic substances produced in the bone marrow.

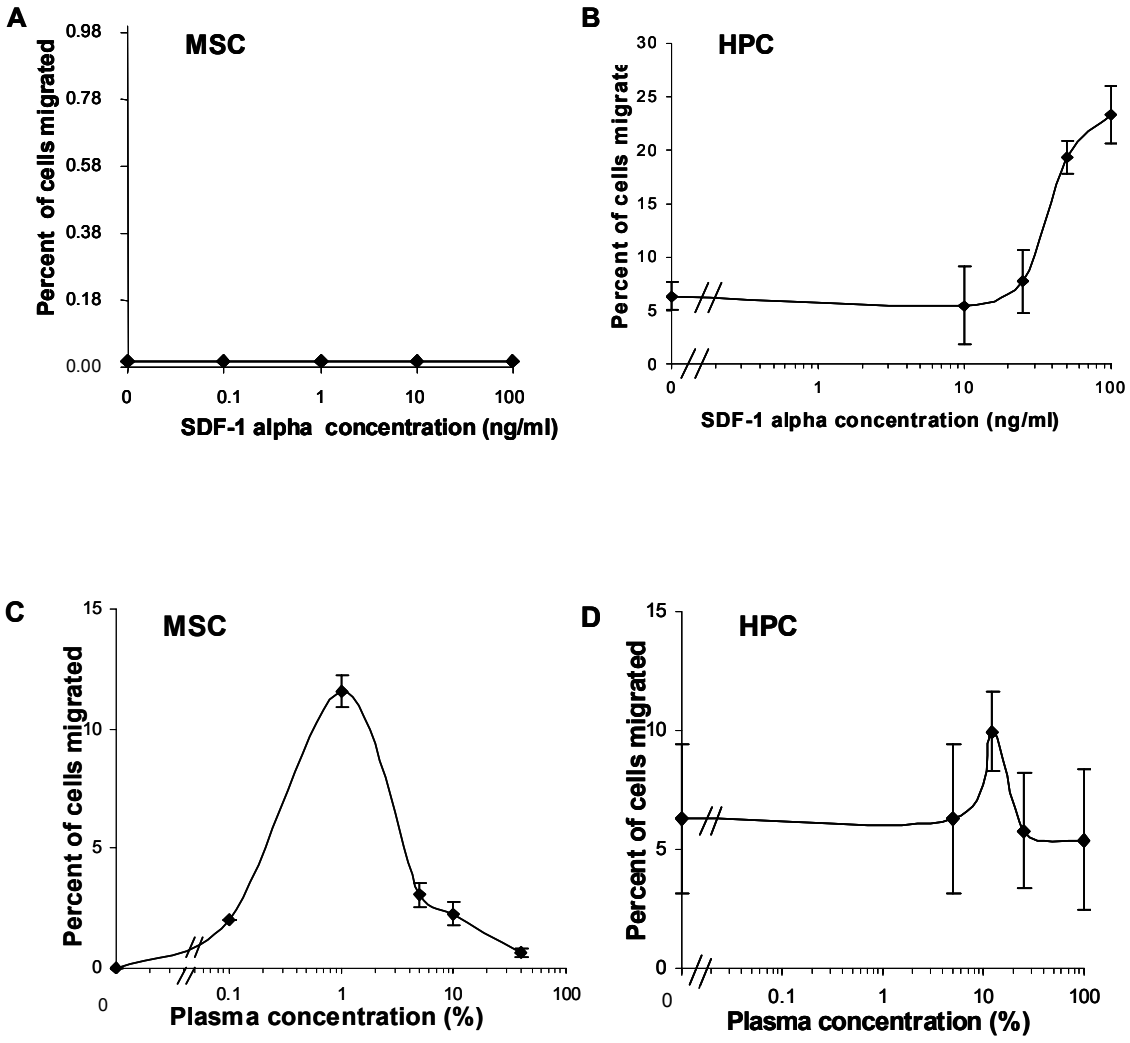


Fig 4.7 Migration induction by plasma and SDF-1α in HPC and MSC. *The transmigration of MSC and HPC towards various concentrations of SDF-1α (0.1 to 100 ng/ml; A, B) and human plasma (0.1 to 100%; C, D) across a porous membrane (5 µm pore size for HPC, 8 µm pore size for MSC) was determined in Boyden chamber type migration assay after 4 hours. Results are expressed as means ± SD, n=6.*

However, since no strong chemotactic agent has been reported yet for migration of MSC, the MSC migration towards SDF-1α, a strong chemoattractant reported for HPC (Aiuti*, et al* 1997) was determined. While more than 20% of the seeded HPC migrated towards SDF-1α (Fig. 4.7 B) there was no detectable migration obtained for MSC (Fig. 4.7 A). In search for a chemotactic agent, human plasma was used to investigate MSC migration. Human plasma was diluted and used as a chemotactic agent in Boyden chamber type assay. MSC were seeded in upper wells of the Boyden type chamber and plasma added to the lower wells. Interestingly, MSC responded to the plasma induced stimuli (Fig. 4.7 C) migrated towards the plasma source as detected by their migration to the lower side of the membrane. The percentage of MSC induced to migrate towards plasma was similar to that obtained for HPC (Fig. 4.7 C and D).

4.3.2 Optimization of experimental conditions for MSC migration in transwell systems

The migration of MSC has not yet been thoroughly investigated. Especially, *in vitro* analysis of transwell migration behaviour of MSC is poorly characterized although i*n vitro* migration assays like Boyden chamber type migration assays have found general acceptance for the study of chemotactic migration of HPC. However, the conditions for such migration for MSC are different from HPC since MSC grow as adherent cells *in vitro* unlike HPC.

PARAMETER	VARIATIONS TESTED
Preincubation of MSC	MSC preincubated for 1 h at 37°C before chemotaxis experiment
Membrane orientation	Shining side facing upwards and non-shining side facing downwards in the chemotaxis chamber
Membrane wiping	After migration assay period, cells on the membrane were fixed with methanol and then non-migrated cells on the upper side were wiped away
Analysis	The migrated cells were fixed, stained and enumerated *in situ* using a microscope

Table 4.2 Experimental conditions for migration of MSC in a 48-well Boyden chamber type migration assay. *Methods were standardized for the pre-incubation, membrane pre-coating with ECM substances, membrane orientation, and fixation of cells on the membrane.*

Therefore, the conditions required to qualitatively and quantitatively visualize and enumerate the migration of MSC were standardized. A summary of these findings is given in Table 4.2. The MSC migrated through the pores of the membrane and found attached to the lower side were enumerated microscopically as shown in Fig. 4.8.

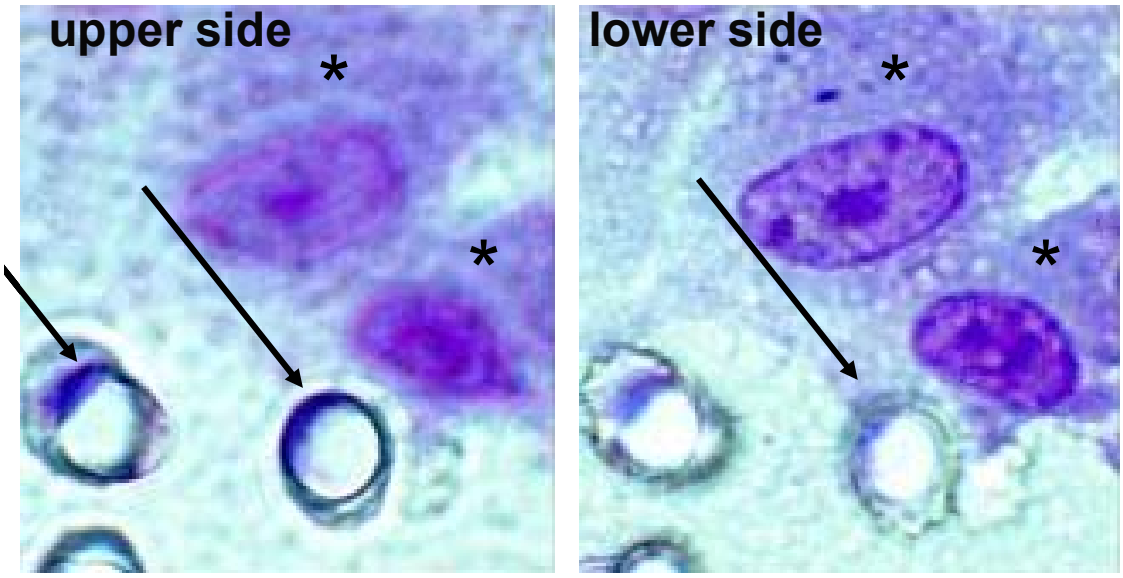


Fig. 4.8 Microscopic image of the membrane after MSC migration focussed on the upper and lower side. *Asterisks represent the migrated cells present on the lower side of the membrane and arrows indicate the pores on the upper surface of the membrane.*

Dependence of MSC transwell migration on plasma concentration

Since the MSC showed a chemotactic migratory response towards human plasma (Fig. 4.9), it was used as a chemotactic agent in this study. The concentration of human plasma to be used for migration experiments was optimized by dose response studies. MSC showed an optimal migration percentage between 0.5 to 1% human plasma as shown in Fig. 4.9 B. Increasing plasma concentrations showed reduced migration rates.

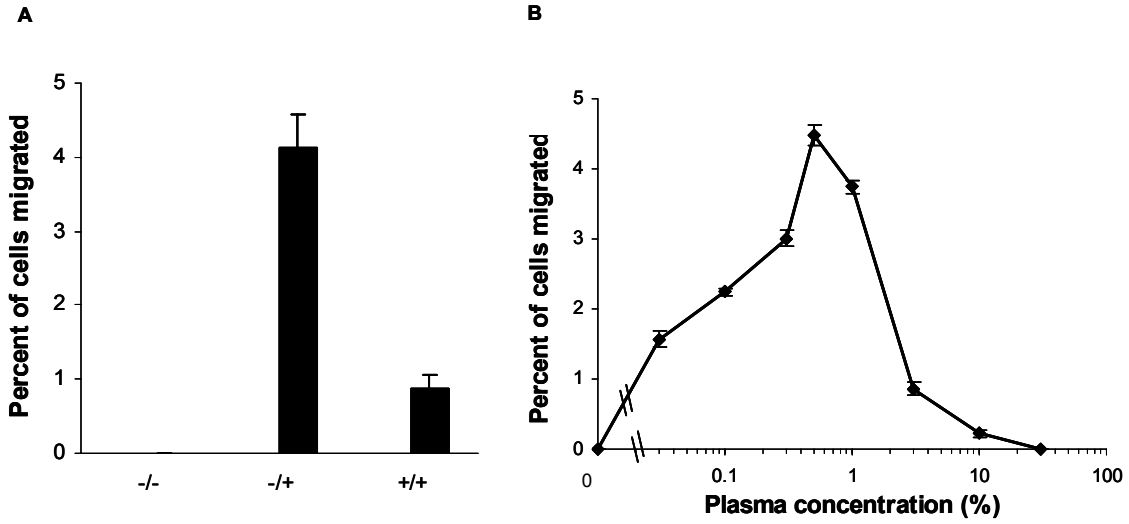


Fig. 4.9 Dose response curve of plasma induced migration of MSC. *Transmigration of MSC across 8 µm pore membrane was determined in a 48 well Boyden chamber type assay towards 1% human plasma (A) or different concentrations of human plasma (B) after 6 hours. -/- represents no chemotactic agent in the upper and lower wells, -/+ the presence of human plasma (1%) in the lower well only, +/+ the presence of human plasma (1%) in both upper and lower wells. Results are shown as percentage of migrated cells. Values are means ± SD, n= 3.*

Influence of membrane pre-coating on transmigration activity of MSC

To test which extracellular matrix substance (ECM) causes best adherence and migration of MSC, the chemotaxis membrane was pre-coated with various extracellular substances. The substances and its concentrations to be tested were narrowed down to human fibronectin (Fn), vitronectin (Vn), laminin (Ln), poly-L-lysine (PLL) depending on the results obtained from the chemokinetic migration experiments (data not shown). Fn, Ln and Vn pre-coatings resulted in quantifiable migration rates whereas pre-coating with PLL showed a reduced migration rate (Fig. 4.10). The spontaneous migration was absent in Vn pre-coating and Fn and Ln pre-coatings showed a lower background migration compared to the plasma induced migration.

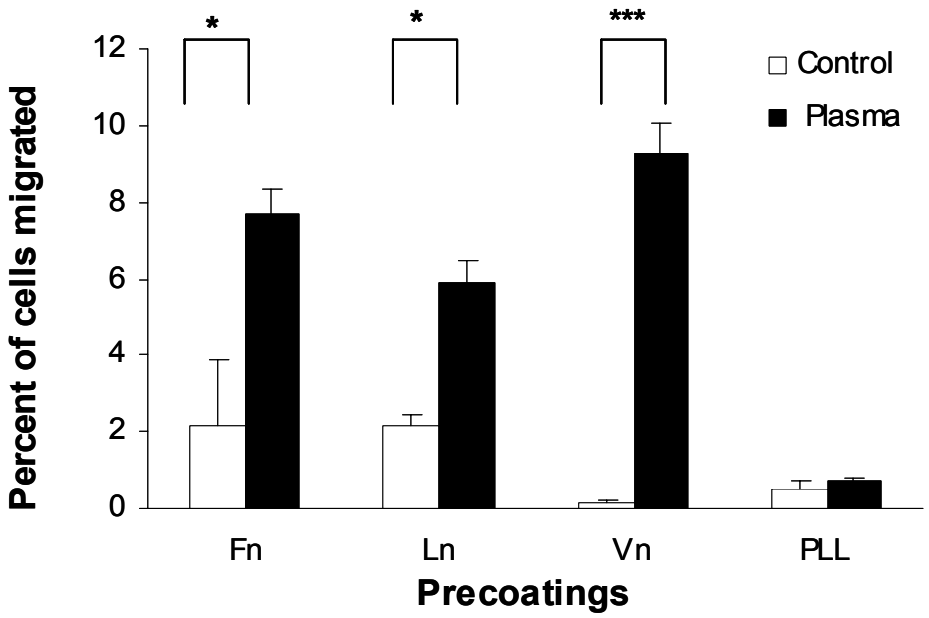


Fig. 4.10 Transmigration of MSC on different pre-coatings. *Chemotaxis membrane (8 µm) was pre-coated with Fn (1 µg/ml) or Vn (100 ng/ml) or Ln (100 ng/ml) or PLL (10 µg/ml) for 2 hours at 37°C. Migration of MSC across the pre-coated membrane towards control substance (DMEM/0.1% BSA) or human plasma (1%, diluted in DMEM) was determined after 6 hours in a Boyden chamber type assay. Results are shown as percentage of cells migrated (Means ±SD, n=3). Statistical significance between the groups are represented by asterisks * p≤0.01, *** p≤0.0001.*

Kinetics of MSC migration

The migration behaviour of MSC was further characterized by the time taken to migrate across Laminin pre-coated membranes towards human plasma. The cells were seeded in equal numbers in each well ($2.5x10^4$ cells/well) and migration percentage was analysed at different time points. The maximum migration was obtained after 4 hours and it plateaus until 8 hours (Fig. 4.11).

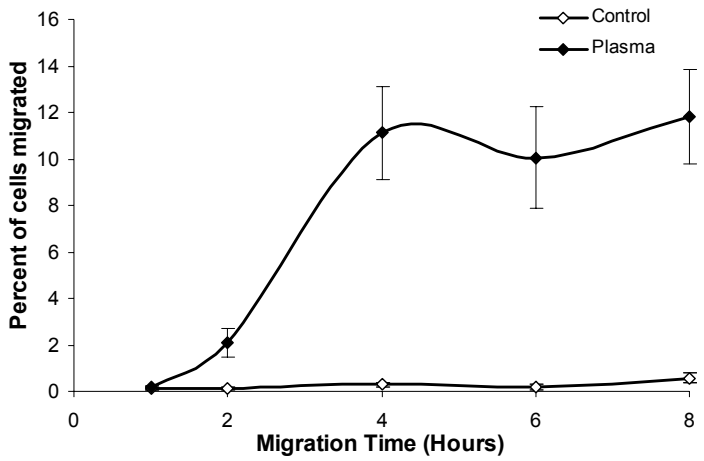


Fig. 4.11 Kinetics of MSC migration*. Transmigration of MSC across Ln (100 ng/ml) pre-coated 8 µm pore sized membrane towards human plasma (1%) in a Boyden chamber type migration assay was determined at the indicated intervals. Results are expressed as percentage of migrated cells (means ± SD, n= 4-5). Statistical significance between the groups are represented by asterisks *** p<0.0001.*

Influence of cell concentration on transmigration of MSC

Cell concentration could influence migration behaviour in many aspects. Certain kinds of cells could migrate only as sheets whereas others migrate individually. In order to find out which concentrations give a valid response in chemotactic migration assay with MSC, different cell numbers were added for a constant migration period and chemotactic agent (human plasma). The cells were seeded in different cell numbers between 500 and $5x10^4$ cells per well in a Boyden chamber type assay and were allowed to migrate towards human plasma for 6 hours. MSC were found to migrate at similar percentages at all cell densities tested. The migration percentage and number of cells seeded were found to be linear, i.e., an increase in cell number caused an increase in the number of detectable migrated cells (Fig. 4.12). In the following experiments, between 20,000 and 50,000 cells were seeded per well.

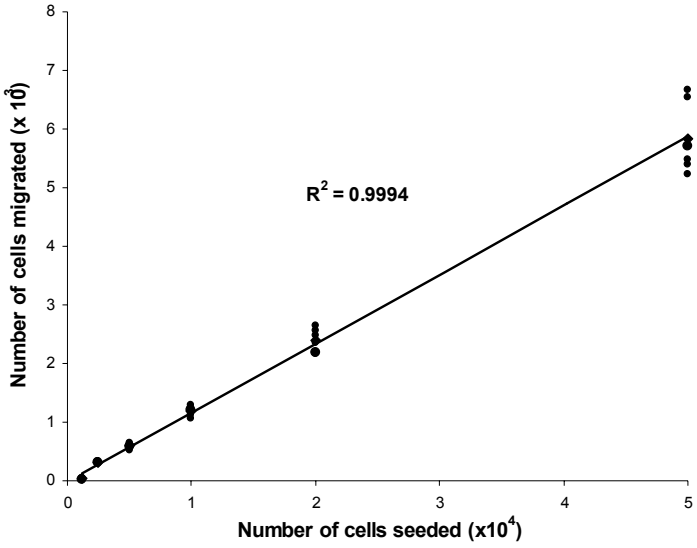


Fig. 4.12 Cell concentration curve of plasma induced migration of MSC. *Transmigration of different cell concentrations of MSC across Ln (100 ng/ml) pre-coated 8 µm pore towards plasma (1%) in a 48 well Boyden chamber type assay was determined. Results are expressed as percentage of migrated cells across the concentration (means ± SD, n= 6).*

4.3.3 Influence of Rho GTPases on chemotactic migration of MSC

Several reports suggested the influence of Rho GTPases in migration of fibroblasts, monocytes and cancer cells (Aepfelbacher*, et al* 1996, Chrzanowska-Wodnicka and Burridge 1996, Fritz*, et al* 2002, Jaffe and Hall 2002, Sawada*, et al* 2002). The effect of inhibition or induction of members of the Rho family (Rho, Rac and Cdc42) on MSC migration (inhibition or induction) was tested. For this, MSC were pre-treated with Rho GTPase inhibiting toxins Toxin B (inhibits Rho, Rac and Cdc42), Lethal toxin (LT, inhibits Rac and Cdc42), and Y27632 (inhibits Rho) and Rho GTPase inducers LPA (Ridley and

Hall 1992), S1P or Cytochalasin D (Cyto D, inhibits actin polymerization) in a Boyden chamber type migration assay. LT, Toxin B, and Cyto D treatment significantly reduced the plasma induced migration (Fig. 4.13). A similar inhibitory effect was obtained with Rho GTPase inducers, LPA, and S1P treatment (Fig. 4.13). However, inhibition of Rho by Y27632 showed a significant increase in the migration rate of MSC (Fig. 4.13).

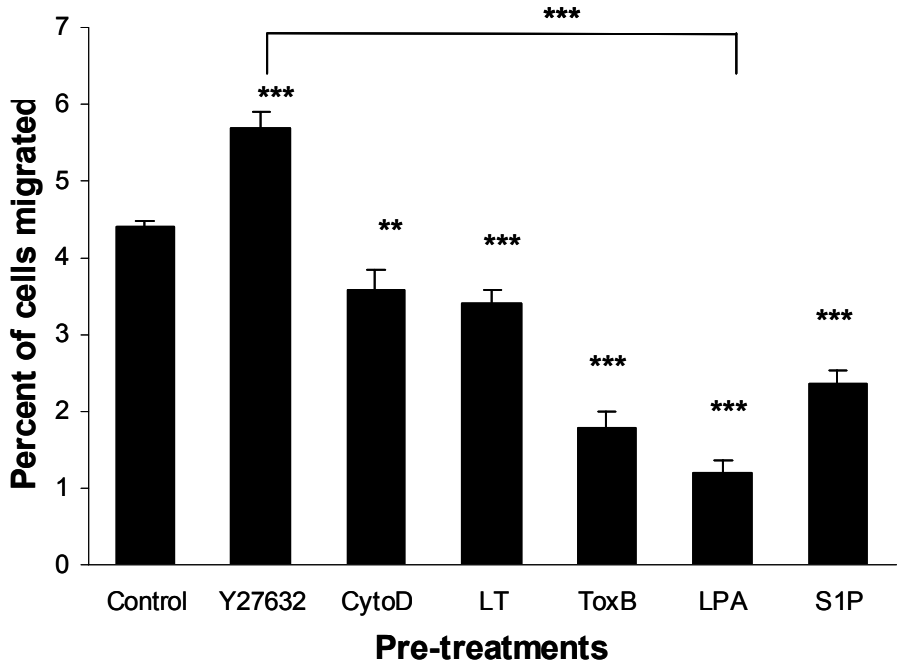


Fig. 4.13 Influence of Rho GTPase inhibition on chemotactic migration of MSC. *MSC were pre-treated with Y27632 (10 µM), Cytochalasin D (10 ng/ml), Lethal Toxin (LT, 100 ng/ml), Toxin B (Tox B, 100 ng/ml), Lysophosphatidic acid (LPA, 25 µM), Sphingosine-1-phosphate (S1P, 10 µM) for 2 hours at 37°C, 5% CO_2. Transmigration of pre-treated or untreated MSC across 8 µm pore membrane towards human plasma (0.5%) in a Boyden chamber type assay was determined after 6 hours (Means ± SD, n= 3-4). All treated groups showed statistically significant differences to control. Statistical significance between the groups is represented by asterisks ** $p \leq 0.001$, *** $p \leq 0.0001$.*

4.3.4 Effect of Rho inhibition on serum induced MSC migration

Since Rho inhibition with Y27632 (chemical Rho activated kinase inhibitor) markedly elevated the migration capacity of MSC, an experiment was done to investigate if a similar increase in migration occurs when Rho is inhibited. To inhibit Rho, the MSC were treated with a cell permeable form of C3 transferase toxin, C2I-C3 (inhibits Rho by ADP-ribosylation), which resulted in a significant rise in the migration rates of MSC in comparable amounts with Y27632 treatment (Fig. 4.14).

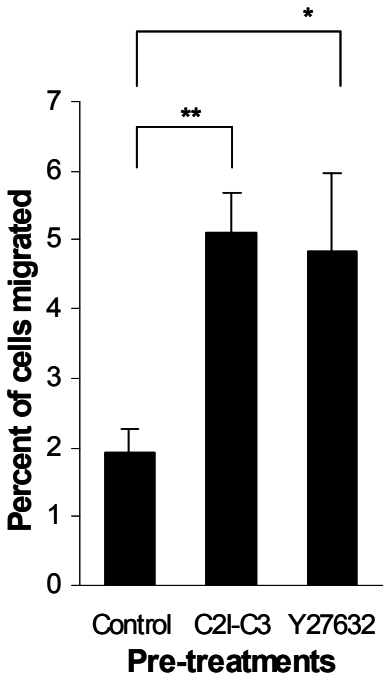


Fig. 4.14 Dose response curve of plasma induced migration of pre-treated and untreated MSC. *MSC were untreated or pre-treated with C2I-C3 (1000 ng/ml) or Y27632 (10 µM) for 2 hours at 37°C, 5% CO_2 and transmigration across Ln (100 ng/ml) pre-coated 8 µm pore membrane towards human plasma (1%) was determined in a Boyden chamber type assay after 6 hours. Results are expressed as percent of cells migrated. Means ± SD, n= 3. Statistical significance between the groups are represented by asterisks * p≤0.01, ** p≤0.001.*

4.3.5 Dose response curve of C2I-C3

The inhibition of Rho by C2I-C3 significantly increased the migration rate of MSC. In order to determine the optimal concentration of C2I-C3 for MSC pre-treatment, dose response studies were carried out. For that, MSC were pre-incubated with different concentrations of C2I-C3 and their migration response was determined by chemotactic migration assay. Surprisingly, the migration percentage of MSC constantly increased over entire dose range of C2I-C3 examined (Fig. 4.15).

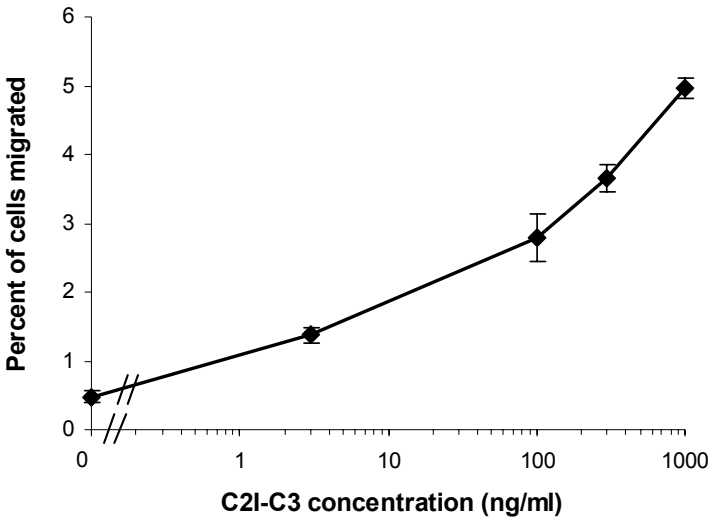


Fig. 4.15 Dose response curve of C2I-C3 pre-incubation and migration of MSC. *MSC were pre-treated with various concentrations of C2I-C3 (3 to 1000 ng/ml) for 2 hours at 37°C, 5% CO_2, and the transmigration across Ln (100 ng/ml) pre-coated 8 µm pore membranes in a Boyden chamber type assay towards human plasma (1%) was determined after 6 hours. Results are expressed as percentage of migrated cells across the concentration. Results are represented as Means ± SD, n= 3.*

4.3.6 Influence of Rho inhibition on chemotactic and chemokinetic migration of MSC

In order to explore if the increased migration rate of MSC after Rho inhibition is rather chemotactic than chemokinetic, migration of MSC with or without a concentration gradient of human plasma was determined in a Boyden chamber type migration assay. For this, MSC were pre-treated with Rho inhibiting toxin C2I-C3 and seeded in the upper wells of the chemotactic chamber. There was a significant increase in the percentage of migrated MSC after pre-treatment with C2I-C3, independent of the plasma gradient (Fig. 4.16). Thus, increased migration of MSC after Rho inhibition is both chemotactic and chemokinetic nature.

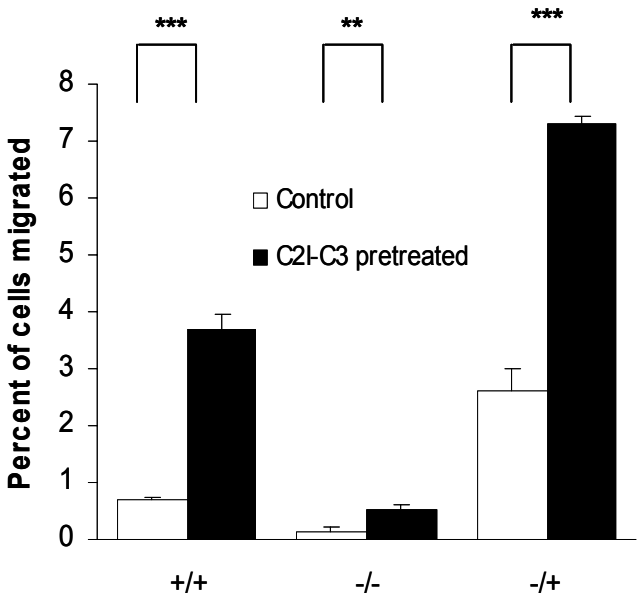


Fig. 4.16 Influence of Rho inhibition on chemotactic and chemokinetic migration of MSC. *MSC were pre-treated with C2I-C3 (1000 ng/ml) for 2 hours at 37°C, 5% CO_2. Migration of MSC untreated or C2I-C3 pre-treated MSC towards human plasma (1%) across 8 µm pore membrane was determined in a Boyden chamber type migration assay after 6 hours. Results are shown as percent of cells migrated. (Means ± SD, n= 3). +/+ indicates presence of human plasma added to both upper and lower wells, -/- indicates absence of plasma in both upper and lower wells, -/+ indicates plasma added only to the lower well. Statistical significance between the groups are represented by asterisks ** $p \leq 0.005$, *** $p < 0.0001$.*

4.3.7 Influence of up or down regulation of Rho GTPases on MSC migration

From the above experiments, it was hypothesized that Rho inhibition induces plasma induced migration of MSC whereas Rac or Cdc42 inhibition caused a decrease in migration (Fig. 4.13). Therefore, to investigate the influence of Rac induction and subsequent Rho inhibition on the migration rate of MSC, the cells were treated with LPA to induce Rho, Rac and Cdc42 and subsequently with C2I-C3 or Y27632 to inhibit Rho. The migration determined by a Boyden chamber type migration assay after pre-treatment with LPA alone showed a 3-fold decrease in the migration rate. In contrast, treatment with LPA and then with C2I-C3 showed a significant 3-fold increase in the migration rate of MSC

(Fig. 4.17). Interestingly inhibiting Rho by treatment with both C2I-C3 toxin and Y27632 showed an increase in the migration rate as either together or alone (Fig. 4.17). However, inducing Rho, Rac and Cdc42 by LPA and subsequent inhibition of Rac and Cdc42 by LT had no impact on the migration potential of MSC (Fig. 4.17).

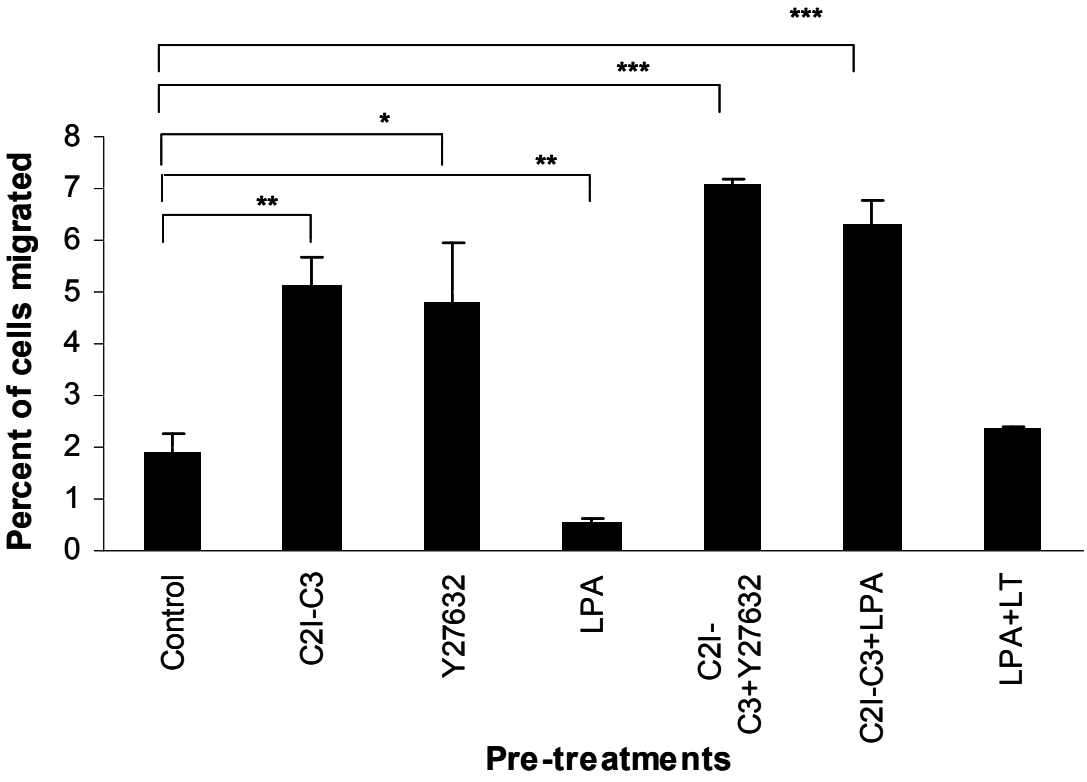


Fig. 4.17 Inhibiting Rho and elevating Rac and Cdc42. *MSC were pre-treated with C2I-C3 (1000 ng/ml), Y27632 (10 µM), LT (100 ng/ml) and LPA (25 µM) for 2 hours at 37°C. In case of pre-treatment with two substances LPA and C2I-C3 or LPA and LT, the cells were pre-treated first with LPA for 2 hours and subsequently with C2I-C3 or LT for another 2 hours at 37°C. In cells treated with C2I-C3 and Y27632, the substances were added together for 2 hours at 37°C. The transmigration of untreated or pre-treated MSC towards human plasma (0.3%) across Ln (100 ng/ml) pre-coated 8 µm membrane was determined after 6 hours. The results are expressed as means ± SD, n= 3. Statistical significance between the groups are represented by asterisks * $p \leq 0.01$, ** $p \leq 0.003$, *** $p < 0.0001$.*

Rho inhibition by C2I-C3 toxin or Y27632 was found to increase significantly the migration rate in MSC. To confirm independently this modulatory role of Rho on MSC migration, MSC were stably transduced with Rho GTPase cDNA isoforms (c.a. and d.n.) and analysed for their chemotactic migration capacity by Boyden chamber type migration assay. MSC transduced with control vector showed similar migration percentage with the non-transduced cells (Fig. 4.18). Interestingly, RhoV14 (c.a. Rho) transduced MSC

showed a significant decrease in the migration rate, whereas, MSC transduced with C3 transferase (d.n. Rho) showed a significant increase migration towards human plasma (Fig. 4.18), a result which correlates with the increased migration percentage obtained with MSC treated with C2I-C3 toxin.

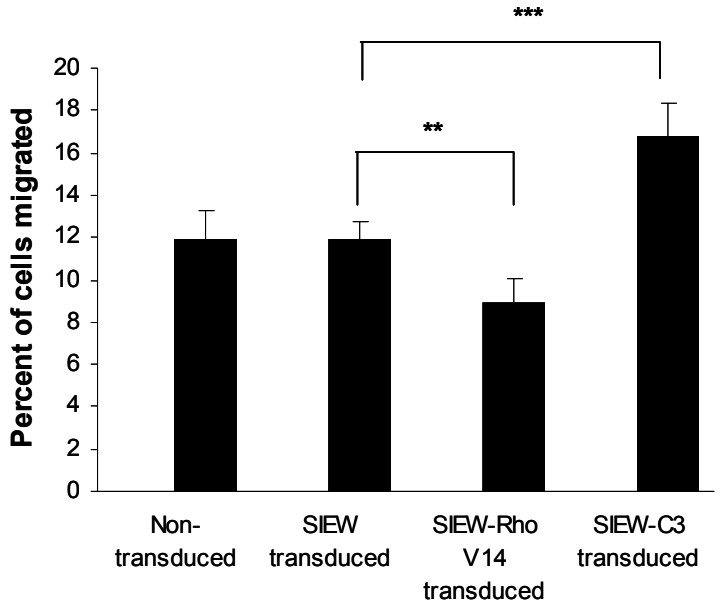


Fig. 4.18 Transduction with C3 elevates chemotactic migration of MSC*. MSC were lentivirally transduced with control vector (SIEW), constitutively active form of Rho (SIEW-RhoV14) and C3 transferase (SIEW-C3) and the transmigration across Ln (100 ng/ml) pre-coated 8 µm pore membrane was determined in a 48 well Boyden chamber type assay towards human plasma (1%) after 6 hours. Results are expressed as percent of cells migrated (Means ± SD, n= 6). Statistical significance between the groups are represented by asterisks ** p≤0.001, *** p≤0.0001.*

4.3.8 Influence of Rho on migration of MSC towards growth factors and phospholipids

In order to examine individual chemoattractants for MSC instead of using a complex mixture of factors in human plasma, the chemotactic activity of growth factors (Platelet derived growth factor; PDGF, Hepatocyte derived growth factor; HGF) or serum phospholipids (LPA, S1P) towards MSC was determined by Boyden chamber type migration assay. In addition, the effect of Rho inhibition on MSC migration towards such factors was determined. MSC were pre-treated with or without the Rho inhibiting toxin C2I-C3 and the cells were analysed for the migration towards human plasma, PDGF, HGF, LPA and S1P. Untreated MSC showed migration response of 0.1 to 0.3% of migrated cells towards PDGF, HGF, LPA, and S1P. Whereas, treatment of MSC with C2I-C3 showed approximately 10-fold increase in the migration percentage towards growth factors (PDGF, HGF) and serum phospholipids (LPA, S1P) shown in Fig 4.19.

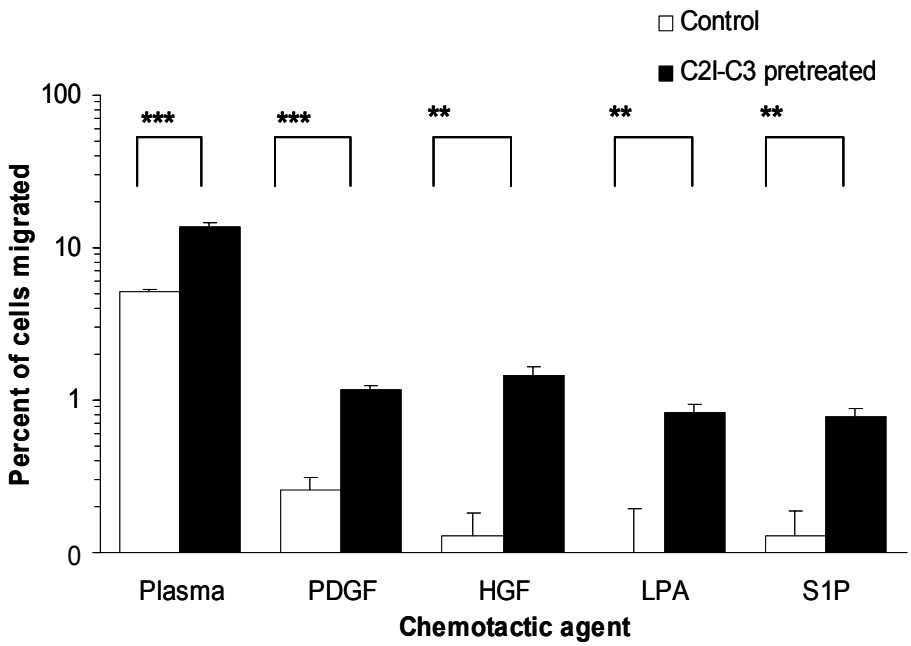


Fig. 4.17 C2I-C3 pre-treatment elevates chemotactic migration of MSC. *MSC were pre-incubated without (open bars) or with C2I-C3 (1000 ng/ml) (closed bars) and the transmigration across Ln (100 ng/ml) pre-coated 8 µm pore membranes in a Boyden chamber type assay was determined towards human Plasma (1%), PDGF (10 ng/ml), HGF (10 ng/ml), LPA (50 µg/ml) and S1P (10 µg/ml) after 6 hours. Results are expressed as percentage of migrated cells across the concentration (Means ± SD, n= 4).). Statistical significance between the groups are represented by asterisks ** p≤0.001, *** p≤0.0001.*

4.3.9 Effect of Rho inhibition on transendothelial migration of MSC

Rho inhibition significantly increased the migration rate of MSC in a chemotactic model where MSC migrated through a membrane. To simulate more closely the physiological situation of transendothelial migration, the ability of MSC to cross endothelial cells (HUVEC) pre-coated on a porous membrane was determined in a transwell migration assay. In this experimental setting, MSC were treated without or with Rho inhibiting toxin C2I-C3 and their migration across an endothelial cell layer through porous membrane coated with collagen (0.1%) towards plasma was determined. This analysis revealed that MSC pre-treated with C2I-C3 significantly elevated the transmigration rate of MSC also through the endothelial layer (HUVEC) towards human plasma as represented in Fig. 4.20.

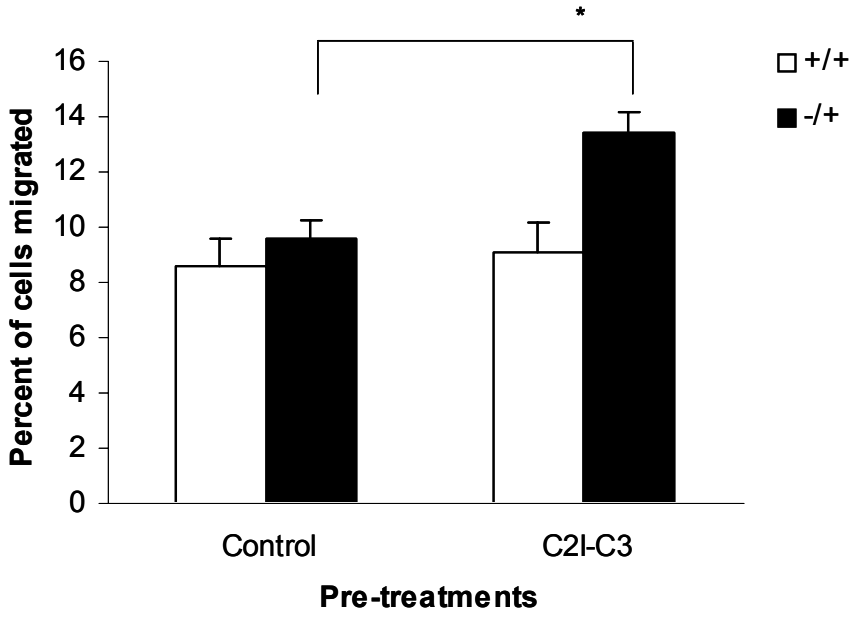


Fig. 4.20 Induction of MSC transendothelial migration. *MSC were pre-treated without or with C2I-C3 (1000 ng/ml) for 2 hours at 37°C, 5% CO_2. Transmigration of MSC across endothelial layer (HUVEC) through 8 µm pore membrane towards human plasma (1%) was determined after 6 hours. Results are expressed as percent of cells migrated (Means ±SD, n=3). +/+ indicates human plasma added to both the upper and lower wells, -/+ indicate human plasma added only to the lower well. Statistical significance between the groups are represented by asterisks ** p≤0.03.*

4.3.10 Effect of Rho GTPases on MSC cytoskeleton

Since Rho GTPases has been reported to be involved in migration and organization of cytoskeleton (Fukata*, et al* 2003, Nobes and Hall 1995), and the chemotactic migration experiments showed a significant difference in the migration rate, the effect of the Rho

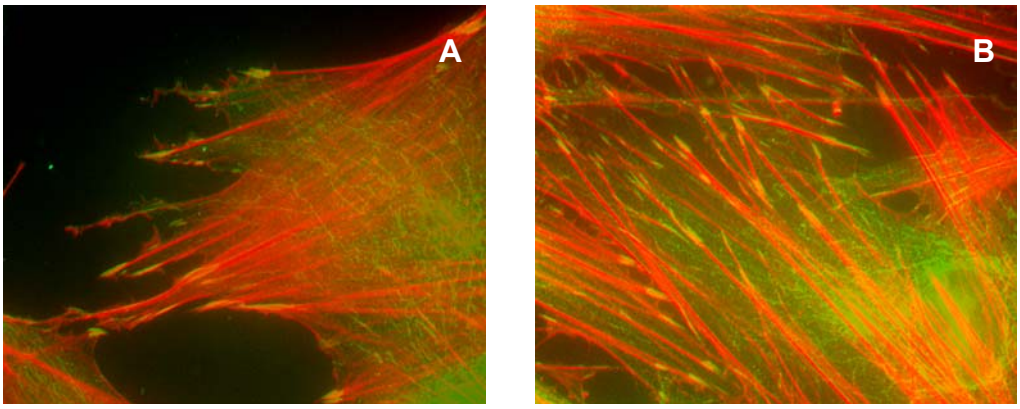


Fig 4.21 Effect of Rho activation on actin organization in MSC. *Actin and paxillin reorganization in MSC treatment without (A) or with LPA (10 µg/ml for 2 hours) treatment (B) was visualized by immunostaining with phalloidin-TRITC (red) for actin and anti-paxillin-FITC (green). Magnification 400 x.*

GTPases on cytoskeletal organization of MSC was determined. For this, MSC were treated without or with lysophosphatidic acid (LPA), and the organization of F-actin and paxillin was visualized by immunostaining. The control cells showed F-actin and paxillin localized at focal adhesion points (Fig. 4.21 A). Whereas in LPA treated cells, the actin filaments are more organized forming stress fibres resulting in increased numbers of focal adhesion points. Thus activation of Rho GTPases in MSC by LPA treatment affects the organization of actin and focal adhesion protein paxillin (Fig. 4.21 B).

The previous experiment showed the influence of Rho GTPase activation (Rho, Rac, and Cdc42) by LPA on MSC cytoskeleton. Since inhibition of Rho function by C2I-C3 or Rho kinase inhibitor Y27632 or d.n. Rho (RhoN19) resulted in a significant increase in chemotactic migration of MSC, its effect on cytoskeletal organization was determined here. MSC treated with Rho inhibitor C2I-C3 toxin was analysed for F-actin organization and paxillin. Staining for F-actin with Phalloidin-TRITC showed untreated MSC with F-actin filaments arranged in an organized fashion running throughout the cell surface (Fig. 4.22 A). Rho inhibition in MSC changed the pattern of actin organization resulting in a more meshwork like arrangement of actin filaments (Fig. 4.22 B).

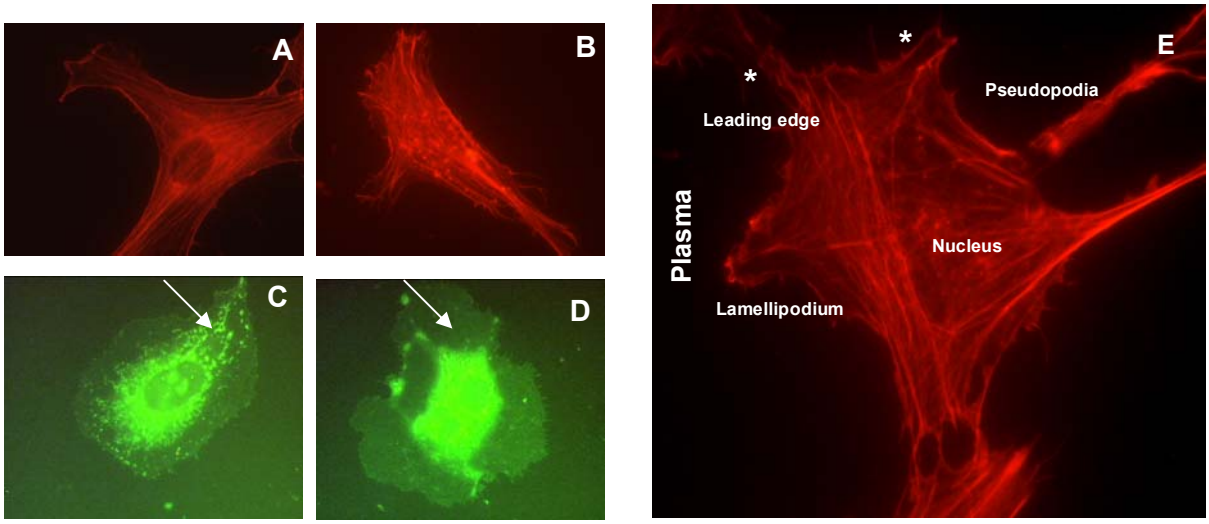


Fig. 4.22 Disruption of F-actin and paxillin by C2I-C3 treatment. *Actin organization in MSC pre-treated without (A) or with (B) Rho inhibitor was visualized by staining with Phalloidin-TRITIC. Paxillin distribution in untreated (C) or Y27632 treated (D) MSC were determined by immunofluorescent staining with anti-paxillin antibodies coupled to fluorescein isothiocyanate (FITC). C2I-C3 (Rho inhibitor) pre-treated MSC were analysed for their migration towards human plasma (1%) and actin organization in the migrating cells was visualized by Phalloidin-TRITC (E). The pre-treatment time for each was 2 hours at 37°C, 5% CO_2. The arrows in C and D indicate the paxillin distribution. Magnification 400 x.*

In order to find out the effect of Rho inhibition on other cytoskeleton associated proteins, the distribution of paxillin in Rho inhibited cells were determined. Consistent with the unorganised actin filaments, the distribution of paxillin staining was altered in Rho inhibitor treated cells (Fig. 4.22 C and D). However, when Rho inhibited MSC (treated with C2I-C3) were induced with plasma, they migrated towards plasma, forming lamellipodia and leading edge like structures (Fig. 4.22 E).

Rho GTPase activation by LPA, or inactivation of Rho by C2I-C3 showed a significant change in the morphology of MSC cytoskeleton. To decipher the effect further and to confirm the cytoskeletal effects caused by Rho GTPase modulation, MSC were microinjected with RhoV14 (c.a. Rho) and C3 (d.n. Rho) cDNAs. Injection was done into the nucleus of MSC. Effective microinjection and expression of the cDNAs was controlled by the expression of eGFP expression, which is present downstream to the Rho GTPase genes. Elevating the amount of active Rho by RhoV14 microinjection led to increased stress fibre formation in MSC (Fig. 4.23 B). On the contrary, inhibiting the Rho function by C3 transferase microinjection led to disoriented actin filaments showing short, thin and unorganized F-actin structures as observed with C2I-C3 toxin treatment (Fig. 4.23 C). These results indicate that one of the biological functions of these Rho proteins is to control actin organization.

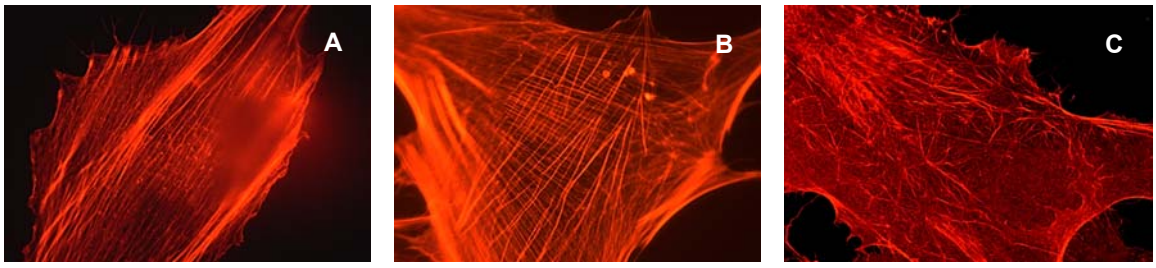


Fig. 4.23 Microinjection of MSC with Rho cDNA isoforms. *Immunofluorescent staining of F-actin of MSC with Phalloidin-TRITC after microinjection with 10 µg/ml of Rho cDNA isoforms. Control vector (A), RhoV14 (B), and C3 (C). Magnification 400 x.*

The previous data indicate that Rho inhibition significantly increased the chemotactic migration of MSC and changed its cytoskeleton (actin) and a related protein, paxillin. To determine the effect of Rho further on MSC morphology, MSC were transduced with pseudotyped lentivirus coding for constitutively active Rho (RhoV14) and C3 transferase (Rho inhibiting toxin gene from *C. limosum*) driven by SFFV promoter, to stably over express or inhibit Rho and their effect on morphology of MSC was determined. The

transduction efficiency was controlled by GFP expression and it was between 20 and 60% of total cells seeded. The transduction of MSC with active Rho or C3 transferase resulted in change in morphology of the cells after 48 hours of transduction as shown in Fig. 4.24. RhoV14 transduced cells were flat and spread (Fig. 4.24 D and E). In contrast, transducing MSC with C3 transferase which inhibits Rho, induced cell retraction, loss of contact with neighbouring cells, resulting in rounded morphology with decreased growth rate (Fig. 4.24 F and G).

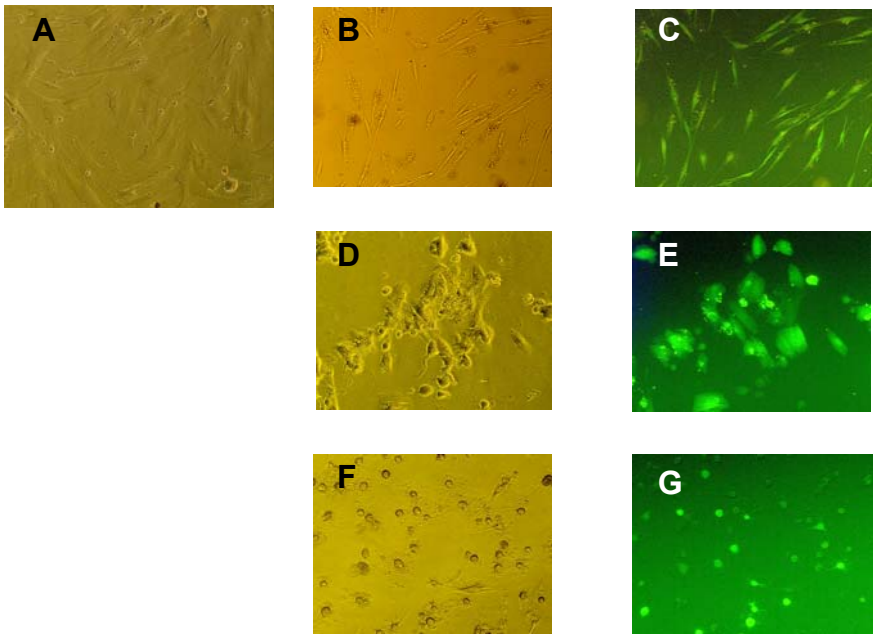


Fig. 4.24 Rho GTPases modify the morphology of MSC*. Morphology of MSC (A). Representative microscopic pictures of lentivirally transduced MSC with control vector (B and C), constitutively active Rho, RhoV14 (D and E), C3 transferase to inhibit Rho (F and G). A, B, D and F represents phase contrast and C, E and G represent fluorescent microscopic images. Transfected cells were identified by the GFP expression in the lentiviral expression vector. Representative pictures are shown for each condition. Magnification 100 x.*

4.4 Role of Rho GTPase activation in endothelial cells for progenitor cell migration and homing

4.4.1 Expression of Rac mutant transgene by retroviral vector

To validate the functionality of the cDNAs used for retroviral expression of mutant GTPases, GTPase activity was determined in a pull down assay based on Western Blot analysis. The active form of Rac was pulled down using GST-Pak1-PBD as described in 3.2.11. GST-Pak1-PBD discriminates between GTP and GDP bound forms of Rac by binding only to GTP bound form of Rac (active Rac). 293T cells were transiently transfected with expression vectors encoding Myc-tagged constitutively active Rac (RacV12) or dominant negative Rac (RacN17) as well as a control expression vector. Cell lysates were prepared and incubated with GST-Pak1-PBD fusion protein, 48 hours after transfection. Binding of the Rac protein was analysed by recovery of the fusion protein on glutathione Sepharose followed by Western Blot analysis in a 12% polyacrylamide gel using an anti-Rac antibody. The active Rac was obtained in cells transfected with vectors encoding active Rac (RacV12) and in control vector transfected cells. The two bands shown in the result are due to the fact that anti-Rac antibody identifies both endogenous and Myc-tagged Rac which has a higher molecular weight than the endogenous form (Fig. 4.25).

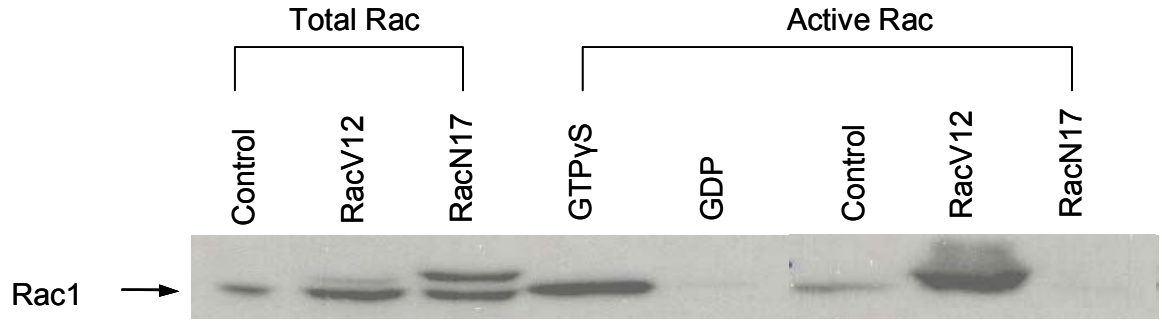


***Fig. 4.25 Immunoblot analysis of Rac expression**. Representative immunoblot of Rac expression in 293T cells transfected with Rac cDNA isoforms (constitutively active and dominant negative). The cells were analysed 48 hours after transfection. Total Rac represents both active and inactive Rac in the transfected cells. To pull down active Rac (GTP bound Rac), the transfected cell lysates were incubated with 20 µg of GST-Pak1-PBD and then precipitated with glutathione-Sepharose beads.*

4.4.2 Expression of Rho mutant transgene by lentiviral vector

The expression and GTPase activity of the mutant Rho cDNA was determined in a Western Blot analysis. The active form of Rho was pulled down using GST-Rhotekin-RBD as described in 3.2.11 GST-Rhotekin-RBD specifically binds to GTP bound form of the Rho GTPase (active Rho). 293T cells were transiently transfected with expression vectors encoding Myc-tagged constitutively active Rho (RhoV14) or dominant negative Rho (RhoN19) as well as a control expression vector. Cell lysates were prepared and incubated with GST-Rhotekin-RBD fusion protein, 48 hours after transfection. Binding of the Rho protein was analysed by recovery of the fusion protein on glutathione Sepharose followed by Western Blot analysis in a 12% polyacrylamide gel using an anti-Rho antibody. The two bands in the result are due to the fact that anti-Rho antibody identifies both endogenous and Myc-tagged Rho which has a higher molecular weight than the endogenous form (Fig. 4.26).

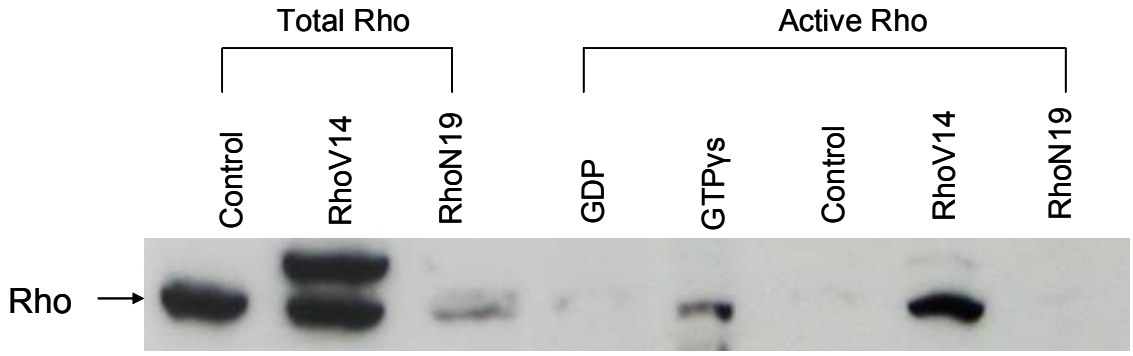


Fig. 4.26 Immunoblot analysis of Rho expression*. Representative immunoblot of Rho expression in 293T cells transfected with Rho cDNA isoforms (constitutively active and dominant negative). The cells were analysed 48 hours after transfection. Total Rho represents both active and inactive Rho in the transfected cells. To pull down active Rho (GTP bound Rho), the transfected cell lysates were incubated with 40 µg of GST-Rhotekin-RBD and then precipitated with glutathione-Sepharose beads.*

4.4.3 Role of Rho in Endothelial cells (HUVEC) during extravasation

Transduction of HUVEC endothelial cells with Rac and Rho cDNA isoforms

The endothelial cells are a major interacting partner during the homing process. In order to determine whether Rho GTPases activity in the endothelial cells might also modulate migration and homing of progenitor cells, HUVEC were subjected to transduction with constitutively active Rac (RacV12) and constitutively active Rho (RhoV14) cDNAs using

pseudotyped retrovirus as described in 3.2.8.2. The transduction efficiency was analysed by flow cytometry for the expression of eGFP reporter gene in the transduced HUVEC (Fig. 4.28).

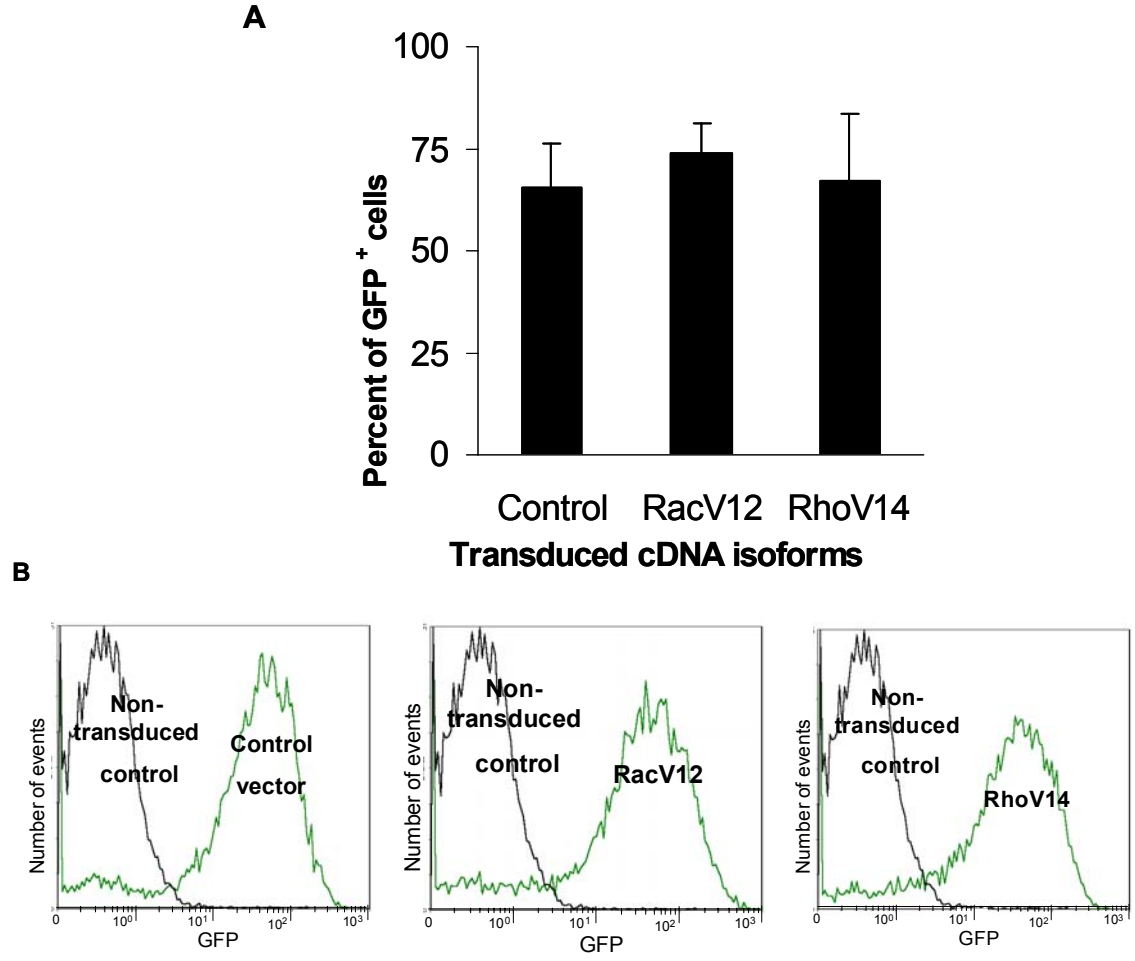


Fig 4.28 Transduction of HUVEC with retroviral expression vectors. *HUVEC transduced with control vector, constitutively active Rac (RacV12) and constitutively active Rho (RhoV14) were analysed for their transduction efficiency by flow cytometry. The results are expressed as means ± SD, n=3. A represents mean values ± SD of triplicate experiments and B shows representative flow cytometric evaluation.*

4.4.4 Regulation of gene expression as determined by microarray analysis

Microarray hybridization technology was used as a tool to determine the modification in the regulation of gene expression after Rho GTPase modulation in endothelial cells (HUVEC). HUVEC were stably transduced with constitutively active Rac (RacV12) and constitutively active Rho (RhoV14) using retroviral expression vectors. 48 hours after transduction, RNA was isolated from the cells and cDNA synthesized with the fluorescent labelled nucleotides and hybridized to a microarray. 20,000 genes were analysed in the

microarray. The threshold was set at 2 fold increase or decrease. Overexpression of active Rho was found to affect a number of genes compared to overexpressed active Rac in HUVEC (Table 4.3).

GTPase mutant	Number of genes upregulated	Number of genes down-regulated
RhoV14 c.a.	303	90
RacV12 c.a.	129	30

Table 4.3 Regulation of gene expression as determined by microarray analysis.
This table shows the number of regulated genes after overexpression of constitutively active Rho (RhoV14) or constitutively active Rac (RacV12), determined by microarray analysis. c.a. represents constitutively active.

4.4.5 Genes Regulated by modulation of Rho GTPase in HUVEC

Modification of Rho GTPase level in HUVEC caused a significant change in the gene expression profile. A number of genes were up regulated. When active Rho (RhoV14) was overexpressed, group of homing molecules, growth factors and receptors, extracellular matrix molecules and cytoskeletal proteins were up regulated (Table 4.4, Table 4.5).

Genes regulated in HUVEC by overexpression of active Rho (RhoV14) as determined by microarray analysis			
Description	**Common Name**	**Genebank**	**Fold expression**
Homing molecules			
CD44 antigen (homing function and Indian blood group system)	CD44	AJ251595	2
E-selectin	ELAM, ESEL, CD62E, LECAM2	NM_000450	4.01
Intercellular adhesion molecule 2	CD102	NM_000873	2.186
Extracellular matrix proteins			
Collagen, type IV, alpha 1	COL4A1	NM_001845	2.178
Procollagen (type III) N-endopeptidase	PRSM1, KIAA0047	NM_002768	2.11
Fibronectin 1	FN, CIG, FINC	AJ276395	-13.79
Growth factors			
Interleukin 8	IL8		2.186
Angiopoietin 1	ANGPT1	AF209975	2.023
Plasminogen activator, urokinase	URK	K03226	2.913
Myeloid leukemia factor 2	MLF2	NM_005439	2.58
Interferon (alpha, beta and omega) receptor 2	IFNABR	L41944	-2.089
Small GTPases associated proteins			
Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	EN-7	NM_002872	4.778
RAB5C, member RAS oncogene family	RAB5C	NM_004583	2.74
Rab acceptor 1 (prenylated)	RABAC1	NM_006423	2.268
G protein beta subunit-like	GBL	NM_022372	2.551
Cytoskeleton associated proteins			
Cytoskeleton-associated protein 1	CKAP1	BC005969	2.822
Cathepsin Z	CTSX	AF073890	2.427
Capping protein (actin filament) muscle Z-line, beta	CAPB, CAPZ, CAPPB	NM_004930	2.365
beta-actin; Human mRNA for beta-actin.	ACTB	X00351	2.333
Talin 1	TLN	NM_006289	3.726
Tubulin, beta, 2	TUBB2	BC004188	2.104
Actin, gamma 2, smooth muscle, enteric	ACTE, ACTA3, ACTSG	NM_001615	2.042
Tropomyosin 2 (beta)	TPM2	NM_003289	2.96
Actin binding LIM protein 1	ABLIM	BC002448	-2.341

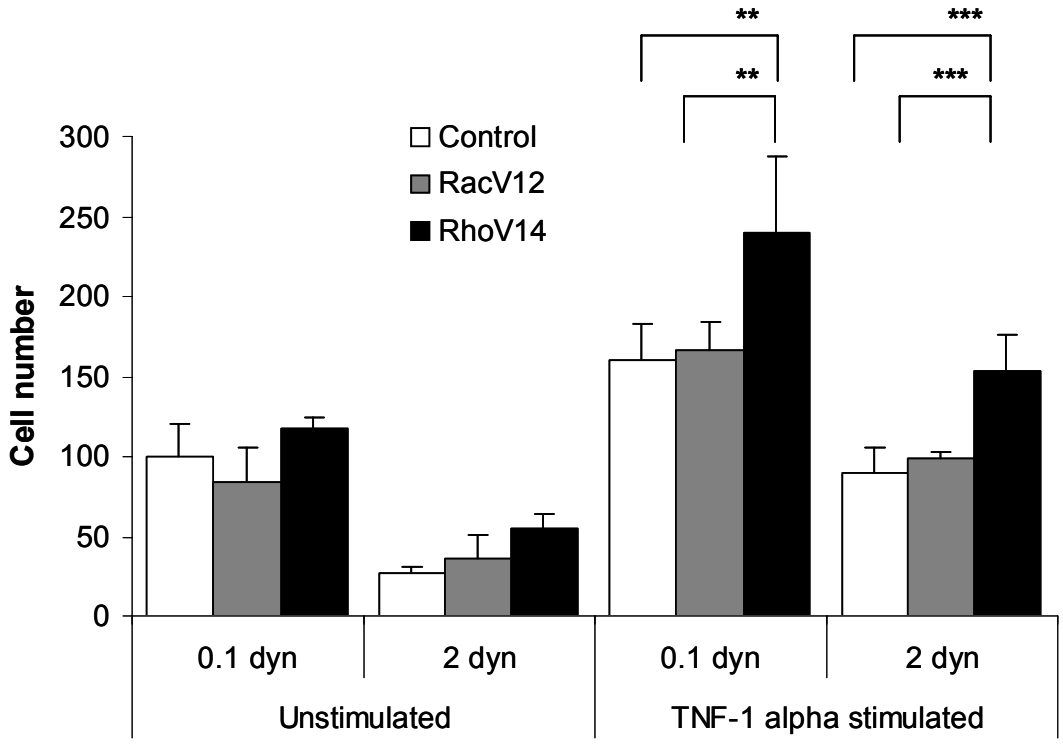
Table 4.4 Genes regulated by RhoV14 (c.a.) in HUVEC. *The table represents the homing, cytoskeleton, growth factors and extracellular matrix protein genes modulated by overexpression of constitutively active Rho (RhoV14) in HUVEC.*

Genes regulated in HUVEC by overexpression of active Rac (RacV12) as determined by microarray analysis			
Description	**Common Name**	**Genebank**	**Fold expression**
Homing molecules			
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	CD61, GP3A, GPIIIa	M35999	2.125
Growth factors			
Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	AF153687	2.715
Plasminogen activator, urokinase receptor	PLAUR	AY029180	2.245
Myeloid leukemia factor 2	MLF2	NM_005439	2.055
Fibroblast growth factor 2 (basic)	BFGF, FGFB, HBGH-2	NM_002006	-2.04
Small GTPases associated proteins			
Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	EN-7	NM_002872	3.971
Rho GDP dissociation inhibitor (GDI) alpha	GDIA1	D13989	3.22
G protein beta subunit-like	GBL	NM_022372	2.033
Cell division cycle 42 (GTP-binding protein, 25kD)	CDC42	NM_015858	-9.386
RAB5C, member RAS oncogene family	RAB5C	NM_004583	-3.333
Membrane associated protein			
Integral membrane protein 1	TMC	NM_002219	2.34
Cytoskeleton associated proteins			
Capping protein (actin filament) muscle Z-line, beta	CAPB, CAPZ, CAPPB	NM_004930	2.639
Talin 1	TLN, KIAA1027	NM_006289	2.556
Plasminogen activator, urokinase	URK	K03226	2.547
Catenin (cadherin-associated protein), beta 1 (88kD)	CTNNB1	NM_001904	2.343
beta-actin; Human mRNA for beta-actin.	ACTB	X00351	2.037
Catenin, beta-interacting protein 1	ICAT	NM_020248	-2.278
Transcription factors			
BCL2-like 1	BCL2L1	NM_001191	2.28
Signal transducer and activator of transcription 2, 113kD	STAT113	NM_005419	2.33

Table 4.5 Genes regulated by RacV12 (c.a.) in HUVEC. *The table represents the homing, cytoskeleton, growth factors and extracellular matrix protein genes modulated by overexpression of constitutively active Rac (RacV12) in HUVEC.*

4.4.6 Modulation of E-selectin dependent HPC rolling and adhesion in endothelial cells

To investigate further if the Rho GTPase mediated change in gene expression of HUVEC as revealed by microarray experiments plays a role in the adhesion or migration of HPC, rolling and adhesion of HPC to the Rho modulated HUVEC was determined under shear stress conditions in a flow chamber assay. Retrovirally transduced HUVEC with either control vector, constitutively active Rac (RacV12) or constitutively active Rho (RhoV14) was coated on a glass slide and mounted on a flow chamber system. HUVEC were treated with TNF-1α for 4 hours to induce the expression of E- Selectin (Doukas and Pober 1990).



4.29 Modulation of E-selectin dependent HPC rolling and adhesion in endothelial cells. *Rolling and adhesion of HPC on the Rho GTPase modulated HUVEC was determined in a flow chamber assay. HUVEC transduced with control vector, constitutively active Rac (RacV12) or constitutively active Rho (RhoV14) was used. HUVEC were stimulated 4 hours prior to the experiment with TNF-1α (10 ng/ml). HPC were flushed over the HUVEC layer at a shear stress of 0.1dyn/cm^2 for 10 minutes and the number of adhered HPC was determined. The shear stress was increased to 2 dyn/cm^2 in the next 10 minutes and the number of cells adhered to the endothelial cell (HUVEC) layer was determined. The results are expressed as number of cells adhered to the endothelial cell layer (Means ±SD, n=3). Statistical significance between the groups are represented by asterisks ** $p \leq 0.006$, *** $p \leq 0.0002$.*

HPC were flushed over the endothelial cell layer at a constant shear stress and the adhered cells were counted. A significant increase in the binding of HPC to active Rho (RhoV14) expressing HUVEC was found (Fig. 4.29). The increased adherence of HPC to Rho activated HUVEC, could be attributed to E-selectin mediated binding since blocking of E-selectin by anti-E-selectin antibody was found to cause suppression in the binding of HPC to HUVEC.

5.0 DISCUSSION

In medicine, autologous and allogeneic stem cell transplantation of hematopoietic stem cells is performed to rescue the hematopoietic system in patients after myelo-ablative chemo- or radiotherapy. A specific requirement for successful long-term engraftment in stem cell transplantation is the migration of transplanted cells from the peripheral blood to the bone marrow in a process termed “homing”. In spite of the importance of these mechanisms, the signalling processes involved in homing of stem cells are not clearly understood. Better understanding of the physiological regulation of stem cell homing is however expected to aid in improving the efficiency of stem cell engraftment. Therefore, the present study investigated the role of Rho GTPases in the migration of hematopoietic and mesenchymal progenitor/stem cells. Furthermore, an approach was taken to analyse the involvement of Rho GTPases in endothelial cells, and its relevance for homing of stem cells.

Induction of chemotactic migration of MSC by plasma

Migration of stem cells to bone marrow or to sites of tissue injury is a necessary key feature for tissue reconstitution. Earlier studies with chemotactic experiments have identified the essential role of the SDF-1α/CXCR4 receptor/ligand pair for induction of migration in HSC (Aiuti*, et al* 1997). In MSC, CXCR4 has been reported to be expressed to a far lesser degree (Wynn*, et al* 2004), which may explain the absence of a clear response of MSC towards SDF-1α observed in this study. However, Wynn et al. found signs of MSC migration towards SDF-1α, which could be attributed to the low level of CXCR4 expressed on the surface of MSC isolates used in the study (Wynn*, et al* 2004). Thus, it was necessary to identify alternative chemotactic substances to study MSC migration under *in vitro* conditions.

It was considered that MSC after leaving the bone marrow would have to be attracted towards the blood circulation by a physiological activity. The present study defines diluted human blood plasma (1%) as a potential chemotactic agent for MSC migration. The fact that diluted human plasma is more efficient as chemotactic agent than pure plasma could be attributed to several reasons. Firstly, the static *in vitro* conditions differ from *in vivo* conditions where there is an involvement of shear stress and the presence of other cells such as erythrocytes, which interact with the stem cells circulating in the peripheral blood to induce their migration and homing. On the other hand, when MSC leave the peripheral

blood to enter the tissues, a plasma concentration gradient between the blood vessels and the tissues is likely to attract MSC towards the tissues. According to the results of this study, human plasma may also contain Rho inhibiting factors that favour the induction of MSC migration. It is important that the individual factors in plasma that influence migration of MSC are deciphered, e.g. by fractionation and analysis of each fraction for their migration inducing potential.

Relevance of methodology used to assess MSC migration

Until now, the literature did not show a methodology which allows induction of MSC migration in transwell systems to a comparable degree as with HSC (Fiedler*, et al* 2004). The present study describes a migration method where absolute numbers of migrated MSC are assessed (Fiedler*, et al* 2004, Ruster*, et al* 2005). This is in contrast to the reports showing only the number of migrated cells per microscopic field (Fiedler*, et al* 2002). The methodology in the present study applies microscopic enumeration of migrated cells on the membrane *in situ* by a two-step staining procedure and the confirmation of the correct transmigrated location of the scored cells. Furthermore, this study shows the technical details to differentiate between chemotactic and chemokinetic migration in MSC transwell assays. Using the above technique, a migration rate as high as 12% during a period of 4 – 6 hours was achieved corresponding to the expected migration efficiency and which is the time period when assessing migration of hematopoietic cells, including HSC (Peled*, et al* 1999, Voermans*, et al* 2001).

Role of extracellular matrix substances in MSC migration

Several reports have demonstrated the role of extracellular matrix (ECM) substances in cell migration during tissue injury or repair (del Pozo*, et al* 1995, Hotchin and Hall 1995, Prockop*, et al* 2000). The question of whether ECM substances might augment MSC migration was addressed in this study by the application of different pre-coating conditions, such as coating with vitronectin, laminin, and fibronectin. The data show that the type of ECM substances influences the induction of chemokinetic and chemotactic migration. Chemokinetic migration, corresponding to an unspecific mobilization of the cells, and true chemotactic migration, i.e. directed migration towards a chemoattractant, have both been determined. Fibronectin or laminin pre-coating have been found to induce both chemokinetic and chemotactic migration, whereas a vitronectin pre-coating favoured the chemotactic migration of MSC. The ability to differentiate between chemotactic and chemokinetic migration brought about by the use of ECM substances should assist the

future discrimination of chemotactic versus chemokinetic factors for the migration of MSC (Ruster, Grace*, et al* 2005). In addition, chemotactic migration of MSC was found to be independent of cell density or concentration of the seeded cells.

Role of Rho GTPases in the regulation of the cytoskeleton and cellular morphology of MSC

Previous studies have reported the involvement of Rho GTPases in the activation of the cytoskeleton in fibroblasts, where Rho induced the formation of stress fibres and focal adhesion complexes. In addition, Rac and Cdc42 have been shown to control the formation of actin ruffles and spikes of the cytoplasmic membrane respectively (Chrzanowska-Wodnicka and Burridge 1996, Kozma*, et al* 1995, Nobes and Hall 1995, Nobes*, et al* 1995, Ridley and Hall 1992). These cytoskeletal changes brought about by Rho GTPases directly relate to the general changes in cell morphology that are associated with migration.

The established specific inhibitor of Rho, C3 transferase (Aktories 1997, Aktories and Hall 1989, Genth*, et al* 1999, Genth*, et al* 2003), was used in this study to down regulate Rho in MSC. The C3 transferase was applied in a cell permeable form, by fusion with the ecto domain of C2 domain of *C. botulinum*, C2I, to allow entry into target cells, C2I-C3. MSC treated with C2I-C3 transferase showed an altered actin organization and finer, shortened actin filaments, which indicating that Rho controls the actin filament organization in MSC. This effect of Rho inhibition on MSC was confirmed after microinjection of MSC with C3 transferase cDNA which also resulted in formation of short and thin actin filaments. Moreover, time-lapse video microscopic pictures of C3 transferase microinjected MSC revealed the emergence of membrane ruffling and lamellipodia like structures in these cells.

Nobes and Hall suggested Rac to be the predominantly responsible molecule controlling cellular actin organization (Nobes and Hall 1995), Also, Rottner et al and Sander et al reported a reciprocal relation of Rho and Rac (Rottner*, et al* 1999, Sander*, et al* 1999). In extension to these previous findings, the current study shows that a selective inhibition of Rho leads to changes in the cytoskeletal structure. However, at this point of time, it cannot be excluded that Rho inhibition itself causes an increase in the level of active Rac in MSC.

Further findings in this study nevertheless indicate that increasing the level of active Rho in MSC by microinjection of RhoV14 cDNA (c.a. Rho) resulted in stress filaments and

focal adhesion-like structures similar to those reported in fibroblasts (Nobes and Hall 1995). Here, the function of Rho in MSC appears similar to that observed in fibroblasts. Moreover, interaction with binding partners of Rho such as guanosine exchange factors(GEFs) and GTPase activating proteins (GAPs) which occur in subcellular compartments may also play a critical role (Boguski and McCormick 1993) in regulating the cytoskeletal organization in MSC.

Rho was reported to have an impact on the formation of focal adhesion complex proteins (Chrzanowska-Wodnicka and Burridge 1996, Nobes and Hall 1995, Nobes*, et al* 1995, Ridley and Hall 1992). Accordingly, the present study shows a decrease in the concentration of the focal adhesion protein, paxillin, in Rho inhibited MSC. This suggests that Rho, in addition to controlling the actin cytoskeleton, is also involved in the regulation of focal adhesion complex substances like paxillin-containing structures in MSC. Focal adhesion complexes contain components of signal transduction (Clark and Brugge 1995). From this, it can be hypothesized that other signalling pathways might be regulated by Rho through signal transduction via focal adhesion complexes, e.g. in neighbouring cells in MSC. Thus, it can be concluded at this point that by controlling activation of actin and paxillin, changes in the activity of Rho result in the altered morphology and migratory behaviour of MSC.

Previous studies have reported that inactivation of Rho leads to clustering, detachment and cell rounding in fibroblasts (Hotchin and Hall 1995). In addition, Aepfelbacher *et al* showed that active Rho acts as a negative regulator of human monocyte spreading, and supports the maintenance of a rounded morphology in those cells (Aepfelbacher*, et al* 1996). Whereas, Vicente-Manzanares et al found in 2002 that RhoA activity induced contraction of the cell cytoskeleton in lymphocytes and caused rounding of the cells (Vicente-Manzanares*, et al* 2002). These previous findings point towards anchoring functions of Rho in fibroblasts, whereas in lymphocytes, Rho activation led to cell rounding. The present study reveals that, hours after inhibition of Rho in MSC by either C3 transferase toxin treatment or by microinjection of C3 transferase cDNA, cell spreading, cell flattening, lamellipodia formation and pseudopodial extension were induced similar to the reports of analogous changes observed in human neuronal cells (Jalink*, et al* 1994) or monocytes (Aepfelbacher*, et al* 1996). In contrast, after stable expression of dominant negative Rho in MSC by lentiviral vectors and analysis several days later, cell clustering, detachment and cell rounding were seen, which is in line with reports in fibroblasts by Hotchin *et al* (Hotchin and Hall 1995, Hotchin and Hall 1996). Therefore, a biphasic role of

Rho-mediated cytoskeletal changes is possible. However, it is also possible that after inhibition of Rho for longer time periods, adaptation events occur in the cells, or that predominance of other Rho GTPases such as Rac determines the phenotypes found. Cancelas, et al speculate that the increased pseudopodial extension observed in Rho inhibited cells might be due to a defect in tail retraction during migration (Cancelas*, et al* 2005). Surprisingly, stable expression of active Rho but not Rac in MSC on the longer term also resulted in cell spreading and flattening. Since the overexpression of active or negative Rho occurs after 48h, adaptive reactions in the cells resulting in altered activation of other Rho GTPases and other signalling chains may have occurred. Although, the morphological changes observed by transient and stable inhibition of Rho in MSC were somewhat dissimilar, the effects of Rho modulation on migration of MSC remain unaltered which suggests that Rho signals via different pathways to alter the morphology and migration.

Effects of Rho inhibition on migration of HPC and MSC

There are several reports about the involvement of Rho GTPases in controlling migration in fibroblasts (Chrzanowska-Wodnicka and Burridge 1996), monocytes (Aepfelbacher*, et al* 1996) and cancer cells (Fritz*, et al* 2002, Jaffe and Hall 2002, Sawada*, et al* 2002). Bacterial toxins from *Clostridial* species were reported to specifically target and inactivate Rho GTPases (Aktories 1997, Aktories and Hall 1989, Genth*, et al* 1999, Genth*, et al* 2003, Waksman 2002) and were used as a tool to study the function of Rho GTPases in migration.

Vicente-Manzanares *et al* reported that simultaneous activation of all the three Rho GTPases (RhoA, Rac1, and Cdc42) exhibited different effects on lymphocyte morphology and inhibited migration (Matozaki*, et al* 2000, Vicente-Manzanares*, et al* 2002). In addition, the present study shows that simultaneous inactivation as well of all the three Rho GTPases (Rho, Rac, and Cdc42) by treatment with Toxin B inhibited SDF-1α and plasma induced migration in HPC and MSC, respectively. However, pre-treatment with the more selective inhibitor, lethal toxin which inhibits Rac and Cdc42 but not Rho, was also found to inhibit the chemotactic migration of HPC and MSC. In contrast, inhibition of Rho alone by C2I-C3 transferase toxin did not affect the chemotactic SDF-1α induced migration of HPC but significantly induced the chemokinetic migration of HPC. Although chemokinetic migration alone does not possibly define chemotaxis, it has been shown to be associated with improved engraftment potential in $CD34^+$ HPC, which showed better

engraftment than their non-migrating counterparts (Peled*, et al* 1999). Analogously, inhibition of Rho also significantly elevated the chemotactic migration response of MSC.

In contrast, stimulation of all the three Rho GTPases by the phospholipids mediators, LPA or S1P resulted in inhibition of chemotactic migration of HPC and MSC. LPA was reported to be a general inducer of Rho GTPases (Rho, Rac and Cdc42) in fibroblasts (Ridley and Hall 1992). LPA synthesized by cells accumulates in extracellular fluids like serum and inflammatory exudates (Goetzl and Lynch 2000), and so the cell response could be of physiological relevance.

The present study shows that activation of Rho GTPases by LPA by pre-incubation of the analysed cells inhibited the SDF-1α and plasma induced migration of HPC and MSC, respectively. Moreover, MSC did not display chemotactic activity towards LPA. This suggests that Rho GTPase activation has an inhibitory effect on MSC migration. Ishii *et al* reported that LPA modulate actin reorganization through activation of Rho (Ishii*, et al* 2000). Furthermore, LPA has been reported to confer chemotactic activation of eosinophils, where it caused a transient actin polymerization (Idzko*, et al* 2004). In addition, LPA and S1P were reported to synergistically function via Rho GTPases (Whetton*, et al* 2003).

In this study, inhibition of Rho thus caused a 10-fold increase in the plasma induced chemotactic migration of MSC towards LPA and S1P. The result of the present study contrasts the report by Sawada et al where LPA induced migration of human ovarian cancer cells was inhibited by Rho inhibition (Sawada*, et al* 2002). This can be explained by different physiological environments where the analysed migration occurs. Transendothelial migration is facilitated by downregulation of Rho and likely predominant Rac activation, whereas interstitial migration which requires closer contacts between neighbouring cells and membrane mediated motility would require predominant activation of Rho.

In addition, the present study describes that rhPDGF-bb and rhHGF induced MSC migration, although less efficient than human plasma. This data corresponds with the findings of Fiedler et al that these factors induce MSC chemotaxis (Fiedler*, et al* 2004, Fiedler*, et al* 2002). The present study however suggests that the migration induction by rhPDGF-bb and rhHGF depends on the activation status of Rho (Eriksson*, et al* 1992, Jiang and Hiscox 1997, Nobes*, et al* 1995, Ridley*, et al* 1992).

Thus, MSC migration is dependent on decreased levels of active Rho. Possibly an antagonism exists between the relative activities of Rho and Rac as described with other cells (Rottner*, et al* 1999, Sander*, et al* 1999). Such antagonism can also explain the increased migration rate of MSC, which was measured, when all the three Rho GTPases (Rho, Rac, and Cdc42) are induced simultaneously by LPA, followed by Rho inhibition with C2I-C3 transferase toxin. It is possible that Rho at least through some part, also functions through inhibiting spontaneous adhesion in MSC, when inducing migration. This would be consistent with the report of Laudanna et al where Rho was found to be involved in integrin mediated cell adhesion in leukocytes (Laudanna*, et al* 1996).

MSC and SDF-1α induced migration

The low or absence of migration of MSC towards SDF-1α and the low surface expression of CXCR4 on MSC reported by Wynn et al correspond to each other (Wynn*, et al* 2004). The latter authors report few migrating MSC towards SDF-1α, which was not seen in this study. This may be due to the fact that these authors did not provide absolute numbers of transmigrated cells and for the data reported it is possible that there was no quantifiable response, such as with 5-10% migrated MSC as in the present study.

SDF-1α has been shown to modify the cytoskeleton in lymphocytes and activate Rho (del Pozo*, et al* 1995). However, RhoA inhibition did not block the SDF-1α induced actin polymerization and formation of filopodia-like structure. This situation is analogous to the findings in MSC migration, showing inhibition of the migratory phenotype. Moreover, SDF-1α is produced by MSC themselves (Bleul and al. 1996) and a gradient of SDF-1α therefore already exists around MSC making them less responsive to the external gradient of SDF-1α in *in vitro* conditions. In addition, migration might only be a minor aspect of SDF-1α function, regulated via Rho, with other major functions left unharmed.

Influence of Rho activation on E-selectin expression

Adhesion of transplanted stem and progenitor cells to the endothelial layer is a pre-requisite for extravasation when they migrate from peripheral blood to enter the tissue (Wojciak-Stothard*, et al* 1999). Several studies reported E-selectin to be involved in initial adhesion, rolling and migration of HPC on bone marrow endothelial cells (Mazo*, et al* 1998, Naiyer*, et al* 1999, Rood*, et al* 2000). Tumour necrosis factor α (TNF-α) was identified to induce E-selectin expression in HUVEC (Nubel*, et al* 2004).

The microarray data of the present study show that overexpression of active Rho (RhoV14), but not Rac, elevated the expression level of E-selectin in HUVEC. Moreover, constitutively active Rho expression in HUVEC increased the adhesion of HPC on HUVEC. Adhesion was chosen as an endpoint since Beekhuizen and van Furth reported that adhesion and spreading are pre-requisites and precede the transendothelial migration (Beekhuizen and van Furth 1993). In addition, Wojciak-Stothard*, et al* reported that adhesion and spreading of monocytes on endothelial cells are dependent on Rho A-regulated clustering of E-selectin, and that Rho inhibition by C3 transferase application inhibited clustering of E-selectin on the endothelial cell surface and Rac was not found to be involved in such regulation (Wojciak-Stothard*, et al* 1999). However, these experiments were not performed under flow conditions like in the present study.

Moreover, the present study proposes in addition to the Wojciak-Stothard et al who identified receptor clustering as the regulated activity, that Rho is also involved in the induction of E-selectin gene expression in HUVEC. These results are consistent with the recent report by Nubel et al that Rho signalling is essential for transcriptional activation of E-selectin gene in HUVEC (Nubel*, et al* 2004). The results of the present study show that the adhesion of HPC on Rho activated HUVEC does not occur at comparable levels as in of TNF-α induced HPC adhesion. However, HPC also showed increased adhesion on TNF-α stimulated HUVEC after Rho activation. This observation points to the situation that TNF-α induced surfacing (externalization) of E-selectin is induced by Rho in HUVEC, which in turn increases HPC adhesion. In sum, the present study suggests that TNF-α acts synergistically with active Rho for increased HPC adhesion on HUVEC through E-selectin and TNF-α signals through the ‘Rho road’ towards E-selectin.

The increased adhesion of HPC to Rho activated HUVEC might be of clinical relevance since E-selectin has been reported to be expressed on small endothelial vessels in hematopoietically active tissues in *in vivo* (Schweitzer*, et al* 1996). Modification of E-selectin expression by Rho-GTPases could therefore be considered as a strategy for modulation of stem cell homing. In addition, up or down regulation of the other homing molecules (CD44, CD102), adhesion and cytoskeletal molecules by constitutive active expression of Rac and Rho suggests that it is not a “yes or no” effect but follows an intertwined regulatory pathway. The deeper investigation of these interactions might reveal further insights on endothelial cell mediated processes in stem cell homing.

Role of Rho GTPases in migration and adhesion of endothelial progenitor cells

Rho GTPases have also been reported to be involved in tumour metastasis, vascularization and angiogenesis (van Nieuw Amerongen and van Hinsbergh 2001). Here, endothelial progenitor cells are thought to give rise and support the growth of tumour associated blood vessels. The present study investigated the function of Rac on embryonic endothelial progenitor cells (eEPC) interacting with adherent endothelial cells under flow. The adhesion and migration behaviour of cells under shear stress conditions in the flow chamber assay simulates an *in vivo* environment. A decreased adhesion of eEPC transduced with active Rac (RacV12) to HUVEC was found. This is in contrast to the results with HPC. This might be due to the relatively decreased Rho level as discussed above, which facilitates migration of adherent cells rather than the adhesion. Complementary to these results employing eEPC transduced with dominant negative Rac showed increased adhesion on HUVEC compared to eEPC transduced with a control vector. This increased adhesion could potentially also be attributable to an increase in cell surface expression of CD44, since this homing molecule was expressed at increased levels in Rac inactivated eEPC. In addition, the cyto-morphological changes induced by active or negative Rac on eEPC are consistent with the results reported by Nobes et al that Rac activation leads to formation of lamellipodia (Nobes and Hall 1995 141).

CONCLUSION

Taken together, migration and homing of stem cells in the tissue is a complex phenomenon dependent on multifactorial influence and that Rho GTPases play an important role in intracellular signalling during various stages of homing, rolling, adhesion, and transmigration of stem cells into the tissues. For a therapeutic model for tissue repair, the results of present study show that by modulating the level of Rho GTPases, homing processes of MSC to bone marrow and other organs could be improved. Several studies reported that transplanted MSC were found circulating or physically trapped in lungs in mice (Anjos-Afonso*, et al* 2004, Barbash*, et al* 2003, Gao*, et al* 2001) and thus engrafted only at low levels in tissues and organs. Rho inhibition might be a useful tool to render MSC more motile and move better through the capillaries such as in the lungs and thus increasing the number of cells arriving at the desired organs such as bone marrow. Thus the elucidation of Rho dependent migration of MSC might promote the development of new therapeutic approaches for bone repair, tissue regeneration and wound healing.

The present study provides insight towards migration and adhesion processes activated by Rho GTPases. The data suggest a model (Fig 5.1) where active Rac is involved in modulating processes such as rolling of stem cells (MSC) in the peripheral blood and the exit of progenitor cells from blood vessels through an endothelial layer. At the sites of tissue injury, induction of endothelial cells by TNF-α activates Rho and results in increased E-selectin expression on endothelial cells pointing to a role of Rho in attachment of stem/progenitor cells to the endothelial layer. Growth factors i.e. PDGF, produced at the injury site, would activate Rac, thereby stem cells are induced to migrate through the endothelium into the tissues. In all these situations, thus a regulated reciprocal balance exists between Rac-induced migration and Rho-induced adhesion which regulate the homing and extravasation of stem and progenitor cells.

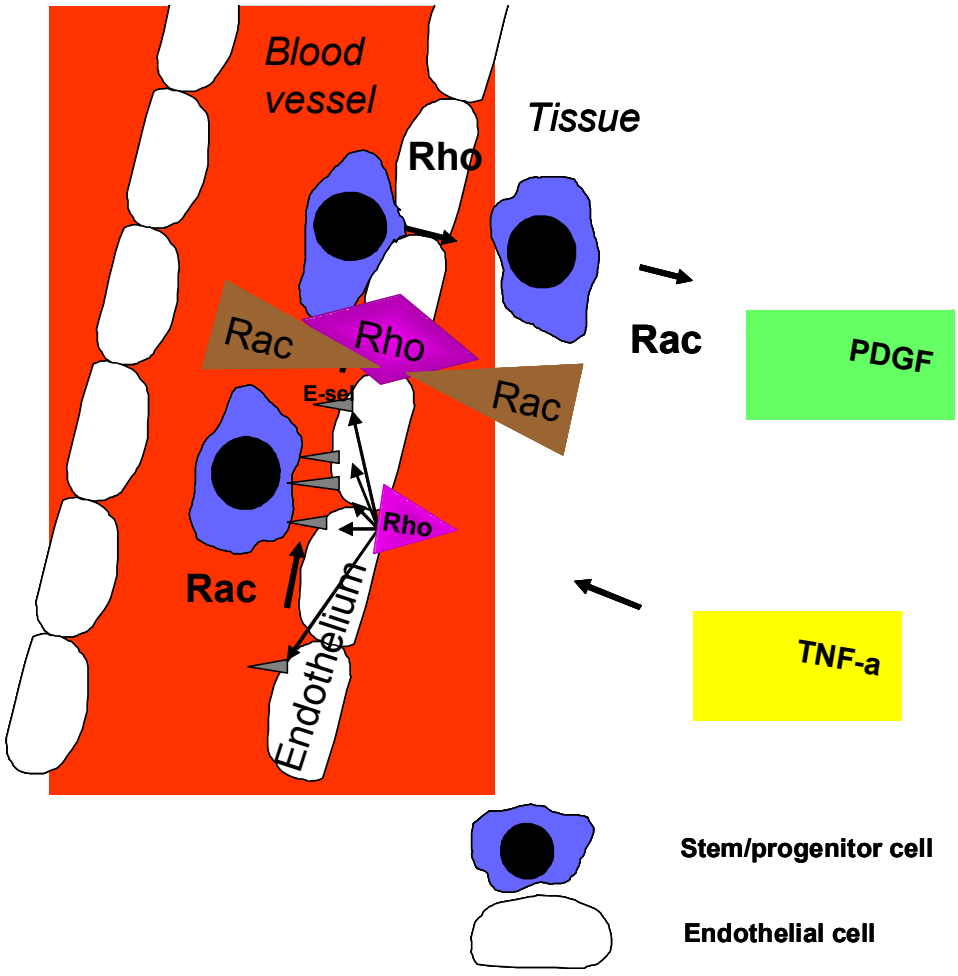


Fig 5.1 Model for the regulation of stem/progenitor cells homing and extravasation by Rac and Rho GTPases. *Triangular type (◄) symbol represent spatial gradient of factors or activity. Arrows indicate directions of blood flow or movement of stem/progenitor cells in the tissue.*

6.0 REFERENCES

Aepfelbacher, M., Essler, M., Huber, E., Czech, A. & Weber, P.C. (1996) Rho is a negative regulator of human monocyte spreading. *J Immunol,* **157,** 5070-5075.

Aiuti, A., Webb, I.J., Bleul, C., Springer, T. & Gutierrez-Ramos, J.C. (1997) The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med,* **185,** 111-120.

Aktories, K. (1997) Bacterial toxins that target Rho proteins. *J Clin Invest,* **99,** 827-829.

Aktories, K. & Hall, A. (1989) Botulinum ADP-ribosyltransferase C3: a new tool to study low molecular weight GTP-binding proteins. *Trends Pharmacol Sci,* **10,** 415-418.

Anjos-Afonso, F., Siapati, E.K. & Bonnet, D. (2004) In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. *J Cell Sci,* **117,** 5655-5664.

Bacigalupo, A. (2004) Mesenchymal stem cells and haematopoietic stem cell transplantation. *Best Pract Res Clin Haematol,* **17,** 387-399.

Barbash, I.M., Chouraqui, P., Baron, J., Feinberg, M.S., Etzion, S., Tessone, A., Miller, L., Guetta, E., Zipori, D., Kedes, L.H., Kloner, R.A. & Leor, J. (2003) Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation,* **108,** 863-868.

Bar-Sagi, D. & Hall, A. (2000) Ras and Rho GTPases: a family reunion. *Cell,* **103,** 227-238.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M. & Peault, B. (1992) Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A,* **89,** 2804-2808.

Beekhuizen, H. & van Furth, R. (1993) Monocyte adherence to human vascular endothelium. *J Leukoc Biol,* **54,** 363-378.

Bevilacqua, M.P. & Nelson, R.M. (1993) Selectins. *J Clin Invest,* **91,** 379-387.

Bishop, A.L. & Hall, A. (2000) Rho GTPases and their effector proteins. *Biochem J,* **348 Pt 2,** 241-255.

Bleul, C. & al., e. (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature,* **382,** 829-733.

Boguski, M.S. & McCormick, F. (1993) Proteins regulating Ras and its relatives. *Nature,* **366,** 643-654.

Campagnoli, C., Roberts, I.A., Kumar, S., Bennett, P.R., Bellantuono, I. & Fisk, N.M. (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood,* **98,** 2396-2402.

Cancedda, R., Bianchi, G., Derubeis, A. & Quarto, R. (2003) Cell therapy for bone disease: a review of current status. *Stem Cells,* **21,** 610-619.

Cancelas, J.A., Lee, A.W., Prabhakar, R., Stringer, K.F., Zheng, Y. & Williams, D.A. (2005) Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. *Nat Med,* **11,** 886-891.

Caplan, A.I. & Bruder, S.P. (2001) Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med,* **7,** 259-264.

Chrzanowska-Wodnicka, M. & Burridge, K. (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol,* **133,** 1403-1415.

Clark, E.A. & Brugge, J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science,* **268,** 233-239.

Cleverley, S.C., Costello, P.S., Henning, S.W. & Cantrell, D.A. (2000) Loss of Rho function in the thymus is accompanied by the development of thymic lymphoma. *Oncogene,* **19,** 13-20.

del Pozo, M.A., Sanchez-Mateos, P., Nieto, M. & Sanchez-Madrid, F. (1995) Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. *J Cell Biol,* **131,** 495-508.

Devine, S.M., Cobbs, C., Jennings, M., Bartholomew, A. & Hoffman, R. (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood,* **101,** 2999-3001.

Dexter, T.M., Allen, T.D., and L.G. Lajtha. (1977) Conditions controlling the proliferation of hematopoietic cells in vitro. *J. Cell. Physiol.,* **91,** 335-344.

Doukas, J. & Pober, J.S. (1990) IFN-gamma enhances endothelial activation induced by tumor necrosis factor but not IL-1. *J Immunol,* **145,** 1727-1733.

DuBridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H. & Calos, M.P. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol,* **7,** 379-387.

Eaves, A.C., and C.J. Eaves (1988) Maintenance and proliferation control of primitive hemopoietic progenitors in long-term cultures of human marrow cells. *Blood Cells,* **14,** 355-368.

Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.H. & Claesson-Welsh, L. (1992) PDGF alpha- and beta-receptors activate unique and common signal transduction pathways. *Embo J,* **11,** 543-550.

Feig, L.A. (1999) Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. *Nat Cell Biol,* **1,** E25-27.

Feig, L.A. & Cooper, G.M. (1988) Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins. *Mol Cell Biol,* **8,** 2472-2478.

Fiedler, J., Etzel, N. & Brenner, R.E. (2004) To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. *J Cell Biochem,* **93,** 990-998.

Fiedler, J., Roderer, G., Gunther, K.P. & Brenner, R.E. (2002) BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. *J Cell Biochem,* **87,** 305-312.

Freeman, J.L., Abo, A. & Lambeth, J.D. (1996) Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. *J Biol Chem,* **271,** 19794-19801.

Frenette, P.S., Subbarao, S., Mazo, I.B., von Andrian, U.H. & Wagner, D.D. (1998) Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc Natl Acad Sci U S A,* **95,** 14423-14428.

Fritz, G., Brachetti, C., Bahlmann, F., Schmidt, M. & Kaina, B. (2002) Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *Br J Cancer,* **87,** 635-644.

Fukata, M., Nakagawa, M. & Kaibuchi, K. (2003) Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr Opin Cell Biol,* **15,** 590-597.

Gao, J., Dennis, J.E., Muzic, R.F., Lundberg, M. & Caplan, A.I. (2001) The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs,* **169,** 12-20.

Gardiner, E.M., Pestonjamasp, K.N., Bohl, B.P., Chamberlain, C., Hahn, K.M. & Bokoch, G.M. (2002) Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. *Curr Biol,* **12,** 2029-2034.

Genth, H., Aktories, K. & Just, I. (1999) Monoglucosylation of RhoA at threonine 37 blocks cytosol-membrane cycling. *J Biol Chem,* **274,** 29050-29056.

Genth, H., Gerhard, R., Maeda, A., Amano, M., Kaibuchi, K., Aktories, K. & Just, I. (2003) Entrapment of Rho ADP-ribosylated by Clostridium botulinum C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex. *J Biol Chem,* **278,** 28523-28527.

Goetzl, E.J. & Lynch, K.R. (2000) Preface: the omnific lysophospholipid growth factors. *Ann N Y Acad Sci,* **905,** xi-xiv.

Goldman, L.A., Cutrone, E.C., Kotenko, S.V., Krause, C.D. & Langer, J.A. (1996) Modifications of vectors pEF-BOS, pcDNA1 and pcDNA3 result in improved convenience and expression. *Biotechniques,* **21,** 1013-1015.

Goodell, M.A., Brose, K., Paradis, G., Mulligan R.C. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med,* **183,** 1797-1806.

Goodell, M.A., Jackson, K.A., Majka, S.M., Mi, T., Wang, H., Pocius, J., Hartley, C.J., Majesky, M.W., Entman, M.L., Michael, L.H., Hirschi, K.K. (2001) Stem cell plasticity in muscle and bone marrow. *Ann N Y Acad Sci.,* **938,** 208-218.

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

Gu, Y., Filippi, M.D., Cancelas, J.A., Siefring, J.E., Williams, E.P., Jasti, A.C., Harris, C.E., Lee, A.W., Prabhakar, R., Atkinson, S.J., Kwiatkowski, D.J. & Williams, D.A. (2003) Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science,* **302,** 445-449.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science,* **279,** 509-514.

Hatzopoulos, A.K., Folkman, J., Vasile, E., Eiselen, G.K. & Rosenberg, R.D. (1998) Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development,* **125,** 1457-1468.

Hotchin, N.A. & Hall, A. (1995) The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J Cell Biol,* **131,** 1857-1865.

Hotchin, N.A. & Hall, A. (1996) Regulation of the actin cytoskeleton, integrins and cell growth by the Rho family of small GTPases. *Cancer Surv,* **27,** 311-322.

Idzko, M., Laut, M., Panther, E., Sorichter, S., Durk, T., Fluhr, J.W., Herouy, Y., Mockenhaupt, M., Myrtek, D., Elsner, P. & Norgauer, J. (2004) Lysophosphatidic acid induces chemotaxis, oxygen radical production, CD11b up-regulation, Ca2+ mobilization, and actin reorganization in human eosinophils via pertussis toxin-sensitive G proteins. *J Immunol,* **172,** 4480-4485.

Ishii, I., Contos, J.J., Fukushima, N. & Chun, J. (2000) Functional comparisons of the lysophosphatidic acid receptors, LP(A1)/VZG-1/EDG-2, LP(A2)/EDG-4, and LP(A3)/EDG-7 in neuronal cell lines using a retrovirus expression system. *Mol Pharmacol,* **58,** 895-902.

Jaffe, A.B. & Hall, A. (2002) Rho GTPases in transformation and metastasis. *Adv Cancer Res,* **84,** 57-80.

Jiang, W.G. & Hiscox, S. (1997) Hepatocyte growth factor/scatter factor, a cytokine playing multiple and converse roles. *Histol Histopathol,* **12,** 537-555.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A. & Verfaillie, C.M. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature,* **418,** 41-49.

Jou, T.S. & Nelson, W.J. (1998) Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J Cell Biol,* **142,** 85-100.

Kiyono, M., Satoh, T. & Kaziro, Y. (1999) G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm). *Proc Natl Acad Sci U S A,* **96,** 4826-4831.

Kjoller, L. & Hall, A. (1999) Signaling to Rho GTPases. *Exp Cell Res,* **253,** 166-179.

Kozma, R., Ahmed, S., Best, A. & Lim, L. (1995) The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol,* **15,** 1942-1952.

Krause, D.S., Theise, N.D., Collector, M.I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., Sharkis, S.J. (2001) Multi-organ, multi lineage engraftment by a single bone marrow derived stem cell. *Cell,* **105,** 369-377.

LaBarge, M.A. & Blau, H.M. (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell,* **111,** 589-601.

Laudanna, C., Campbell, J.J. & Butcher, E.C. (1996) Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science,* **271,** 981-983.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. & McKay, R. (2001) Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science,* **292,** 1389-1394.

Mackay, D.J. & Hall, A. (1998) Rho GTPases. *J Biol Chem,* **273,** 20685-20688.

Majka, S.M., Jackson, K.A., Kienstra, K.A., Majesky, M.W., Goodell, M.A. & Hirschi, K.K. (2003) Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. *J Clin Invest,* **111,** 71-79.

Malliri, A. & Collard, J.G. (2003) Role of Rho-family proteins in cell adhesion and cancer. *Curr Opin Cell Biol,* **15,** 583-589.

Martin, M.J., Muotri, A., Gage, F. & Varki, A. (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med,* **11,** 228-232.

Matozaki, T., Nakanishi, H. & Takai, Y. (2000) Small G-protein networks: their crosstalk and signal cascades. *Cell Signal,* **12,** 515-524.

Mazo, I.B., Gutierrez-Ramos, J.C., Frenette, P.S., Hynes, R.O., Wagner, D.D. & von Andrian, U.H. (1998) Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *J Exp Med,* **188,** 465-474.

Metcalf, D. (1984) *The hematopoietic colony-stimulating factors.*

Metcalf, D. (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature,* **339,** 27-30.

Michaelson, D., Silletti, J., Murphy, G., D'Eustachio, P., Rush, M. & Philips, M.R. (2001) Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol,* **152,** 111-126.

Moolenaar, W.H. (1999) Bioactive lysophospholipids and their G protein-coupled receptors. *Exp Cell Res,* **253,** 230-238.

Moolenaar, W.H. (2000) Development of our current understanding of bioactive lysophospholipids. *Ann N Y Acad Sci,* **905,** 1-10.

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

Naiyer, A.J., Jo, D.Y., Ahn, J., Mohle, R., Peichev, M., Lam, G., Silverstein, R.L., Moore, M.A. & Rafii, S. (1999) Stromal derived factor-1-induced chemokinesis of cord blood CD34(+) cells (long-term culture-initiating cells) through endothelial cells is mediated by E-selectin. *Blood,* **94,** 4011-4019.

Nimnual, A.S., Taylor, L.J. & Bar-Sagi, D. (2003) Redox-dependent downregulation of Rho by Rac. *Nat Cell Biol,* **5,** 236-241.

Nobes, C.D. & Hall, A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell,* **81,** 53-62.

Nobes, C.D., Hawkins, P., Stephens, L. & Hall, A. (1995) Activation of the small GTP-binding proteins rho and rac by growth factor receptors. *J Cell Sci,* **108 (Pt 1),** 225-233.

Nolan Retroviral transduction: Second generation helper free Phoenix cells. http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html.

Nubel, T., Dippold, W., Kleinert, H., Kaina, B. & Fritz, G. (2004) Lovastatin inhibits Rho-regulated expression of E-selectin by TNFalpha and attenuates tumor cell adhesion. *Faseb J,* **18,** 140-142.

Oostendorp, R., Ghaffari, S. & Eaves, C. (2000) Kinetics of in vivo homing and recruitment into cycle of hematopoietic cells are organ-specific but CD44-independent. *Bone Marrow Transplant,* **26,** 559-566.

Orkin, S.H. & Morrison, S.J. (2002) Stem-cell competition. *Nature,* **418,** 25-27.

Osada, S., Izawa, M., Koyama, T., Hirai, S. & Ohno, S. (1997) A domain containing the Cdc42/Rac interactive binding (CRIB) region of p65PAK inhibits transcriptional activation and cell transformation mediated by the Ras-Rac pathway. *FEBS Lett,* **404,** 227-233.

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

Passegue, E., Jamieson, C.H., Ailles, L.E. & Weissman, I.L. (2003) Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci U S A,* **100 Suppl 1,** 11842-11849.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D. & Lapidot, T. (1999) Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science,* **283,** 845-848.

Pereira, R.F., O'Hara, M.D., Laptev, A.V., Halford, K.W., Pollard, M.D., Class, R., Simon, D., Livezey, K. & Prockop, D.J. (1998) Marrow stromal cells as a source of

progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci U S A,* **95,** 1142-1147.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. & Marshak, D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science,* **284,** 143-147.

Pouyani, T. & Seed, B. (1995) PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell,* **83,** 333-343.

Prockop, D.J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science,* **276,** 71-74.

Prockop, D.J., Azizi, S.A., Colter, D., Digirolamo, C., Kopen, G. & Phinney, D.G. (2000) Potential use of stem cells from bone marrow to repair the extracellular matrix and the central nervous system. *Biochem Soc Trans,* **28,** 341-345.

Qiu, R.G., Chen, J., McCormick, F. & Symons, M. (1995) A role for Rho in Ras transformation. *Proc Natl Acad Sci U S A,* **92,** 11781-11785.

Quesenberry, P.J. & Becker, P.S. (1998) Stem cell homing: rolling, crawling, and nesting. *Proc Natl Acad Sci U S A,* **95,** 15155-15157.

Quesenberry, P.J., Stewart, F.M., Zhong, S., Habibian, H., McAuliffe, C., Reilly, J., Carlson, J., Dooner, M., Nilsson, S., Peters, S., Stein, G., Stein, J., Emmons, R., Benoit, B., Bertoncello, I., Becker, P. (1999) Lymphohematopoietic stem cell engraftment. *Ann N Y Acad Sci.,* **872,** 40-47.

Raftopoulou, M. & Hall, A. (2004) Cell migration: Rho GTPases lead the way. *Dev Biol,* **265,** 23-32.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L. & Verfaillie, C.M. (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood,* **98,** 2615-2625.

Ridley, A.J. & Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell,* **70,** 389-399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. & Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell,* **70,** 401-410.

Roberts, A.W., Kim, C., Zhen, L., Lowe, J.B., Kapur, R., Petryniak, B., Spaetti, A., Pollock, J.D., Borneo, J.B., Bradford, G.B., Atkinson, S.J., Dinauer, M.C. & Williams, D.A. (1999) Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity,* **10,** 183-196.

Rood, P.M., Dercksen, M.W., Cazemier, H., Kerst, J.M., Von dem Borne, A.E., Gerritsen, W.R. & van der Schoot, C.E. (2000) E-selectin and very late activation antigen-4 mediate adhesion of hematopoietic progenitor cells to bone marrow endothelium. *Ann Hematol,* **79,** 477-484.

Rossman, K.L., Der, C.J. & Sondek, J. (2005) GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol,* **6,** 167-180.

Rottner, K., Hall, A. & Small, J.V. (1999) Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr Biol,* **9,** 640-648.

Ruster, B., Grace, B., Seitz, O., Seifried, E. & Henschler, R. (2005) Induction and detection of human mesenchymal stem cell migration in the 48-well reusable transwell assay. *Stem Cells Dev,* **14,** 231-235.

Sahai, E. & Marshall, C.J. (2002) RHO-GTPases and cancer. *Nat Rev Cancer,* **2,** 133-142.

Saltiel, A.R. (2003) Muscle or fat? Rho bridges the GAP. *Cell,* **113,** 144-145.

Sander, E.E., ten Klooster, J.P., van Delft, S., van der Kammen, R.A. & Collard, J.G. (1999) Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J Cell Biol,* **147,** 1009-1022.

Sawada, K., Morishige, K., Tahara, M., Kawagishi, R., Ikebuchi, Y., Tasaka, K. & Murata, Y. (2002) Alendronate inhibits lysophosphatidic acid-induced migration of human ovarian cancer cells by attenuating the activation of rho. *Cancer Res,* **62,** 6015-6020.

Schweitzer, K.M., Drager, A.M., van der Valk, P., Thijsen, S.F., Zevenbergen, A., Theijsmeijer, A.P., van der Schoot, C.E. & Langenhuijsen, M.M. (1996) Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. *Am J Pathol,* **148,** 165-175.

Spangrude, G.J., Heimfeld, S. & Weissman, I.L. (1988) Purification and characterization of mouse hematopoietic stem cells. *Science,* **241,** 58-62.

Spooncer, E., Boettiger, D. & Dexter, T.M. (1984) Continuous in vitro generation of multipotential stem cell clones from src-infected cultures. *Nature,* **310,** 228-230.

Spooncer, E., Heyworth, C.M., Dunn, A. & Dexter, T.M. (1986) Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. *Differentiation,* **31,** 111-118.

Springer, T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell,* **76,** 301-314.

Strobel, E.S., Mobest, D., von Kleist, S., Dangel, M., Ries, S., Mertelsmann, R. & Henschler, R. (1997) Adhesion and migration are differentially regulated in hematopoietic progenitor cells by cytokines and extracellular matrix. *Blood,* **90,** 3524-3532.

Szilvassy, S.J., Lansdorp, P. M., Humphries, R. K., Eaves, A. C., Eaves, C. J. (1989) Isolation in a single step of a highly enriched murine hematopoietic stem cell population with competitive long-term repopulating ability. *Blood,* **74,** 930-939.

Taupin, P. & Gage, F.H. (2002) Adult neurogenesis and neural stem cells of the central nervous system in mammals. *J Neurosci Res,* **69,** 745-749.

Texeido, J., Hemler M.E., Greenberger J.S., Anklesaria P.. . . . : (1992) Role of beta1 and beta2 integrins in the adhesion of human CD34hi stem cells to bone marrow stroma. *J. Clin. Invest,* **90,** 358-367.

Thomas, E.D. (1999a) Bone marrow transplantation: a review. *Semin Hematol.,* **36,** 95-103.

Thomas, E.D. (1999b) A history of haemopoietic cell transplantation. *Br J Haematol,* **105,** 330-339.

Thomas, E.D., Storb, R. (1970) Technique for human marrow grafting. *Blood.,* **36,** 507-515.

Thomas, E.D., Storb, R., Clift, R.A., Fefer, M. Johnson F.L., Neiman, P.F., Lerner, K.G., Glucksberg, H., Buckner, C.D. (1975) Bone marrow transplantation. *N. Engl. J. Med.,* **292,** 832.

Tuan, R.S., Boland, G. & Tuli, R. (2003) Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther,* **5,** 32-45.

Uchida, N., Aguila, H. L., Fleming, W. H., Jerabek, L. Weissman, I. L. (1994) Rapid and sustained hematopoietic recovery in lethally irradiated mice transplanted with purified Thy-1.1lo Lin-Sca-1+ hematopoietic stem cells. *Blood,* **83,** 3758-3779.

Uchida, N., Weissman, I. L. (1992) Searching for hematopoietic stem cells: Evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *Journal of Experimental Medicine,* **175,** 175-184.

van Bekkum, D. (1978) The rhesus monkey as a preclinical model for bone marrow transplantation. *Transplant Proc.,* **10,** 105-111.

van Buul, J.D., Voermans, C., van den Berg, V., Anthony, E.C., Mul, F.P., van Wetering, S., van der Schoot, C.E. & Hordijk, P.L. (2002) Migration of human hematopoietic progenitor cells across bone marrow endothelium is regulated by vascular endothelial cadherin. *J Immunol,* **168,** 588-596.

van Hennik, P.B., de Koning, A.E., Ploemacher, R.E. (1999) Seeding efficiency of primitive human hematopoietic cells in nonobese diabetic/severe combined immune deficiency mice: implications for stem cell frequency assessment. *Blood,* **94,** 3055-3061.

van Nieuw Amerongen, G.P. & van Hinsbergh, V.W. (2001) Cytoskeletal effects of rho-like small guanine nucleotide-binding proteins in the vascular system. *Arterioscler Thromb Vasc Biol,* **21,** 300-311.

Verfaillie, C.M., Benis, A., Iida, J., McGlave, P.B. & McCarthy, J.B. (1994) Adhesion of committed human hematopoietic progenitors to synthetic peptides from the C-terminal heparin-binding domain of fibronectin: cooperation between the integrin alpha 4 beta 1 and the CD44 adhesion receptor. *Blood,* **84,** 1802-1811.

Vicente-Manzanares, M., Cabrero, J.R., Rey, M., Perez-Martinez, M., Ursa, A., Itoh, K. & Sanchez-Madrid, F. (2002) A role for the Rho-p160 Rho coiled-coil kinase axis in the chemokine stromal cell-derived factor-1alpha-induced lymphocyte actomyosin and microtubular organization and chemotaxis. *J Immunol,* **168,** 400-410.

Voermans, C., Kooi, M.L., Rodenhuis, S., van der Lelie, H., van der Schoot, C.E. & Gerritsen, W.R. (2001) In vitro migratory capacity of CD34+ cells is related to hematopoietic recovery after autologous stem cell transplantation. *Blood,* **97,** 799-804.

Waksman, G. (2002) Hijacking the host cell proteasome. *Dev Cell,* **3,** 763-764.

Webb, D.J. & Horwitz, A.F. (2003) New dimensions in cell migration. *Nat Cell Biol,* **5,** 690-692.

Weiner, O.D., Servant, G., Welch, M.D., Mitchison, T.J., Sedat, J.W. & Bourne, H.R. (1999) Spatial control of actin polymerization during neutrophil chemotaxis. *Nat Cell Biol,* **1,** 75-81.

Weissman, I.L. (2002) Stem cells--scientific, medical, and political issues. *N Engl J Med,* **346,** 1576-1579.

Westwick, J.K., Lee, R.J., Lambert, Q.T., Symons, M., Pestell, R.G., Der, C.J. & Whitehead, I.P. (1998) Transforming potential of Dbl family proteins correlates with transcription from the cyclin D1 promoter but not with activation of Jun NH2-terminal kinase, p38/Mpk2, serum response factor, or c-Jun. *J Biol Chem,* **273,** 16739-16747.

Whetton, A.D., Lu, Y., Pierce, A., Carney, L. & Spooncer, E. (2003) Lysophospholipids synergistically promote primitive hematopoietic cell chemotaxis via a mechanism involving Vav 1. *Blood,* **102,** 2798-2802.

Williams, D.A., Rios, M., Stephens C., Patel V.P. . . : (1991) Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature,* **352,** 438-441.

Wojciak-Stothard, B., Potempa, S., Eichholtz, T. & Ridley, A.J. (2001) Rho and Rac but not Cdc42 regulate endothelial cell permeability. *J Cell Sci,* **114,** 1343-1355.

Wojciak-Stothard, B., Williams, L. & Ridley, A.J. (1999) Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *J Cell Biol,* **145,** 1293-1307.

Woodbury, D., Schwarz, E.J., Prockop, D.J. & Black, I.B. (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res,* **61,** 364-370.

Wynn, R.F., Hart, C.A., Corradi-Perini, C., O'Neill, L., Evans, C.A., Wraith, J.E., Fairbairn, L.J. & Bellantuono, I. (2004) A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood,* **104,** 2643-2645.

Xu, X., Barry, D.C., Settleman, J., Schwartz, M.A. & Bokoch, G.M. (1994) Differing structural requirements for GTPase-activating protein responsiveness and NADPH oxidase activation by Rac. *J Biol Chem,* **269,** 23569-23574.

Yang, F.C., Atkinson, S.J., Gu, Y., Borneo, J.B., Roberts, A.W., Zheng, Y., Pennington, J. & Williams, D.A. (2001) Rac and Cdc42 GTPases control hematopoietic stem cell shape, adhesion, migration, and mobilization. *Proc Natl Acad Sci U S A,* **98,** 5614-5618.

Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P. & Hedrick, M.H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng,* **7,** 211-228.

CURRICULUM VITAE

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EDUCATIONAL QUALIFICATION

October 2002- September 2005	Ph.D.	Department of Stem Cell Biology DRK-Blutspendedienst Frankurt, Germany
June 1997- May 2002	M.Sc (Life sciences) Five year integrated course (Specialization Microbiology)	Bharathidasan University Department of Microbiology School of life sciences, India
June 1995- May 1997	Higher Secondary School	Tamil Nadu State Board Amala Matric Hr. Sec. School, India
June 1990- May 1995	High School	Matriculation Exam Amala Matric Hr. Sec. School, India

RESEARCH AND PROFESSIONAL EXPERIENCE:

October 2005 – present:
Postdoctoral Research Fellow on a project to study the "**Biology of Mesenchymal Stem Cells**" at Haematopoietic Stem Cell Lab, Cancer Research UK, London, United Kingdom.

October 2002 - September 2005:
Ph.D. Dissertation
Ph.D. student on a project entitled "**Role of Rho GTPases in migration of stem and progenitor cells"** at the Institute for Transfusion Medicine and Immune Hematology, DRK-Blutspendedienst, Frankfurt, Germany.

January 2002 - April 2002:
M.Sc. Dissertation
"Molecular Genetic Analysis of Familial Hypertrophy Cardiomyopathy (FHC) in India" under the supervision of Dr.M.D Bashyam, Head, Lab of Molecular Oncology at Centre for DNA Fingerprinting & Diagnostics (CDFD), Hyderabad, India.

June 2001 - July 2001:
Summer Project
"16srRNA Sequence Analysis of Non-culturable Microorganisms" under the supervision of Dr. Tapan Chakrabarti, Microbial type culture collection (MTCC) & Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India.

May 2000 - June 2000:
Summer Project
"Microbial Xylanases and Proteases" under the supervision of Dr. Seeta Laxman, Division of Biochemical Sciences, **National Chemical Laboratory (NCL),** Pune, India.

December 1999 – April 2000:
Short term project ***** **"To study the effect of growth regulators on callus induction in *Momordica charantia*. L"** under the supervision of Dr.M. V. Rao, Plant Tissue Culture Laboratory, Department of Plant Science, Bharathidasan University, Trichy, India.

***** The paper was presented at the National Symposium on RECENT TRENDS IN PLANT SCIENCE RESEARCH, April 2000, University of Kerala, Thiruvananthapuram, India.

July 1998-December 1998:
Worked on a Student Computer Project for "**RFLP** and **Restriction Enzymes Analysis"** in **C** language, Bioinformatics Centre, Bharathidasan University, Trichy, India.

ACHIEVEMENTS

1. Cleared **All India combined entrance examination** for doing Master of Life Science at School of Life Sciences, Bharathidasan University through entrance exam conducted by Department of Biotechnology, comes under Ministry of science and technology, New Delhi, India.
2. Submitted a review on "**Functional Genomics and Proteomics - Emerging Trends** " to Department of Microbiology, Bharathidasan University Research Notes (October 2002).
3. **First Class with distinction** in all years of the study.
4. **Top student** in the class of Microbiology.

PUBLICATIONS:

1. Brigitte Rüster*, **Bithiah Grace***, Oliver Seitz, Erhard Seifried, Reinhard Henschler. (2005) Induction and detection of human mesenchymal stem cell migration in the 48-well reusable transwell assay. *Stem Cells Dev,* **14,** 231-235. Transwell Assay, *Stem Cells and Development*. Apr 2005, Vol. 14, No. 2: 231-235.

2. Stephan Göttig, Dietrich Möbest, Brigitte Ruester, **Bithiah Grace**, Sylvia Winter, Erhard Seifred, Jens Gille, Thomas Wieland, Reinhard Henschler. (2006) Role of the monomeric GTPase Rho in hematopoietic progenitor cell migration and transplantation. *Eur J Immunol,* **36,** 180-189.

3. **Bithiah Grace**, Lars Dressel, Brigitte Rüster, Oliver Seitz, Erhard Seifried, Martin Ruthardt, Reinhard Henschler: Rho and Rac GTPases Antagonistically Regulate the Migration of Mesenchymal Stem Cells (MSC) Induced by Lysophospholipids or Polypeptide Growth Factors (in process).

* Equal contribution

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Declaration

I hereby declare that, I composed the present study without any unlawful help. Quotations from other authors are indicated as such.

Frankfurt am Main, 20.03.2006 ……..……

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