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Hematopoietic stem cell differentiation and lineage selection control by GADD45G

Die Funktion von GADD45G in der Differenzierung von hämatopoetischen Stammzellen und deren Linienentscheidung

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1. Introduction

1.1 The blood system

Blood has been attributed as symbol of life in many cultures for ancient times. Johann Wolfgang von Goethe stated in his novel Faust I that "Blood is a quite peculiar juice." (Goethe, 1808). Indeed, blood is essential for life in all vertebrates. It transports the required oxygen from the lung to all parts of the body and distributes essential nutrients and minerals. It also removes metabolic waste for the clearing in liver and kidney. The immune defense is permitted by specialized blood cells and factors transported in blood. Hormones and cytokines that circulate through the blood stream enable signaling between distant organs. 55% of the blood volume is liquid, known as serum, which transports the soluble parts (Alberts, Wilson 2008). The remaining volume comprises of hematopoietic cells, also known as the hematocrit, and consists of erythrocytes, thrombocytes and leukocytes. Erythrocytes, so-called "red blood cells", transport oxygen and CO_2 bound to hemoglobin for the oxidative respiration. Thrombocytes or platelets adhere specifically to the endothelial cell lining of damaged blood vessels, where they aid in blood clotting and healing (Alberts, Wilson 2008). Leukocytes, so-called "white blood cells", built up the immune defense of the organism. They are divided in myelocytes and lymphocytes. Myelocytes are cells of the innate immunity, which are the phagocytic macrophages and granulocytes as well as dendritic cells (Immunobiology 2001). Lymphocytes reside in the lymph nodes, where they survey the blood and lymphatic fluids for invading pathogens and transformed cells. The two types of lymphocytes, T cells and B cells provide the majority of the adaptive immune response. T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for antibody-mediated humoral immunity. Both cell types generate specific responses against invading pathogens such as bacteria or viruses, and serve for the long-lasting immune memory. Natural killer (NK) cells are part of the innate immune system and help defending against virally infected or transformed cells (Immunobiology 2001).

Blood is a highly regenerative organ. Since many mature blood cell types are short lived, they have to be constantly replenished by new cells. The life time of circulating erythrocytes in mice is around 40 days, and 2.4×10^8 new erythrocytes are produced every day; this is approx. 1000-fold higher in humans, where more than 10^{11} mature blood cells are produced daily (Metcalf 1989; Wagers et al. 2002). This regeneration of blood cells is called hematopoiesis. Excessive blood loss or an infection rapidly increases the blood cell production making blood a very plastic organ (Orkin, Zon 2008). All the cells of the blood

system derive from a very small number of multipotent hematopoietic stem cells (HSCs), which reside in the bone marrow (BM).

1.1.1 Embryonic hematopoiesis

The first primitive hematopoietic cells occur in the yolk sack through differentiation of so-called “hemangioblasts” at embryonic day E7.5. The primitive hematopoiesis first gives rise to erythrocytes providing tissue oxygenation as the embryo grows, followed by myeloid cells building a primitive immune system (Orkin, Zon 2008). The primitive hematopoietic system is only transient and is rapidly replaced by definitive hematopoiesis. The first definitive HSCs develop de novo from hemogenic endothelial cells of the aortal walls in the aorta-gonad-mesonephros (AGM) region (Muller et al. 1994) and placenta at embryonic day E10.5 (Gekas et al. 2005; Ottersbach, Dzierzak 2005). Definitive HSCs migrate into placenta and fetal liver soon after their generation, where they massively expand and produce progenitors of all lineages for definitive hematopoiesis. They migrate into the spleen and finally into the BM shortly after birth, which is the major site of adult hematopoiesis (Bowie et al. 2007).

1.1.2 The adult hematopoietic differentiation hierarchy

The hematopoietic system is the best understood somatic stem cell system in mammals. Profound knowledge was gained by the fact that the hematopoietic system is not a solid organ, but consists of individually free-floating cells that can easily be accessed by blood withdrawal. Furthermore, the mature blood cell lineages can be distinguished by a variety of morphological and histological features. Blood cells are robust and can survive strong shear forces as they naturally need to squeeze themselves through small blood vessels and endothelial cell layers. This property makes them resistant to harsh procedures like centrifugation and flow cytometry. The surface protein profile (“cluster of differentiation”, CD) of distinct hematopoietic cell populations and lineages has been unraveled, and the combination of antibodies against these surface markers with FACS (fluorescence-activated cell sorting) made the prospective isolation of distinct populations technically possible (Morrison, Weissman 1994; Osawa et al. 1996).

The first evidence for the existence of hematopoietic progenitors, which give rise to mature blood cells, came from experiments in the 1960s, when injected BM cells of donor mice formed myelo-erythroid colonies in irradiated mice (Becker et al. 1963; Till, McCulloch 1961). Development of functional tests for clonogenicity, lineage potential, and self-renewal potential enabled the prospective isolation of distinct hematopoietic stem and progenitor cell (HSPC) populations and the elucidation of their relationship, known as the hierarchy of

hematopoietic differentiation (Figure 1; (Rieger, Schroeder 2012). Constant refinement of the populations led to the complex picture of the hierarchy nowadays (Okada et al. 1992; Morrison, Weissman 1994; Osawa et al. 1996; Kondo et al. 1997; Akashi et al. 2000; Kiel et al. 2005; Inlay et al. 2009) A major achievement was the finding that immature multipotent progenitors express only low levels of the surface markers that are characteristic for mature cells. The progenitors therefore were described as lineage marker negative. Some of them also express stem cell antigen 1 (SCA-1) and CD117 (c-KIT) (Okada et al. 1992; Uchida, Weissman 1992; Morrison, Weissman 1994) and were therefore called LSK (lineage$^-$, SCA-1$^+$, c-KIT$^+$) cells. LSKs comprise only 0.06% of the BM and in-vivo blood reconstitution studies revealed that only one in 30 LSK is a true stem cell (0.002% in BM). There is no HSC activity detected outside the LSK fraction (Figure 1; LSK= left panel, Morrison, Weissman 1994). Additional surface markers for improved prospective HSC identification were subsequently described. The maximum enrichment is one in two true murine HSCs with the currently used marker combination of CD150$^+$, CD48$^-$, CD34^{low}, LSK (Kiel et al. 2005). These markers also helped to dissect functionally distinct HSCs: the long-term (LT-), intermediate-term (IT) and short-term (ST) repopulating HSCs, according to their temporal ability to reconstitute the blood of recipient animals (Yang et al. 2005). Importantly, only LT-HSCs provide life-long self-renewal and multipotent differentiation potential, while the latter ones have limited self-renewal potential and are therefore better called multipotent progenitors (MPPs) (Benveniste et al. 2010). Even single transplanted LT-HSCs are able to reconstitute the blood system of recipient mice life-long (Boitano et al. 2010; Ema et al. 2005). LT-HSCs reside in BM predominantly in a quiescent state, namely in the G0 phase of the cell cycle (Cheshier et al. 1999; Qian et al. 2007). Once activated, LT-HSCs start diving, but later return back to quiescence. The LT-HSC division is called self-renewal, when at least one of the daughter cells remains a stem cell. Once committed for differentiation, the LT-HSCs lose the capacity for self-renewal and become MPPs. More differentiated progenitors have an accelerated proliferation and restricted lineage potential (Wagers et al. 2002; Reya et al. 2001).

The LT-HSC population is yet not as homogeneous as originally anticipated (Muller-Sieburg et al. 2004). Single LT-HSCs exhibited predisposed myeloid- or lymphoid-biased reconstitution patterns (Dykstra et al. 2007). Using the increased efflux of the dye Hoechst 33342 allows sorting of the so-called side population (SP) (Goodell et al. 1996). Separating “side population” lower and higher HSCs allowed prospective isolation of myeloid- and lymphoid- biased LT-HSCs, respectively (Challen et al. 2010). Another factor which allowed

discrimination of those subpopulations was the level of CD150 expression on LT-HSCs (Beerman et al. 2010; Morita et al. 2010), indicating that both sorting schemes purified the same functional HSC fraction. Similar expression of CD135 on the surface is successively increasing as the HSC differentiates and CD135^{high} expression discriminates lymphoid primed MPPs (LMPPs) form the more immature fractions (Adolfsson et al. 2005).

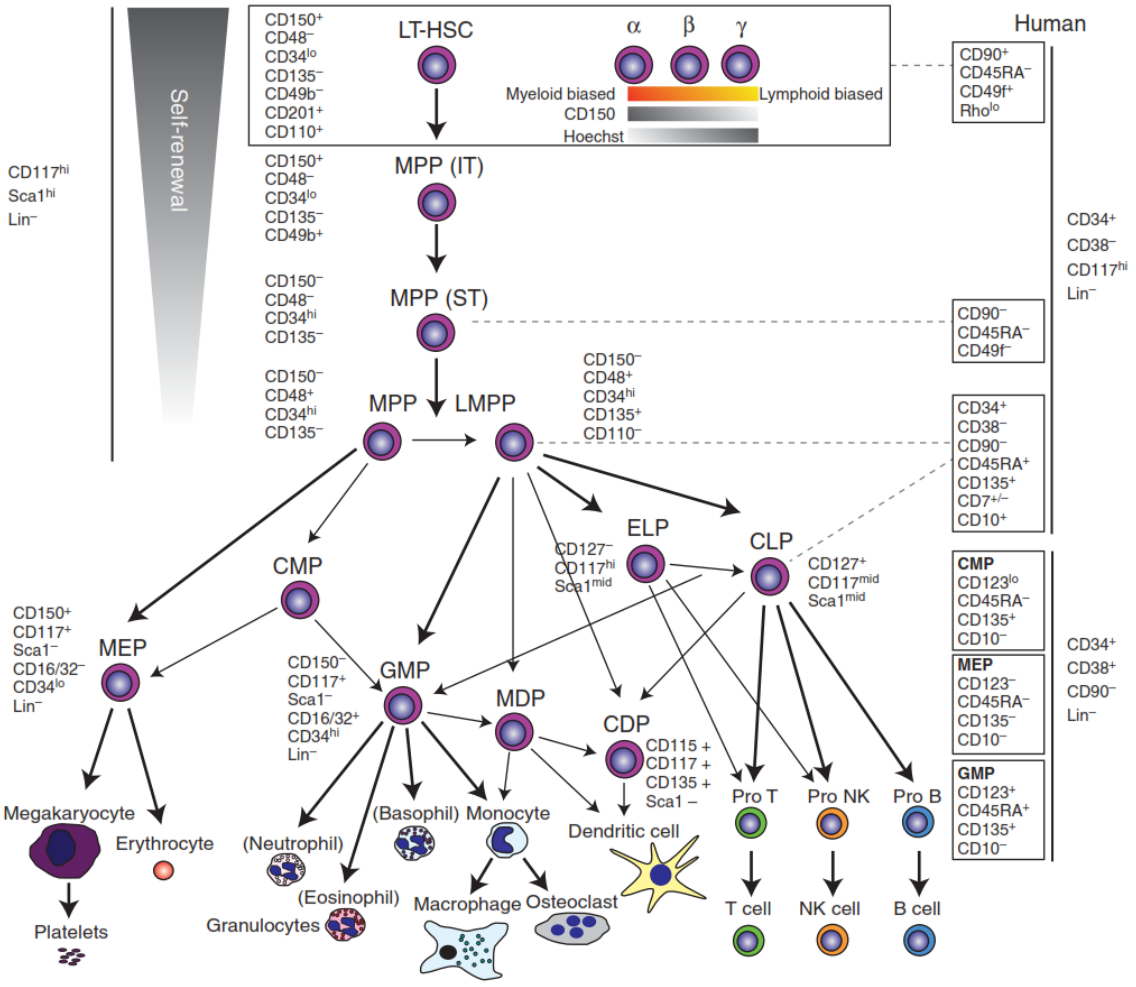

Figure 1: Adult hematopoietic differentiation hierarchy.

Long-term self-renewing HSCs are at the apex of a hierarchy of multiple progenitor cell stages giving rise to all blood cell lineages. Distinct HSPC stages have been described by correlating surface marker expression and functional properties for prospective isolation. The markers for murine hematopoietic progenitors are displayed in the graph; corresponding human HSPC populations with their markers are indicated at the right. HSCs differentiate into all blood cell lineages via long described (bold arrows) and potentially also or alternatively more recently described differentiation routes (thin arrows). It is important to point out that this model is only a simplified representation of current knowledge and will continue to change. HSC, hematopoietic stem cell; MPP, multipotent progenitor; LT-, long-term repopulating; IT-, intermediate-term repopulating; ST-, short-term repopulating; LMPP, lymphoid-primed MPP; ELP, early lymphoid progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte–macrophage progenitor; MEP, megakaryocyte –erythrocyte progenitor; CDP, common dendritic progenitor; MDP, monocyte–dendritic cell progenitor; NK, natural killer cell. Adapted from (Rieger, Schroeder 2012)

Multipotency is gradually lost during differentiation. MPPs develop into megakaryocyte-erythrocyte progenitors (MEPs) or LMPPs, that can only differentiate into common lymphoid progenitors (CLPs) or granulocyte-macrophage progenitors (GMPs) but have lost their megakaryocyte/ erythrocyte potential (Adolfsson et al. 2005). CLPs are restricted to the lymphoid lineages of NK, B, and T cells as well as dendritic cells. GMPs differentiate into cells of the myelomonocytic branch including various kinds of granulocytes, macrophages, osteoclasts, and some dendritic cells. Common myeloid progenitors (CMPs) are restricted to the myeloid lineage and differentiate into GMPs and MEPs. MEPs themselves are restricted to erythrocytes and megakaryocytes, the latter produce platelets. The generation of the mature cells from their committed progenitors also involves many stages of differentiation, which are beyond the scope of this thesis.

1.1.3 Long-term maintenance of HSCs: the importance of the niche

The LT-HSCs reside in the BM in certain areas known as the HSC niche. The niche provides optimal conditions for LT-HSC maintenance and gives the proper signals for LT-HSCs to self-renew or differentiate. This is achieved by a complex interplay of soluble and surface bound ligands as well as extracellular matrix components (Ehninger, Trumpp 2011; Ding et al. 2012). The BM contains at least two HSC niches, which may have different functions in hematopoiesis (Figure 2). It was proposed that the endosteal niche provides the optimal conditions for LT-HSC quiescence, while the perivascular niche allows active LT-HSCs to self-renew and generate MPPs (Ehninger, Trumpp 2011). However, their exact function is still discussed in the field (Morrison, Scadden 2014).

Osteoblasts (OBs), Nestin^{+} mesenchymal stem cells (MSCs) as well as CXCL12 abundant reticular (CAR) cells are considered important players in LT-HSC maintenance as they express many factors necessary for LT-HSC survival and quiescence (Figure 2), such as stem cell factor (SCF), thrombopoietin (TPO), CXCL12, Angiopoietin 1 (ANG-1) and various cell adhesion molecules (CAMs) (Calvi et al. 2003; Zhang et al. 2003; Kiel et al. 2005; Sugiyama et al. 2006; Méndez-Ferrer et al. 2010; Omatsu et al. 2010). Endothelial cells of the sinusoid blood vessels are believed to activate LT-HSCs since they express Notch-ligands, which promotes HSC self-renewal and regeneration after BM injury (Butler et al. 2010; Kobayashi et al. 2010). Osteomacs belong to macrophages and are crucial for HSC maintenance as they deliver survival signals for both OBs and MSCs (Winkler et al. 2010). Loss of osteomacs by granulocyte colony stimulating factor (G-CSF) treatment leads to mobilisation of LT-HSCs into the blood stream (Forsberg et al. 2010; Winkler et al. 2010; Christopher et al. 2011). This mechanism is utilized for mobilisation of HSCs in stem cell

donations for transplantation (Liu et al. 2000). The maintenance of LT-HSC dormancy also depends on Ca^{2+} concentration and mechanical support by the extracellular matrix (Adams et al. 2006), hypoxia (Jang, Sharkis 2007), high rates of glycolysis and inhibition of mitochondrial oxidative phosphorylation reducing the production of reactive oxygen species (ROS) (Simsek et al. 2010). The importance to keep LT-HSCs in a dormant state was shown in a cooperation project analysing that DNA damage is a direct consequence of HSCs entering the cell cycle (Walter et al. 2015). Repeated activation of HSCs from fanconi anemia (FA) mice with 5FU but also physiologic stress led to complete collapse of the hematopoietic system similar to FA patients due to DNA damage induced HSCs loss.

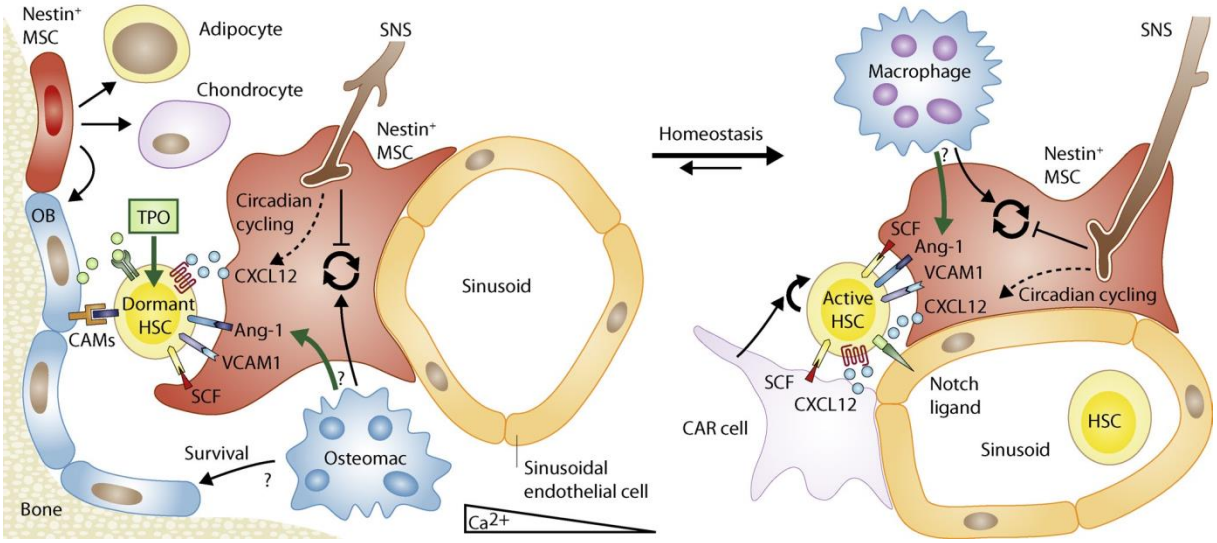

Figure 2: Model illustrating the quiescent endosteal and the active perivascular HSC niche

Deeply quiescent (dormant) HSCs in the endosteal niche are likely in close contact with Oseoblasts (OBs) and nestin+ Mesenchymal stem cells (MSCs), both of which supply HSC maintenance and quiescence factors, including CXCL12, SCF, ANG-1, VCAM-1, and TPO, and cooperate to retain HSCs in their niche. MSCs can generate OBs, adipocytes, and chondrocytes. The perivascular niche is more distant from the endosteum and does not contain OBs, but includes perivascular CAR cells that secrete factors that promote self-renewal of active HSCs, which are significantly more abundant than dormant HSCs. Self-renewal is also stimulated by Notch ligands expressed by sinusoidal endothelial cells. Both niches contain perivascular nestin+ MSCs as a key component. Different subtypes of phagocytes support the maintenance of OBs (osteomacs) and maintenance and proliferation of MSCs (macrophages). They also induce the expression of HSC maintenance factors. The sympathetic nervous system (SNS) inhibits MSC proliferation and induces circadian oscillations of CXCL12expression. ANG-1, Angiopoietin-1; CAMS, cell adhesion molecules. (Ehninger, Trumpp 2011)

Many factors expressed by the niche cells control HSC behavior. Soluble SCF and CXCL12 attract the LT-HSCs to the niche (Nervi et al. 2006). The CXCL12- CXCR4 interaction provides homing of HSCs into the niche after HSC mobilization or after transplantation (Sugiyama et al. 2006). Under steady state, the circadian CXCL12 cycling in

nestin^{+} MSC controls the migration of LT-HSCs within the BM and also mobilisation into the blood stream (Méndez-Ferrer et al. 2008). Stimulation of the receptor C-KIT with SCF enhanced HSCs self-renewal in-vivo and survival of HSCs and other C-KIT expressing progenitors (Keller et al. 1995). Soluble and membrane bound SCF may have different functions. Transgenic mice expressing only soluble SCF are anaemic and die due to a loss of HSPCs (Barker 1994; Thoren et al. 2008). Membrane-bound SCF may be responsible tethering LT-HSC to their niche, together with ANG-1 and other surface proteins such as integrins, cadherins and CAMs (Wang, Wagers 2011).

The function of TPO on HSPCs is controversial (Yoshihara et al. 2007; de Graaf, Metcalf 2011). TPO is widely used in vitro to maintain LT-HSC stemness for at least some days (Cheshier et al. 1999). TPO is critical for HSC maintenance and keep the HSC in an quiescent state (Qian et al. 2007) by activating cyclin-dependent kinase inhibitors (CDKIs) such as p57kip2 (Arai et al. 2009; Yoshihara et al. 2007). Controversially, during severe thrombocytopenia, elevated TPO levels cause a higher HSC activity that replenishes lost cells (Ng et al. 2014). Interestingly, TPO signaling stimulates DNA repair in HSCs by increasing DNA-PK-dependent nonhomologous end-joining preserving the integrity of the LT-HSC genome upon activation (Laval et al. 2013).

These examples indicate that the level of stimulation and also the interplay between the niche factors may influence the decision of an LT-HSC. However, the integration of these signals into the LT-HSCs and the intricate pathways through which the final fate decisions are brought about require further elucidation. Much effort has been spend in mimicking the niche for efficient in vitro HSCs expansion. Several factor combinations and co-culture systems have been used to improve stemness in vitro, at least for some days (Zhang, Lodish 2005; Cook et al. 2013). Also small molecule inhibitors are used in combination with cytokines to allow human HSC expansion (Boitano et al. 2010; Fares et al. 2014). However, so far a robust culture system to maintain or expand LT-HSCs for many days needs further development.

1.1.4 Intrinsic and extrinsic factors regulate HSC self-renewal and differentiation

Hematopoietic cell fate decisions have to be tightly controlled and are governed by a complex interplay of extrinsic and intrinsic factors regulating LT-HSCs quiescence, self-renewal and differentiation as well as the proliferation and differentiation of progenitors.

LT-HSCs intrinsically differ in cell activity and linage potential (Trumpp et al. 2010). These differences can be inherited as shown by serial transplantations (Dykstra et al. 2007; Kent et al. 2009; Morita et al. 2010). Only recently a few genes have been identified that

separate these populations. Myeloid factors such as PU.1 were higher expressed in myeloid-primed LT-HSC, while Ikaros, which plays a pivotal role in B cell development is higher expressed on lymphoid-primed LT-HSC (Challen et al. 2010). Cells with a higher self-renewal capacity expressed higher levels of PRNP, GATA-1 and BMI1 indicating that those factors are important for HSC maintenance (Kent et al. 2009). A variety of transcription factors have been described to be important for HSC development, most were identified by gene knock out studies in mice. Interestingly, many of them including MLL1, RUNX1, TAL1 and NOTCH turned out to be important in fetal HSC development, but dispensable for adult HSCs survival (Orkin, Zon 2008; Rieger, Schroeder 2012). In Cooperation with the group of Dr. Martin Zörnig at the Georg-Speyer-Haus, Frankfurt, Germany we identified the DNA-binding transcriptional regulator FUSE Binding Protein 1 (FUBP1) as an essential factor of fetal and adult HSC self-renewal (Rabenhorst, Thalheimer, Gerlach, *et al.*, submitted manuscript). *Fubp1* deficient mice die in utero with a lethal anemic phenotype at E15.5 due to loss of LT-HSCs. Adult LT-HSCs with a knock-down of *Fubp1* had defects in HSC maintenance and failed in long-term blood reconstitutions. Other factors like WNT or NOTCH signaling exhibited a benefit in HSC expansion in vitro but their essential role in vivo could not be proven (Reya et al. 2003; Benveniste et al. 2014). The *Hox* genes are known intrinsic factors that promote HSC self-renewal. Ectopic expression of MEIS1 and HOXB4, A9 and A10 enhanced HSC numbers in vitro, especially when expressed as fusion constructs with Nucleoporin 98 (NUP98–HOXB4 and NUP98–HOXA10 homeodomain), while still maintaining multi-lineage potential without induction of leukaemia in vivo (Antonchuk et al. 2002; Ohta et al. 2007).

Some factors promote self-renewal of LT-HSCs. EVI-1 supports self-renewal by preventing differentiation (Kataoka et al. 2011) and the loss of the oncogenic transcription factor C-MYB leads to an accelerated and aberrant differentiation with exhaustion of the adult HSC pool (Lieu, Reddy 2009). Other factors are important to induce HSC quiescence. BMI-1, the FOXO family and GFI-1 return the cycling HSCs back into the dormant state by inhibiting the cell cycle (Wilson et al. 2009). The preservation of HSC-specific DNA methylation patterns via de novo methylation activity by DNMT3A and DNMT3B may be crucial for HSC self-renewal (Tadokoro et al. 2007; Challen et al. 2012). The complexity to study HSC self-renewal at a molecular level comes into play as various intrinsic factors influence and regulate each other. Further, the deletion of one factor may be compensated by others.

One well-studied network of transcription factors that regulate lineage choice is the GATA switch. GATA-1 expression forces the down-regulation of the self-renewal promoting

GATA-2, which is mainly expressed in HSCs, causing the differentiation of immature cells towards the megakaryocyte-erythrocyte lineage (Figure 3A) (Orkin, Zon 2008). Similar systems of two opposing transcription factors control the differentiation of progenitors into committed lineages (Figure 3A). GATA-1 induces differentiation into erythrocytes and megakaryocytes, while PU.1 promotes the GM lineages (Arinobu et al. 2007). The balance of EKLF and FLI-1 regulate erythroid versus megakaryocytic lineage choice, while Gfi1 and PU.1 antagonize the outcome of neutrophils versus monocytes. Other examples of direct antagonism of transcription factors are C/EBP and FOG1 for eosinophil or multipotential cell fates, as well as GATA-3 and T-BET for TH1and TH2 cells (Orkin, Zon 2008).

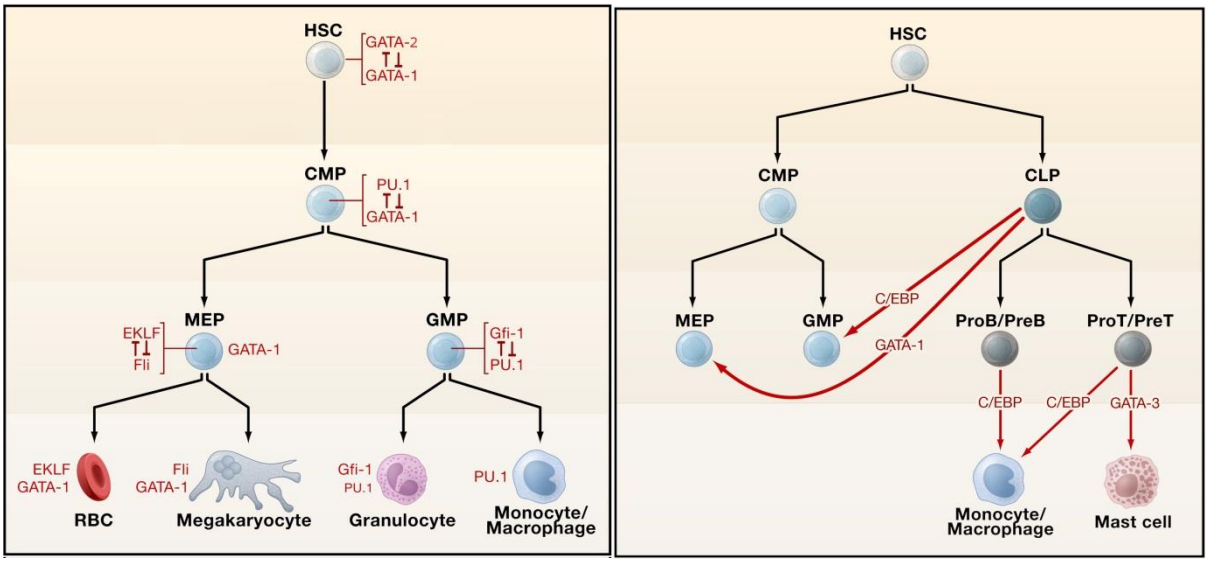

Figure 3: Lineage choice control by transcription factors

A) Examples of antagonistic transcription factors (in red) present in the mature precursors following choice of specific lineage are shown at the bottom in black. B) Reprogramming of hematopoietic lineages by transcription factors GATA-1, C/EBP, or GATA-3 depicted by orange arrows. Adapted from (Orkin, Zon 2008)

GATA-1 is also a prime example for the potency of transcription factors to instruct lineage choice. Ectopic expression of GATA-1 in myeloid progenitors and also in CLPs induces differentiation into MEPs, even across lineage borders (Figure 3B). This reprogramming process is called trans-differentiation (Kulessa et al. 1995; Iwasaki et al. 2003). The most potent trans-differentiation factor in the hematopoietic system is C/EBP alpha. C/EBPA is essential for development of granulocytes and macrophages and acts synergistically with PU.1. Most progenitor cells committed to various lineages, which were forced to express C/EBPA or C/EBPB were reprogrammed into functional monocytes and macrophages (Xie et al. 2004). Also committed pro B and T cells trans-differentiated into functional macrophages, when C/EBPA was introduced (Laiosa et al. 2006). However, there are only spare examples that link cell extrinsic stimulation directly with the expression and activation of intrinsic

signaling molecules and transcription factors inducing distinct cell fate choices (Rieger et al. 2009; Sarrazin et al. 2009; Mossadegh-Keller et al. 2013).

Extrinsic factors are cell-cell-contacts, contacts to the extra cellular matrix and soluble factors such as cytokines, as discussed in chapter 1.1.3. However, the functions of extrinsic factors are complex and still controversial as they sometimes have opposing roles depending on the type and differentiation stage of the cell (Hirano 1998; Metcalf 2003; Metcalf 2008). Cytokines are essential and sufficient for hematopoiesis. They regulate proliferation, survival, differentiation, maturation and function of hematopoietic cells in a stage cell type specific manner (Metcalf 2008). Among the many cytokines the most relevant ones for hematopoiesis are Interleukins (IL) IL2, IL3, IL4, IL5, IL6, IL7, Granulocyte macrophage colony stimulating factor (GM-CSF), G-CSF, M-CSF, TPO, EPO and SCF. To date, it is still unclear, if cytokines can instruct the differentiation of LT-HSCs or uncommitted progenitors into a certain lineage. IL3 and IL6 support proliferation and differentiation in MPPs promoting the delineation into myeloid cells (Zhang et al. 1999a). Lymphoid development is supported by stimulation with FLT-ligand on LMPPs, up-regulating the IL7 receptor alpha chain (Adolfsson et al. 2005). IL7 stimulation can act on LMPP or ELP and promotes lymphoid development into B cell precursors (Nutt, Kee 2007). Notch signaling on lymphoid restricted progenitors promotes differentiation into T cell development (Rothenberg 2007). Several cytokines stimulate the same cells and cause the same lineage outcome. For example, GM-CSF, IL3 and IL6 on GMPs have largely overlapping functions leading to both mature granulocytes and macrophages. In contrast, G-SCF and M-CSF have lineage instructive potential on bipotent GMPs (Rieger et al. 2009). The continuous observation via video-microscopy based cell tracking showed, that M-CSF instructs GMPs to differentiate into monocytic and macrophage cells, whereas G-CSF stimulation instructed the development of granulocytes. A similar finding was reported in *MafB*$^{-/-}$ HSCs, which display a prolonged M-CSF receptor signaling, that instructs uncommitted progenitors to develop into monocytic cells (Sarrazin et al. 2009).

1.2 Growth arrest and DNA damage inducible 45 family

The growth arrest and DNA damage inducible 45 (Gadd45) family consists of three genes, *Gadd45 alpha, beta* and *gamma* in mouse and men. The *Gadd45* genes are evolutionary conserved among species and code for 18kDa, highly acidic proteins. GADD45A was the first of the GADD45 proteins identified as growth arrest and DNA damage-inducible (*Gadd*) gene that was rapidly induced after UV radiation in Chinese hamster ovary (CHO) cells (Zhan et al.

1994). *Gadd45b* was found as myeloid differentiation primary response gene (MyD118) in M1 myeloblastic leukemia cells after treatment with differentiation inducing agents (Selvakumaran et al. 1994). *Gadd45g* was discovered as cytokine response gene 6 (CR6) upon IL2 stimulation in T cells, which resulted in their clonal expansion (Beadling et al. 1993). The proteins have a high similarity in amino acid sequence of 55-57% homology in between the different isoforms, while the identities between the human proteins and their mouse counterparts are 93%–95% (Figure 4B). The GADD45 proteins form a beta- sheet core surrounded by five alpha-helical structures. While the sequence of the beta-sheet core is quite conserved, the N-terminal region and the alpha-helices 1, 4 and 5 (Figure 4A, teal areas) are variable between the three family members presumably exerting the different functions of the GADD45 isoforms.

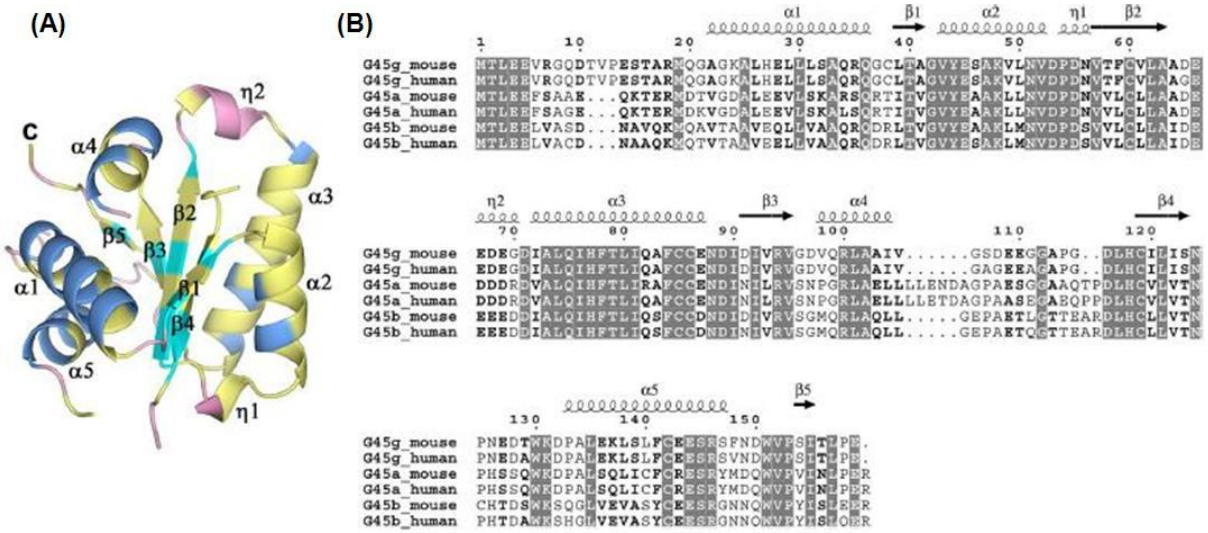

Figure 4: Structure and sequence conservation of GADD45 isoforms.

(A) Ribbon representation of the GADD45G monomer. Secondary structural elements are marked, and residues that are identical among all GADD45 isoforms are colored yellow. This figure was created by using PyMOL (53). (B) Amino acid sequence alignment of GADD45 family members. Identical residues are boxed in gray. Secondary structural elements observed in GADD45 crystals are marked above the sequences. Adapted from (Schrag et al. 2008).

The genes encoding for the *Gadd45a, Gadd45b and Gadd45g* isoforms are located on chromosome 1p31.2, 19p13.3 and 9q22.1, respectively, in the human genome and on chromosome 6 C1.6, 10 C1 and 13A5-B, respectively, in the mouse. They have independent promoters and regulatory elements (Kastan et al., 1992; Jung et al., 2000; Lefort et al., 2001; Furukawa-Hibi et al., 2002; Thyss et al., 2005). GADD45A is the most abundant of the three proteins, with high levels seen in spleen, heart, lung, brain and liver, and highest levels in kidney and skeletal muscle (Zhang et al. 1999b). GADD45B is expressed at low levels in

heart, brain and spleen, and highly expressed in lung, liver and skeletal muscle. GADD45G levels are high in skeletal muscle, lung, liver and kidney. A comprehensive comparison of GADD45A, B and G expression during mouse embryonic development was performed using in situ hybridization (Kaufmann et al. 2011). GADD45A is expressed on the tip of the closing neural tube wall and the eye sockets of E8.5 to E10.5 old embryos. GADD45B can be found in higher abundances at the tips of the limps and in the front brain, while GADD45G is strongly expressed in neural tube and the developing brain suggesting a prominent role in neurogenesis. All three GADD45 proteins are expressed in the somites. The fact that expression levels of the proteins are different in various tissues and that all *Gadd45* genes have their own regulatory elements indicate distinct functions in different tissues. *Gadd45a* was rapidly induced after ultraviolet (UV) radiation (Zhan et al. 1994) and is the only one regulated by p53 (Carrier et al. 1994; Zhan et al. 1999). *Gadd45b* is the only family member regulated by TGF-B (Selvakumaran et al. 1994). *Gadd45g* and *Gadd45b* are both primary response genes of IL3, IL6, G-CSF and GM-CSF stimulation in M1 myeloblastic leukemia (Zhang et al. 1999b). Furthermore, expression of all three *Gadd45a, Gadd45b* and *Gadd45g* is induced in response to multiple environmental and physiological stresses, including methyl-methanesulfonate (MMS), G -irradiation (IR), UV, VP-16, daunorubicin (DNR), and inflammatory cytokines (Gupta et al. 2005; Liebermann, Hoffman 2008). Even though all *Gadd45* genes are induced by those stressors; their pattern of expression remains unique, consistent with each GADD45 family member playing distinct roles in response to each source of stress or cell type.

1.2.1 The function of GADD45 proteins

GADD45 proteins are involved in many fundamental cellular processes such as cell cycle arrest, apoptosis or cell survival and DNA damage repair by acting primarily as stress sensors (Liebermann, Hoffman 2008). GADD45 proteins do not harbor catalytic activity or transcription factor function; they modulate the cellular processes by interaction with other factors involved in cell cycle regulation and stress responses (Figure 5). They form homo- or heterodimers and interact with other proteins thereby modulating their structure and/or activity, modifying the biological outcomes depending on the GADD45 isoform and their expression levels in a cell type and differentiation state specific manner (Liebermann, Hoffman 2008).

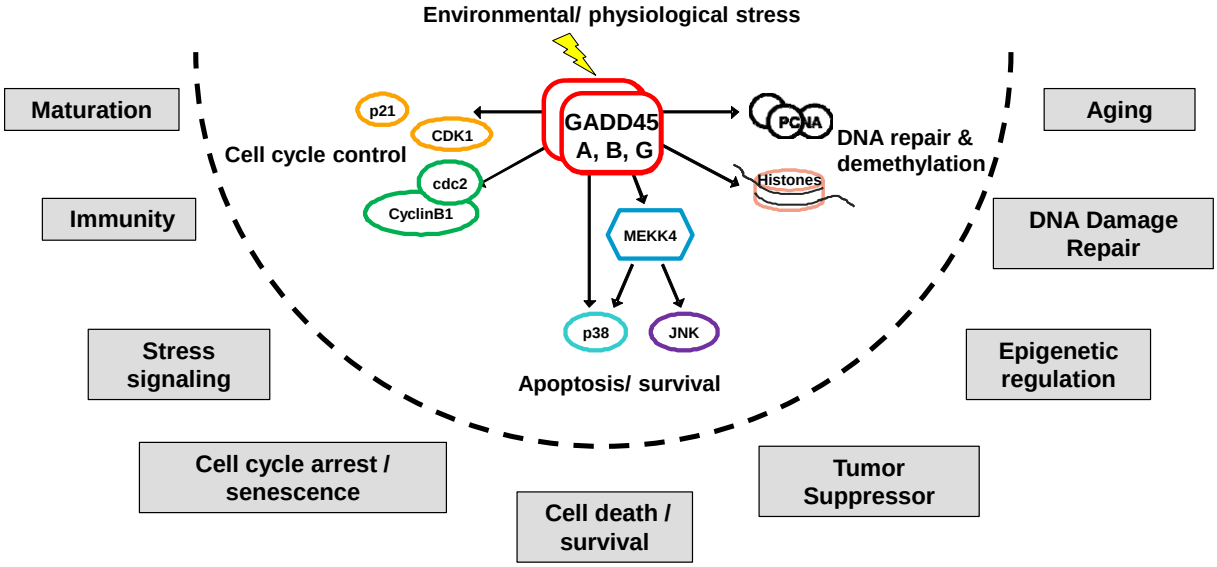

Figure 5: Reported GADD45 functions upon environmental/physiological stress

Summary of the various protein-protein interactions of GADD45 proteins that affect cellular processes such as cell cycle arrest, survival, apoptosis, senescence, DNA repair as well as epigenetic gene activation. Implications of GADD45 family members in a wide variety of different cellular processes displayed in grey boxes.

A cell usually arrests the cell cycle to withhold DNA synthesis and repair the DNA damage upon genotoxic stress. The GADD45 proteins regulate the cell cycle by physically interacting with cell cycle proteins like p21, CDK1 and CDC2 (Hoffman, Liebermann 2007). The direct interaction of all three GADD45 isoforms with p21 augments the universal cyclin dependent kinase inhibitor function of p21 causing both G1/S and G2/M cell cycle arrest (Vairapandi et al. 1996; Fan et al. 1999). GADD45A and GADD45B can bind to CDC2/CyclinB1, which results in the dissociation of the complex and inhibition of CDC2 kinase activity leading to G2/M call cycle arrest (Wang et al. 1999; Yang et al. 2000). GADD45G also inhibits the CDC2/CyclinB1 complex without disrupting the complex(Vairapandi et al. 2002). Interestingly, all three isoforms co-operatively inhibit the CDC2/CyclinB1 complex by forming heterodimers in RKO lung carcinoma cell lines upon exposure to UV irradiation (Kovalsky et al. 2001).

GADD45 proteins also regulate DNA repair. They directly interact with proliferating cell nuclear antigen (PCNA) (Chen et al. 1995; Vairapandi et al. 1996; Vairapandi et al. 2000; Azam et al. 2001). PCNA plays a pivotal role in DNA replication and repair (Jónsson, Hübscher 1997). The ring-shaped homotrimeric PCNA complex encircles and slides along double-stranded DNA acting as a so-called "sliding clamp" and serves as a scaffold for other proteins (Essers et al. 2005). Following a single strand break in the DNA, GADD45 recruits PCNA to the lesion by which in turn recruits repair DNA polymerase delta to repair the single

strand break by nucleotide excision repair (NER) (Smith et al. 1994). GADD45A is also implicated in the epigenetic activation of genes through a repair-mediated active DNA demethylation (Barreto et al. 2007; Niehrs, Schäfer 2012).

GADD45 proteins also regulate the stress induced mitogen-activated protein kinase (MAPK) pathway. Gadd45 proteins can bind to and activate MAP3K4, an upstream activator of the stress induced p38/JNK kinases (Takekawa, Saito 1998). GADD45 proteins bind to the N-terminal auto-inhibitory domain of MAP3K4 leading to a conformational change that results in an auto-phosphorylation of MAP3K4 (Mita et al. 2002). Phosphorylated MAP3K4 then activates down-stream MAP kinase kinases (MKK), mainly MKK3 and MKK6, which then phosphorylate p38 MAPK and c-jun N-terminal kinases (JNKs). The latter are effector kinases, which phosphorylate a variety of transcription factors and other downstream kinases that trigger apoptosis. Interestingly, GADD45B can also inhibit JNK activation by directly blocking the catalytic activity of MKK7, the kinase upstream of JNK (Papa et al. 2004; Tornatore et al. 2008). Tumor necrosis factor alpha (TNFA) stimulation induces GADD45B expression via NF-κB, leading to the inhibition of JNKs and a decline in apoptosis (Papa et al. 2004). Furthermore GADD45A or G may directly activate p38 by physically interaction (Bulavin et al. 2003; Zhu et al. 2009).

A unique function of GADD45G in male sex determination has been previously identified (Gierl et al. 2012). Sex determination in humans and most mammals occurs by activation of the Y-chromosome-linked sex determining region of Y (*Sry)* gene in the embryonic gonads, resulting in testis formation. Male mice deficient in *Map3K4* or *Gadd45g* were born with a complete sex reversal to a female phenotype similar to what is seen in mice with *Sry* deletion (Warr et al. 2012; Johnen et al. 2013). Sex reversal in both mutants is associated with reduced phosphorylation of p38 MAPK and GATA4 in the gonad region during embryonic development.

1.2.2 The role of GADD45 proteins in cancer

Since the GADD45 proteins have an important role as stress sensors, it is likely that they are also instrumental in monitoring oncogenic stress and thereby influence tumor development. More than 50% of human tumors have a deletion or mutation in the *TP53* gene (Hollstein et al. 1994), coding for p53 as the best studied tumor suppressor and the “guardian of the human genome”. Various genotoxic stresses can activate p53, which in turn initiates several downstream targets that set up the cellular “checkpoints” of cells cycle arrest and apoptosis. Pausing the cell cycle allows the cell to repair DNA damage and in cases with fatal damages, to induce apoptosis (Bookstein et al. 1993). Activation of GADD45A by p53 triggers the pro-

apoptotic JNK signaling pathways. JNK itself can phosphorylate p53 stabilizing it creating a positive feedback loop (Jin et al. 2003). When the p53 signaling is disrupted by p53 mutation, cells can escape the cellular checkpoints and accumulate additional mutations for further transformation (Drayton, Peters 2002).

The anti-tumor activity of GADD45 proteins has been shown *in vitro* and *in vivo*. *Gadd45a*- and *Gadd45b*-deficient mice show a higher susceptibility to mutagenic chemicals, UV- and γ- irradiation, resulting in genomic instability (Gupta et al. 2005; Gupta et al. 2006a). These mice also display a higher mutation rate (Hollander et al. 1999), because they exhibit abnormal nucleotide- and base-excision DNA repair (Hollander et al. 2001). Furthermore, it was shown that expression of the oncogene H-Ras in *Gadd45a*-deficient mouse embryo fibroblasts was sufficient for malignant transformation underscoring the importance of GADD45 proteins as tumor suppressors (Bulavin et al. 2003; Tront et al. 2006). Deletions within the *Gadd45a* gene recurrently occur in breast cancer tissues (Hoggard et al. 1995) as well as in pancreatic cancer (Yamasawa et al. 2002). The *Gadd45b* gene locus is a common target of retroviral integration that resulted in a clonal transformation in a variety of cancers (Engelmann et al. 2008).

Similar to other tumor suppressors, the GADD45 proteins are also actively down-regulated in tumor cells. The *Gadd45a* promoter is methylated in the majority of breast cancers, resulting in reduced expression when compared with normal breast epithelium (Wang et al. 2005). Hyper-methylation of the *Gadd45b* promoter occurred in 50% of human hepatocellular carcinoma (Qiu et al. 2004). Similar *Gadd45a* and *Gadd45g* were down-regulated in 77% (Gramantieri et al. 2005) and 65% (Sun et al. 2003) of analyzed hepatocellular carcinomas, compared to corresponding normal liver tissues. In both cases, the extend of gene silencing correlated with the stage of hepatocellular carcinoma differentiation (Gramantieri et al. 2005; Zhu et al. 2009; Guo et al. 2013a). *Gadd45g* seems to be commonly silenced in tumor tissues (Bahar et al. 2004; Sun et al. 2003; Ying et al. 2005; Zhang et al. 2010; Guo et al. 2013a). In 67% of the patients with pituitary adenomas, the *Gadd45g* gene was epigenetically silenced through CpG-methylation (Bahar et al. 2004; Guo et al. 2013b). Reversal of this epigenetic modification resulted in re-expression and increased tumor responsiveness (Bahar et al. 2004). Ectopic expression of any one of the GADD45 proteins resulted in a G2/M cell cycle arrest or apoptosis in several cancer cell lines, i.e. M1 myeloblastic leukemia, H1299 lung carcinoma, HeLa cervical cancer, RKO colon carcinoma, as well as in non-transformed NIH3T3 mouse embryonic fibroblasts (Zhang et al. 2001; Sun et al. 2003; Jiang, Wang 2004; Ying et al. 2005). GADD45A and G are negatively regulated

by NF-κB in various cancer cell lines such as prostate cancer, glioma, melanoma and hepatocellular carcinoma (Karin et al. 2002; Zerbini et al. 2004), which prevents JNK activation and protects the cancer cells from apoptosis (Zerbini et al. 2004; Zhu et al. 2009). Ectopic expression of one GADD45 protein activate both p38 and JNK and induce G2/M cell cycle arrest and apoptosis in Hep-G2 (Zhu et al. 2009).Also inhibition of NF-κB restores *Gadd45a and g* expression leading to the activation of JNK and induction of apoptosis (Zerbini et al. 2004; Yang et al. 2009).

These studies further reiterate the important role which is played by the GADD45 proteins in tumor development. Ectopic expression of GADD45 can improve sensitivity to stress signals and resulted in the induction of cell cycle arrest or apoptosis of the cancer cells making the GADD45 proteins or their interaction partners very promising targets for anti-tumor treatment.

1.2.3 The role of GADD45 in hematopoiesis

Life-long hematopoiesis needs an intact pool of HSCs. Disturbed hematopoiesis may cause a failure of differentiation into mature blood cells and eventually a progression into leukemia or other hematologic malignancies. As discussed in previous sections, steady state as well as stress-induced hematopoiesis is regulated by a fleet of hematopoietic cytokines, although the molecular mechanisms behind this regulatory circuit remain to be deciphered (Wagers et al. 2002). Several studies have shown that the GADD45 proteins have an important part on the hematopoiesis regulatory mechanism.

Gadd45g was initially discovered as cytokine response gene up-regulated upon IL2 stimulation of T cells (Beadling et al. 1993). GADD45G and B also modulate interferon gamma (IFN G) production in CD4+ T cells following CD3, IL12 and IL18 stimulation (Chi et al. 2004). Thereby both GADD45G and B are involved in TH1 cell differentiation and successful activation of CD4 T cells for clonal expansion. Myeloid cells treated with pro-myeloid cytokines like IL-3, GM-CSF, G-CSF or M-CSF showed an induction of all three *Gadd45* genes. Expression of the isoforms varied in duration and intensity during the myeloid developmental program (Zhang et al. 1999b; Gupta et al. 2006b; Ju et al. 2009). The transcription of *Gadd45g* is dependent on the cytokine-receptor signal transduction and transcription factor STAT5 (Hoffmeyer et al., 2001).

However, germ-line deletion of any of the *Gadd45* genes did not affect the production of blood cell lineages under steady state conditions (Lu et al. 2001; Hoffmeyer et al. 2001; Gupta et al. 2005). This might be explained by some redundancy of the GADD45 family members. Additionally, since the GADD45 proteins are known to be stress sensors, it was

reasoned that they may be dispensable for steady-state hematopoiesis, but important during hematopoietic stress. BM cells (BMCs) from *Gadd45a*- or *Gadd45b*-deficient mice are more sensitive to genotoxic stress induced by either UV irradiation or DNR and exhibit higher apoptosis rates compared to wild-type cells (Gupta et al. 2005). To investigate if GADD45 proteins are also important for physiological stress induced hematopoiesis, the same authors isolated myeloid enriched BMCs from *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice and stimulated them with cytokines secreted during hematopoietic stress (GM-CSF, IL-3, M-CSF, or G-CSF), both *in vivo* and *in vitro* (Gupta et al. 2006b). The *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ BMCs had reduced outputs of granulocytes and macrophages and higher apoptosis four days after cytokine stimulation compared to the wild-type cells. The BMCs of the mutant mice also showed an initial decrease in colony formation followed by a higher proliferative capacity suggesting an impaired or delayed response of either *Gadd45a*- or Gadd45b-deficient myeloid cells to hematopoietic stress (Gupta et al. 2006a). BMCs from *Gadd45a*$^{-/-}$ mice exhibited an enhanced and prolonged colony-forming ability and an increased self-renewal capacity in serial competitive transplantation (Chen et al. 2014). Furthermore DNA damage accumulated in *Gadd45a*$^{-/-}$ HSCs eventually promoted development of leukemia in aged mice (Chen et al. 2014).

Gadd45 genes are persistently down-regulated in leukemias (Selvakumaran et al. 1994; Valk et al. 2004; Perugini et al. 2009; Ju et al. 2009; Liebermann et al. 2011) suggesting a function in terminal differentiation. Along this line, re-expression of either GADD45G and GADD45B in M1 myeloblastic leukemia cell line induced cell cycle arrest and differentiation (Zhang et al. 1999b). Similar treatment with a novel histone deacetylase inhibitor LBH589 caused an increased expression of all three *Gadd45* genes in acute lymphoblastic leukemia (ALL) cells lines T-cell MOLT-4 and pre-B-cell Reh (Scuto et al. 2008). Expression of *Gadd45* was accompanied by growth arrest and increased cell death.

Moreover, GADD45G is upregulated in LT-HSCs of aged mice (Rossi et al. 2005). In aged mice the HSC pool is increased with more HSCs in active cell cycle. The aged HSCs have accumulated DNA damage and suffer from reduced reconstitution ability and a myeloid linage bias (Beerman et al. 2010). HSC aging therefore believed to be central to myelo-based diseases in the elderly such as acute myeloid leukemia (AML) development. Further, shRNA-mediated knockdown of GADD45G improved survivability of aged HSCs in serial transplantation (Wang et al. 2012), suggesting an important function of GADD45G in HSC biology.

1.3 Aims and work plan of the thesis

HSCs with their unique properties of self-renewal and multi-lineage differentiation provide blood cell regeneration life-long. The enormous potency of HSCs to completely reconstitute the hematopoietic system of recipients becomes apparent in stem cell or BM transplantations - prime examples for successfully applied regenerative medicine. A critical aspect of successful stem cell therapies may be the limiting number of functional HSCs in the donor source, besides the search for a suitable HLA-matched donor; this particularly holds true for cord blood-derived grafts. However, despite of decades of research, so far there are no conditions which allow robust *in vitro* maintenance or even expansion of HSCs without inducing differentiation. To this aim a better understanding of the molecular processes governing the early fate decision between self-renewal and differentiation in HSCs is needed, and of how extrinsic signals can control these fate decisions. To identify molecules controlling this balance in HSCs a transcriptional screen for immediate early response genes under the self-renewal promoting thrombopoietin signaling via the transcription factors STAT5A/B was conducted. The DNA-damage response and tumor suppressor gene *Gadd45g* was identified. Nothing was known so far about the function of GADD45G in HSCs. Consequently, the role of GADD45G in regulating early cell fate decisions of HSCs in adult hematopoiesis will be elucidated in this thesis.

In order to investigate whether various hematopoietic cytokines can induce the expression of the Gadd45 family members alpha, beta, and gamma, qPCR-based Gadd45 expression will be assessed. Lentiviral expression systems that efficiently transduce prospective isolated HSPC populations will be used to simulate the cytokine-induced expression. To assess the differentiation kinetics of LT-HSCs over time in culture, the expression of differentiation markers via FACS will be analyzed, the cell morphology will be scored by May-Grünwald staining, and serial re-platings of colony-forming cells will be performed. To analyze potential influences of Gadd45 family members on the lineage choice of HSCs, colony-formation assays in semisolid medium will be performed. The colony size (clonal proliferative capacity) and the lineage composition can be read out by this assay and retrospectively allows functional conclusions on the seeded stem/progenitor cells. The proliferative capacity and the cell cycle propagation of a cell will be assessed either by cell counting, a luminometric cell viability assay, or BrdU incorporation.

Whether GADD45G-expressing LT-HSCs show any changes in their self-renewal can only be determined by transplanting them into irradiated recipients *in vivo*. Only HSCs have the ability to maintain multilineage blood production for more than 20 weeks after

transplantation, and can also be serially transplanted into further recipients, which is the golden standard assay for adult stem cells. Therefore competitive serial-transplantations of lentivirally transduced HSCs into lethally irradiated mice are planned. Short-term transplantations (2-4 weeks) with subsequent BM analysis will determine changes in the stem and progenitor composition of GADD45G-expressing cells. The physiologic function of GADD45G in hematopoiesis will be investigated in *Gadd45g*-deficient mice during steady state and stress hematopoiesis in BM, peripheral blood and spleen. *Gadd45g*$^{-/-}$ HSCs will be challenged in cytokine stimulation experiments and competitive serial-transplantation.
Insights in the dynamic process of HSC differentiation and lineage choice upon GADD45G expression will be gained by time-lapse microscopy and continuous cell tracking at single cell resolution, which allow the real time determination of cell cycle progression, transgene expression, differentiation stage and cell fate of individual HSCs and their progeny over many days.

In order to elucidate the molecular mechanism of Gadd45G action in HSCs, known interaction partners and downstream molecules of GADD45G will be investigated by functional assays using loss and gain of function approaches. Changes in signaling will be assessed by phosphoflow cytometry at the single cell level. Gene expression profiling of GADD45G-expressing cells will further enlighten downstream genes altered by GADD45G action.

Mis-regulated self-renewal and differentiation block are the main drivers of hematopoietic malignancies and development of leukemia. Since GADD45G was also proposed as tumor suppressor the effect of re-expression of GADD45G pathway in leukemic samples to reduce the leukemic burden will be investigated.

2. Material and methods

2.1 Material

2.1.1 Chemicals

Table 1: Chemicals for molecular biology

Chemical	Source
Agarose	Roth, Karlsruhe, Germany
Ampicillin (sodium salt)	Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Serva, Heidelberg, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
LB Broth	Roth, Karlsruhe, Germany
LE Agarose	BioRad, Hercules, California, USA
Methanol	Roth, Karlsruhe, Germany

Table 2: Chemicals for cell culture and animal experiments

Chemical	Source
Anisomycin	Sigma Aldrich, St.Louis, Missouri, USA
Bovine serum albumin (BSA)	Sigma, St.Louis, Missouri, USA
Bromodeoxyuridine (BrdU)	BD bioscience, Franklin Lakes, New Jersey, USA.
Calcium chloride ($CaCl_2$)	Merck, Darmstadt, Germany
Cyclohexemid (Chx)	Sigma, St.Louis, Missouri, USA
Dimethyl sulfoxide (DMSO)	Sigma, St.Louis, Missouri, USA
Disodium phosphate (Na_2HPO_4)	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma, St.Louis, Missouri, USA
Formaldehyde (37 %)	Roth, Karlsruhe, Germany
Glycerin 88% for cell culture	Gerbu, Heidelberg, Germany
Hydroxyethylpiperazineethanesulfonic acid (HEPES)	Sigma, St.Louis, Missouri, USA
Isofluran/Enfluran	Abbott, Wiesbaden, Germany
Isopropanol	Merck, Darmstadt, Germany
JNK inhibitors: JNK-IN-8, SP600125	Merck, Darmstadt, Germany
Murine and human cytokines	Peprotech, Rock Hill, New Jersey, USA
P38 ihibitors: BIRB-796, Ly2228820, SB 203580, VX-702	Selleckchem, Munich, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, St.Louis, Missouri, USA
Sodium azide (NaN_3)	Sigma, St.Louis, Missouri, USA
Trypan blue	Sigma, St.Louis, Missouri, USA
B-mercapto-ethanol	Gibco, Life Technologies, Darmstadt, Germany

2.1.2 Buffers and solutions

Table 3: Buffers and solutions for molecular biology

Buffer/Solution	Ingredients/Source
DNA loading buffer (6x)	New England Biolabs, Ipswich, Massachusetts, USA
Giemsa-solution	Sigma Aldrich, St.Louis, Missouri, USA
LB medium	Roth, Karlsruhe, Germany
May-Grünwald concentrated solution	Sigma Aldrich, St.Louis, Missouri, USA
P1 resuspension buffer (500 ml)	50 mM Tris HCl pH 8; 10 mM EDTA; 500 µl RNAse A; in 500 ml ddH_2O

P2 alkaline lysis buffer (500 ml)	200 mM NaOH, 1% SDS in ddH_2O
P3 neutralisation buffer (500 ml)	300 ml potassium acetate; 57.5 ml acetate; 142.5 ml ddH_2O
Restriction enzyme buffer	New England Biolabs, Ipswich, Massachusetts, USA
10x TAE buffer	Roth, Karlsruhe, Germany
1x TE buffer	Gibco, Life Technologies, Darmstadt, Germany

Table 4: Buffers and solutions for cell culture (and animal experiments)

Buffer/Solution	Composition/Source
BSA stock solution (10 %)	10 g BSA in 100 ml PBS, stored at 4°C
Cyclohexemide (100x)	1 mg/ml in ddH_2O, stored at 4°C
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Life Technologies, Darmstadt, Germany
Dulbecco's Phosphate Buffered Saline (PBS)	PAA Laboratories, Pasching, Austria
FACS buffer	PBS, 2 % FBS, 0.05 % NaN_3, stored at 4°C
FACS fixation solution (2x)	2 % Paraformaldehyde in PBS, stored at 4°C
Fetal bovine serum (FBS)	PAA Laboratories, Pasching, Austria
Ficoll-Histopaque 1083	Sigma Aldrich, St.Louis, Missouri, USA
Hanks balanced salt solution (HBSS)	Sigma, St.Louis, Missouri, USA
HEPES buffer (2x)	281 mM NaCl, 100 mM HEPES, 5 mM Na_2HPO_4 in ddH_2O, pH 7.0, stored at -20°C
100 mM L-Glutamine	Gibco, Life Technologies, Darmstadt, Germany
Methocult supplemented medium M3434	Stem cell technologies, Vancouver, Canada
Penicillin/Streptavidin	Gibco, Life Technologies, Darmstadt, Germany
Roswell Park Memorial Institute (RPMI) 1640 Medium	Gibco, Life Technologies, Darmstadt, Germany
StemSpan; serum free expansion medium (SFEM)	Stem cell technologies, Vancouver, Canada
Trypsin-EDTA solution (0.05%)	Invitrogen, Carlsbad, California, USA

Table 5: Cell culture media

Medium	Composition
DMEM, complete	10 % (v/v) Fetal bovine serum (FBS), 20 mM HEPES, 3.5 mM L-glutamine
MEP Methocult M3434	Methocult M3434 ; 100 ng/ml TPO
RPMI 1640, complete	10 % (v/v) Fetal bovine serum (FBS), 2 % (v/v) L-glutamine,
RPMI 1640-leukemic medium	30 % (v/v) Fetal bovine serum (FBS), 2 % (v/v) L-glutamine, 0.5 µM B-mercaptoethanol
SFEM differentiation medium	SFEM; 100 ng/ml SCF, 100 ng/ml TPO, 20 ng/ml IL3, 20 ng/ml IL6
SFEM permissive medium	SFEM; 100 ng/ml SCF, 100 ng/ml TPO

Self-made buffers and solutions were sterilized by either autoclaving or filtration

2.1.3 Technical equipment

Table 6: Plastic material and consumables

Material	Source
Combitips plus (1, 5 and 10 ml)	Eppendorf, Hamburg, Germany
Cryotubes (2 ml)	Sigma, St.Louis, Missouri, USA
FACS tubes	Sarstedt, Nümbrecht, Germany
FACS tubes (sterile, polypropylene)	Greiner Bio-One, Frickenhausen, Germany
Falcon sterile centrifuge tubes (5, 15 and 50 ml)	Greiner Bio-One, Frickenhausen, Germany
Freezing box	neoLab, Heidelberg, Germany
Insulin syringe	BD, Franklin Lakes, New Jersey, USA
NUNC tissue culture plates seal tight (24 well)	Thermo-Fischer Scientific, Waltham, MA, USA

Pasteur pipettes (glas)	Roth, Karlsruhe, Germany
Pipette tips (10, 100, 200 and 1000 µl)	Gilson, Limburg-Offheim, Germany
Protection gloves (Latex, Nitril)	Meditrade, Kiefersfelden, Germany
Reaction tubes (0.2, 1.5 and 2 ml)	Eppendorf, Hamburg, Germany
PCR reaction tubes and PCR stripes (0.2 ml)	Sarstedt, Nümbrecht, Germany
RealTime PCR 96-well plates	4titude, Dorking, United Kingdom
Scalpels	Mediware Servoprax, Wesel, Germany
Silicon stem cell inserts	IBIDI, München, Germany
Sterile cell strainer (100 and 70 µm)	BD, Franklin Lakes, New Jersey, USA
Sterile filters (0.22 and 0.50 µm)	Merck Millipore, Billerica, Massachusetts, USA
Sterile pipettes (2, 5, 10 and 25 ml)	Costar, Corning, New York, USA
Syringes (5,10 and 20 ml)	Braun, Melsungen, Germany
Tissue culture dishes (3.5 and 10 cm in diameter)	Greiner Bio-One, Frickenhausen, Germany
Tissue culture plates (6, 12, 24, 96 well)	Sarstedt, Nümbrecht, Germany

Table 7: Instruments and devices

Instrument	Source
Analytical scales Ac210s, AX2202m	Sartorius Stedim Biotec, Göttingen, Germany
CellObserver 430 optical microscope	Carl Zeiss AG, Oberkochen, Germany
Centrifuge, Heraeus Megafuge 1.0R	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Centrifuge Rotana 460R	Hettich, Tuttlingen, Germany
Centrifuge, tabletop (Rotanta 200, 220R)	Hettich, Tuttlingen, Germany
Clean bench, HERAsafe KSP	Thermo Fisher Scientific, Waltham, Massachusetts, USA
CO_2 Incubator, HERAcell 150i	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Digital PH caliper	Mettler Toledo, Greifensee, Switzerland
Dissection set	Heiland, Hamburg, Germany
DNA electrophoresis chamber	BioRad, Hercules, California, USA
FACS AriaI or AriaIII cell sorter	BD, Franklin Lakes, New Jersey, USA
FACS CantoII	BD, Franklin Lakes, New Jersey, USA
FACS FortessaII	BD, Franklin Lakes, New Jersey, USA
Freezer -80°C, Heraeus	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Freezer -20°C and refrigerators	Liebherr, Bulle, Switzerland
Incubator, Heraeus	Thermo Fisher Scientific, Waltham, Massachusetts, USA
LightCycler® 480 II quantitative PCR cycler	Roche Life Science, Rotkreuz, Switzerland
Nano-Drop 1000 spectrometer	Thermo Fischer Scientific, Waltham, MA, USA
Thermal cycler MyCycler	BioRad, Hercules, Berkeley, CA, USA
Thermomixer	Biometra, Analytik Jena, Jena, Germany
Ultracentrifuge Optima L-90K	Beckman Coulter, Pasadena, CA, USA
UV-Transilluminator GelDoc 2000	BioRad, Hercules, Berkeley, CA USA
Vacuum pump	Integra Biosciences, Fernwald, Germany
Vortex	Roth, Karlsruhe, Germany

2.1.4 Enzymes and kits

Restriction enzymes AgeI, BamHI, BsrGI, Eco47III, EcoRI, FseI, HindIII, NheI, NotI, XbaI and XhoI were purchased from New England Biolabs, Ipswich, Massachusetts, USA and used with the recommended restriction enzyme buffer. Restriction enzymes BcuI (SpeI), MreI were order from (Fermentas, now Thermo-Fischer Scientific, Waltham, MA, USA) and used according to the manufacturer's protocol.

DNA ladders for agarose gel electrophoresis 100 bp-Standard marker and 1 kb-Standard marker were obtained from New England Biolabs (Ipswich, MA, USA).

Table 8: commercial kits

Kit	Source
M-MuLV Reverse Transcriptase (RT) Kit	Thermo-Fischer Scientific, Waltham, MA, USA
BD Fix Buffer I	BD bioscience, Franklin lakes, NJ, USA
BD Perm Buffer III	BD bioscience, Franklin lakes, NJ, USA
BD Pharm lyse™ buffer	BD bioscience, Franklin lakes, NJ, USA
BD Phosflow starter kit	BD bioscience, Franklin lakes, NJ, USA
BD proliferation assay APC BrdU Flow Kit	BD bioscience, Franklin lakes, NJ, USA
HotStar HiFidelity Polymerase Kit	QIAGEN, Venlo, Nederland
pGEM®-T easy cloning vector kit	Promega, Madison, WI, USA
NucleoBond® Xtra maxi kit	Macherey-Nagel, Düren, Germany
QIAquick Gel Extraction kit	QIAGEN, Venlo, Nederland
Ribolock (RNase-Inhibitor)	Fermentas, Waltham, MA, USA
RNeasy® Mini Kit	QIAGEN, Venlo, Nederland
TaqMan® Gene Expression Cells-toCT™ Kit	Life Technologies, Carlsbad, USA
TaqMan® PreAMP Cells-toCT™ Kit (ambion)	Life Technologies, Carlsbad, USA
TaqMan® Gene Expression master mix	Life Technologies, Carlsbad, USA
ViaLight Plus Cell Proliferation and Cytotoxicity BioAssay Kit	Lonza Group AG, Basel, Switzerland

2.1.5 Gene specific oligomers

Table 9: DNA oligomers

Description	Sequence	Melting temp
Multiple cloning site fw	GATCCTCGAGGCCGGCCGCGCGCCGGCGACTAGTCCGGATTTAAATCTAGACGCGTA	93°C
Multiple cloning site rev	CCGGTACGCGTCTAGATTTAAATCCGGACTAGTCGCCGGCGCGCGGCCGGCCTCGAG	94°C
Gadd45g fw xFseI	TTAAggccggccAGCCATGACTCTGGAAGAAGTCCGTGGC	69°C
Gadd45g rev xMreI	TTAAcgccggcgGATCGATCAGACCAAGGTCCCTGC	68°C
Gadd45a fw xFseI	TTggccggccGAGGGACTCGCACTTGCAATATG	64°C
Gadd45a rev xSpeI	TTactagtTGAACTCGGCCCCTTGACAT	64°C
Gadd45b fw xFseI	TTggccggccCTGCATCATGACCCTGGAAGAG	63°C
Gadd45b rev xSpeI	TTactagtTCACGGGTAGGGTAGCCTTTGA	64°C
Gadd45g T79E fw	ACATagcgctGCAGATCCATTT**CGA**GTTGATTCAGGCGTTC	74°C
Gadd45g L80E fw	ACATagcgctGCAGATCCATTTCAC**GGA**GATTCAGGCGTTC	75°C
Gadd45g mutant rev	GTACGCGTCTAGATTTAAATCCGGactagtCGCCG	74°C
caMAP3K4 fw xBamHI	CGATACCGTCGACggatcc**ATG**GATACCCCTAAGTCCTATGATAACG	60°C
MAP3K4 rev xSpeI	CGTCTAGATTTAAATCCGGactagtCTTCACTCTTCATCTGTGC	65°C

Oligomer sequence for polymerase chain reaction (PCR) restriction enzyme recognition sides are shown in small letters. Alterations in the original sequence are underlined and written in bold.

Table 10: TaqMan gene expression assays

Assay ID	Gene Symbol	Assay ID	Gene Symbol
Mm00432802_m1*	Gadd45a	Mm00434561_m1	Jak2
Mm00435123_m1*	Gadd45b	Mm01232838_g1	Khsrp (Fubp2)
Mm00442225_m1*	Gadd45g	Mm00517492_m1	kras
Mm00445212_m1	c-Kit	Mm00725832_s1	Mcl1
Mm00437783_m1	Bcl2l1 (Bcl-XL)	Mm00514820_m1	Mecom ((Mds1-) Evi1)
Mm00463355_m1	Bcl2l13 (Mil1)	Mm00491303_m1	Mecom (Mds1)
Mm00476123_m1	Bik	Mm00487664_m1	Meis1

Mm03053893_gH	CcnB1 (Cyclin B1)	Mm00440310_m1	Mpl
Mm00432359_m1	Ccnd1 (Cyclin D1)	Mm00482418_m1	Nkap
Mm00438070_m1	Ccnd2 (Cyclin D2)	Mm00836412_m1	Oas1a
Mm00455932_m1	CD48	Mm00451763_m1	Pmaip1 (Noxa)
Mm00846882_s1	Cdc42	Mm01206465_s1	Prmt6
Mm04208750_m1	cdc42ep3	Mm00477208_m1	Pten
Mm00726334_s1	Cdk4	Mm00488142_m1	Pu.1
Mm04205640_g1	Cdkn1a (p21)	Mm00505017_m1	Puf60 (Fir)
Mm00438168_m1	Cdkn1b (p27)	Mm01201653_mH	Rac1
Mm01272135_g1	Cdkn1c (p57)	Mm00485472_m1	Rac2
Mm00483241_m1	Cdkn2b (p16)	Mm01213405_m1	Runx1
Mm00486943_m1	Cdkn2d (p19)	Mm01196721_g1	Sh2B3 (Lnk1)
Mm00486943_m1	Cdkn2d (p19)	Mm00443316_m1	Slamf1 (CD150)
Mm00487804_m1	c-Myc	Mm00850544_g1	Socs2
Mm01311417_m1	Fubp1	Mm03053818_s1	Stat5a
Mm01328093_m1	Fubp3	Mm00839889_m1	Stat5b
Mm01352636_m1	Gata1	Mm01187033_m1	Tal1
Mm00492301_m1	Gata2	Mm04197910_g1	Tcea1
Mm00515855_m1	Gfi1	Mm01265659_g1	Txnip
Mm00492318_m1	Gfi1b	Mm00446968_m1	Hprt
Mm00433966_m1	HoxA10	Mm00607939_s1	β-Act
Mm04213381_s1	HoxA5	Mm99999915_g1	Gapdh
Mm00439364_m1	HoxA9	Mm01197698_m1	Gusb
Mm00657964_m1	HoxB4	Mm01180596_m1	Pum1
Mm00439653_m1	Il6Ra	Mm00437762_m1*	B2m

List of TaqMan assays used for qPCR-based gene expression analysis.

2.1.6 Lentiviral plasmids

Table 11: Plasmids for lentiviral particle production

Plasmid name	Description	Reference
pMDLg.HIVGag-Pol.pRRE	Expression plasmid for structural genes (*gag/pol*) of HIV-1; for lentiviral particle assembly; CMV promoter	Zufferey *et al.*[128]
pRSV.HIV-REV	Expression plasmid for lentiviral reverse transcriptase (*rev*) of HIV-1; driven by RSV promoter	(Dull et al. 1998)
pMD2.G.VSV-G	Expression plasmid for VSV-G (*env*); pseudo typing of lentiviral particles; CMV promoter	Yee *et al.*[127]
pRRL.PPT.SFFV.IRES.eGFP.wPRE	Lentiviral vector with a 5' packaging signal coding for the fluorescent protein eGFP; lentiviral gene transfer; SFFV promoter	Schambach *et al.*
pRRL.PPT.SFFV.MCS.IRES.VENUSnucmem.wPr	Plasmid vector with a 5' packaging signal for lentiviral gene transfer; coding for a fusion protein of hImportin and the fluorescent protein VENUS behind an IRES domain; driven by SFFV promoter	Cloned from pRRL.PPT.SFFV.IRES.eGFP.wPRE
pRRL.PPT.SFFV.MCS,IRES.tdTOMATOnucmem.wPre.	Plasmid vector with a 5' packaging signal for lentiviral gene transfer; coding for a fusion protein of hImportin and the fluorescent protein tdTOMATO behind an IRES domain; driven by SFFV promoter	Cloned from pRRL.PPT.SFFV.IRES.eGFP.wPRE
pCMV5 MEKK4alpha WT	Expression plasmid for HA tagged murine MEKK4 (*MAP3K4);* CMV promoter	Addgene #12187 (Gerwins et al. 1997)
pCDNA3-Flag MKK6(glu)	Gene transfer vector for cloning of flag tagged human constitutive active MKK6 (*MKK6(glu)*)	Addgene #13518 (Raingeaud et al. 1996)
pRRL.PPT.SFFV.Gadd45g.IRES.tdTOMATOnucmem.wPreor	Plasmid vector with a 5' packaging signal for lentiviral gene transfer; coding for murine Gadd45g and a fluorescent reporter tdTOMATOnucmem or	Cloned from pRRL.PPT.SFFV.MCS,IRES.tdTOMAT

pRRL.PPT.SFFV.Gadd45g.IRES.VENUSnucmem.wPre	VENUSnucmem behind an IRES domain; driven by SFFV promoter	Onucmem.wPre.
pRRL.PPT.SFFV.Gadd45gL80E.IRES.VENUSnucmem.wPre or pRRL.PPT.SFFV.Gadd45gT79E.IRES.VENUSnucmem.wPre	Plasmid vector with a 5' packaging signal for lentiviral gene transfer; coding for mutated murine Gadd45g L80E or T79E and VENUSnucmem behind an IRES domain; driven by SFFV promoter	Cloned from pRRL.PPT.SFFV.Gadd45g.IRES.VENUSnucmem.wPre
pRRL.PPT.SFFV.Gadd45a.IRES.VENUSnucmem.wPre or pRRL.PPT.SFFV.Gadd45b.IRES.VENUSnucmem.wPre	Plasmid vector with a 5' packaging signal for lentiviral gene transfer; coding for murine Gadd45a or b and a fluorescent reporter VENUSnucmem behind an IRES domain; driven by SFFV promoter	Cloned from pRRL.PPT.SFFV.MCS,IRES.tdTOMATOnucmem.wPre.
pRRL.PPT.SFFV.caMAP3K4.IRES.VENUSnucmem.wPre or pRRL.PPT.SFFV.caMKK6.IRES.VENUSnucmem.wPre	Plasmid vector with a 5' packaging signal for lentiviral gene transfer; coding for murine constitutive active *Map3K4* or *MKK6(glu)* and VENUSnucmem behind an IRES domain; driven by SFFV promoter	Cloned from pRRL.PPT.SFFV.MCS,IRES.tdTOMATOnucmem.wPre.

CMV = cytomegalovirus; eGFP= eukaryotic green fluorescent protein; HIV-1 = human immunodeficiency virus type-1; IRES= internal ribosome entry side; RSV = Rous Sarcoma Virus; SFFV = spleen focus-forming virus; VSV = vesicular stomatitis virus; VSV-G = glycoprotein G of VSV; WPRE=Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element

2.1.7 Antibodies for flow cytometry

Table 12: Antibodies for flow cytometry

Antigen	Clone	Conjugate	Company
CD3	145-2C11	Biotin, PE-Cy7	eBioscience
CD3e	500A2	V500	BD Bioscience
CD11b	M1/70	Biotin	eBioscience
CD11b	M1/70	PE, AlexaFluor® (AF)-647	Biolegend
CD16/32	2.4G2	FITC, V450	BD Bioscience
CD19	1D3	Biotin	eBioscience
CD34	RAM34	eFluor®660	eBioscience
CD41	MWReg30	Biotin	eBioscience
CD48	HM48.1	PE, FITC	Biolegend
CD45.1	A20	eFluor®450, FITC	eBioscience
CD45.2	104	PerCP-Cy5.5, V500	eBioscience
CD45R (B220)	RA3-6B2	Biotin, PE	eBioscience
CD117 (c-KIT)	2B8	PE-Cy7	eBioscience
CD150	TC15-12F12.2	PE, PerCP-Cy5.5	Biolegend
SCA-1	D7	PerCP-Cy5.5, Pacific Blue	eBioscience
Gr-1	RB6-8C5	Biotin, AF-647, AF-700	eBioscience
TER119	TER-119	Biotin, APC-eFlour®780	eBioscience
Streptavidin		APC-eFluor®780	eBioscience
Fixable viability dye		eFluor®780, eFluor®506	eBioscience
P- p38 MAPK	36/p38	PE	BD Bioscience
P-MK2	P24-694	AF-647	BD Bioscience
BrdU	BU20A	PE	eBioscience

2.2 Molecular biology Methods

2.2.1 DNA preparation methods

2.2.1.1 Transformation of bacteria

Plasmid DNA was propagated and multiplied in TOP10 chemically competent *Escherichia coli (E.coli)* from Invitrogen (Carlsbad, California, USA) with following genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara- leu)7697 galU galK rpsL (StrR) endA1 nupG. Chemically competent *E.coli* bacteria in 100 µl stocks were thawed on ice and mixed with 50- 100 ng DNA by finger flipping. After 30 min incubation on ice DNA uptake was mediated by heat shock for 45 sec at 42°C followed by 2 min chill on ice. After 45 min incubation in LB medium at 37°C under 200 rpm shaking, 10- 100 µl of bacteria suspension was plated on LB-Agar plates supplemented with ampicillin and incubated overnight at 37°C. Single bacteria colonies grown on ampicillin were picked and expanded in 4 ml LB-medium containing 100 µg/ml ampicillin and cultivated at 37°C and continuous shaking at 300 rpm. 2 ml bacteria suspension was harvested for analytical plasmid DNA preparation (2.2.1.2). The remaining culture were stored at 4°C as starting culture for larger scale plasmid DNA preparation in 250 ml LB medium (100 µg/ml ampicillin) after the identity of plasmid DNA was confirmed (2.2.1.3). Two times 220 µl bacteria suspension were mixed with 780 µl 80% glycine solution and stored at -80°C to preserve bacterial clones.

2.2.1.2 Analytical plasmid-DNA preparation

To analyze the identity of plasmid-DNA via control digest DNA was routinely isolated from chemical competent *E. coli* bacteria by alkaline lysis. From an overnight culture 2 ml bacteria suspension was harvested by centrifugation (4000 x g, 10 min, 4°C), resuspended in 250 µl P1 buffer followed by adding 250 µl buffer P2 for alkaline lysis and incubation for 5 min at room temperature. Lysis was stopped with 250 µl of neutralising buffer P3 incubating on ice for 5 min, followed by centrifugation for 15 min at 18200 x g at 4°C to get rid of cell debris, protein and genomic DNA. Supernatant was mixed with 700 µl of isopropanol and centrifuged again for 20 min at 18200 x g at 4°C to precipitate the plasmid-DNA. Plasmid DNA was washed once with 500 µl 70% ethanol for 5 min with 18200 x g at 4°C. DNA pellet was dried at room temperature for 15-20 min, before 30 µl endotoxin-free water was added to resuspended the plasmid DNA. Concentration and quality of the purified plasmid DNA was analyzed with a spectrophotometer (Nanodrop 1000, Thermo Fischer Scientific).

2.2.1.3 Preparative plasmid DNA preparation

Larger scale plasmid DNA preparation was performed from 250 ml over-night culture using the NucleoBond® Xtra maxi kit (Macherey-Nagel) according to the manufacturer´s instructions. Plasmid DNA was stored at -20°C.

2.2.1.4 DNA restriction digest

For analytical validation of plasmid DNA and for preparative purposes DNA restriction enzyme digest were routinely performed according to the instructions of the restriction enzymes manufacturer (section 2.1.4). For analytical digests typically 0.5 µg of plasmid DNA were digested with a combination of appropriated restriction enzymes (3 units) for 1 hour at 37°C in recommended buffer. If multiple digest was not possible a sequential digest was performed with an intermediate DNA purification step (chapter 2.2.1.6). For preparative DNA digest 5 µg of plasmid DNA was digested with the combination of desired restriction enzymes either with 3 units over night or with 10 units for 2 hours at 37°C followed by an inactivation of the restriction enzymes for 10 min at 65°C or 85°C.

2.2.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used either to analyze restriction pattern controlling plasmid DNA for subsequent methods or to separate specific fragments from preparative DNA restriction digests from unwanted cutting products. Agarose gels contained 0.7 to 2% (w/v) agarose heat dissolved in 1x TAE buffer. After cooling to roughly 60°C 2 µg/ 50 ml ethidium bromide was added enabling visualization of DNA under UV light. Polymerized gels were then placed into an electrophoresis chamber, covered by 1x TAE buffer and loaded with DNA samples mixed with 6x loading dye (New England Biolabs). To determine DNA fragment size, a reference DNA ladder was also loaded on the gels. DNA fragments were separated for 30 – 60 min applying 100 – 120 V and restriction pattern documented by a UV-transilluminator (GelDoc 2000, Biorad). For preparative digests the desired DNA band was cut using a scalpel and transferred into a pre-weighted 2 ml reaction tube.

2.2.1.6 DNA extraction and purification

QIAquick Gel Extraction Kit (QIAGEN) was used to extract and clean plasmid DNA fragments from agarose gels following the manufacturer’s protocol. In short gel fragments were dissolved in 3 times volume (w/v) QG buffer heated to 50°C for 15 min under constant shaking (300 rpm). DNA was bound onto a QIAquick gel extraction column in presences of one volume isopropanol. The column was washed twice with 500 µl QG buffer and once with 700 µl PE buffer by centrifugation at 17000 x g for 15 sec, the run through was discarded.

The purified DNA fragment was eluted from the column membrane incubating the column with 30 µl pre-warmed, nuclease-free water for 5 min and centrifugation at 17000 x g for 1 min. The elution step was repeated to increase the yield of extracted DNA.

2.2.1.6.1 Column purification

To purify linear DNA fragments or PCR products the samples were mixed with 5 volumes of PB buffer and loaded onto the QIAquick gel extraction columns (QIAGEN) by centrifugation for 1 min at 17000 x g. The column membrane was washed once with 700 µl PE buffer. The purified DNA fragment was eluted from the column membrane incubating the column with 30 µl pre-warmed, nuclease-free water for 5 min and centrifugation at 17000 x g for 1 min. The elution step was repeated to increase the yield of extracted DNA.

2.2.1.7 DNA ligation

Ligation of double stranded linear DNA fragments were performed using T4-DNA- ligase (Thermo-Fischer-Scientific) using the manufacturer's protocol. In short a 50 ng vector and a vector to insert ration of 1:3 was used. Ligation either was performed at room temperature for 1 hour or overnight at 16°C. T4-DNA ligase was inactivated heating the sample to 65°C for 15 min.

2.2.1.8 DNA sequencing

Identity of the purified plasmid DNAs were routinely confirmed by Sanger based DNA sequencing. Sequencing was performed at GATC biotech AG, Cologne, Germany. The results were compared with the digital reference sequence using the Clone manager 9 software (Sci-Ed Software, Morrisville, NC, USA).

2.2.2 Generation of lentiviral expression vectors

2.2.2.1 Production of multiple cloning side containing lentiviral expression vectors

A third generation self-inactivating HIV-1 based lentiviral vector system (lentiviral particles) was used for gene transfer into primary murine hematopoietic cells or standard culture cell lines. At first the open reading frame (ORF) of a fluorescent reporter protein (either VENUS-hImportin subunit α1 (AA2-67) or tdTOMATO-hImportin subunit α1 (AA2-67)) was inserted into the third generation self-inactivating lentiviral vector pRRL.PPT.SFFV.IRES.eGFP.wPRE by replacing the ORF of green fluorescent protein (Schambach et al. 2006). Therefore the ORF of all three fluorescent reporter proteins as well as the lenitviral backbone were cut with PmlI and BsrGI out of their plasmid backbones and cleaned by DNA gel extraction 2.2.1.5.

The purified linear DNA was ligated together building the new VENUS-hImportin or tdTOMATO-hImportin containing lentiviral vectors and validated by sequencing.

In a second cloning step an *in silico* self-constructed multiple cloning site (MCS) containing 10 restriction enzyme recognition sides was inserted after the SFFV promoter for multiple cloning techniques.

```
5′GATCCTCGAGGCCGGCCGCGCGCCGGCGACTAGTCCGGATTTAAATCTAGACGCGTA-3‘
3‘    GAGCTCCGGCCGGCGCGCGGCCGCTGATCAGGCCTAAATTTAGATCTGCGCATGGCC-5‘
  BamHI     FseI   MauBI  MreI  XhoI  SpeI  SwaI  XbaI MluI  AgeI
```

The two oligomers (each 10 µl of 100 µM) plus 2.2 µl NEB buffer 2 were incubated at 95°C for 10 min in a water bath and slowly cooled down against the room temperature, allowing optimal annealing of the two oligos. The lentiviral expression vectors were linearized using BamHI and AgeI and purified as described above. The annealed oligomers were ligated with their overlapping 5’ ends building new lentiviral expression vectors.

2.2.2.2 Generation of complementary DNA from isolated RNA

For isolation of mRNA primary murine cells or NIH 3T3 cell were harvested by spin down centrifugation for 5-10 min at 290 x g and 4°C and depending on the cell number resuspended in 350 µl (for less than 5 x 10^6 cells) or 700 µl (for more than 5 x 10^6 cells) buffer RLT for cell lysis. Pulling the lysate through a 0.9 mm syringe enhanced the lysis of the cells and homogenized the sample. Isolation of RNA was performed with RNeasy® Mini Kit (QIAGEN) following the manufacturer’s manual. To increase the RNA yield the elution step with 30 µl of RNase-free water was performed twice. Concentration and quality of the RNA was analyzed with a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific). RNA was stored at -80°C. To translate RNA into complementary DNA (cDNA) M-MuLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (RT) Kit (Thermo Fischer scientific) was used according to the manufacturer’s instructions.

2.2.2.3 Generation of Gadd45 expression vectors from cDNA

To generate lentiviral constructs expressing the open reading frame (ORF) of murine Gadd45 proteins gene specific cDNAs were generated using reverse DNA oligomers of the *Gadd45* genes as described in chapter 2.2.2.2. To amplify and modify the cDNA for subsequent cloning into lentiviral expression vectors polymerase chain reaction (PCR) was used. The PCR method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the sample for DNA melting and enzymatic replication of the DNA. The PCR technique is based on the thermal resistant DNA polymerase enzyme of the bacterium *Thermus aquaticus*

which stands heating to 95°C. Two gene specific primers covering approx. 20 base pairs flanking the amplified gene product were generated with modified 5'-ends containing appropriate restriction enzyme recognition sides (Table 9). For the PCR amplification of murine Gadd45 genes 2 µl of reverse transcribed cDNA (20-100 ng) and 2 µl of gene-specific forward and reverse primer at a concentration of 10µM were added into nuclease-free PCR-tubes. A master mix of 4 µl (5x) Hotstar HiFi Polymerase buffer und 1 µl Hotstar HiFi-Polymerase was added together with 9 µl nuclease-free water to a final volume of 20 µl. A MyCycler Thermal Cycler (Biorad) with gradient function was used for thermal cycling. A gradient of different annealing temperatures from 7°C below to the calculated melting temperatures of the DNA oligomers were used as depicted in Table 13.

Table 13: Program of Hotstar HiFi PCR reaction cycles

Temperature	95°C	94°C	54-68°C	68°C	72°C	10°C
Time	5 min	15 sec	1 min	2 min	15 min	∞
Program	Hold	40 cycles			once	hold
Reaction	initialisation	Denaturation	annealing	elongation	extension	storage

The PCR products were separated by agarose gel electrophoresis confirming their size and yield 2.2.1.5 and purified as described in chapter 2.2.1.6. The purified PCR products were cloned into the pGEM-T easy expansion vector (Promega) by TA-tailing using the poly-adenosine overhang from the PCR reaction and the Thymidine-tailing in the pGEM-T vector (2.2.1.7). The ligated vector was expanded in TOP10 bacteria and purified as described above. To clone the gene of interest into lentiviral expression vector both the pGEM-T vector containing mGadd45 sequence and pRRL.PPT.SFFV.MCS.IRES.VENUSnucmem were cut with the indicated restriction enzymes FseI, MreI or SpeI. The steps of agarose gel electrophoresis and gel extraction were repeated for both the Gadd45 gene cassette and the linearized lentiviral vector. The ORF of the Gadd45 genes were then ligated into the lentiviral expression vectors using T4-ligase 2.2.1.7. After transformation in TOP10 bacteria plasmid DNA was isolated, purified and sequenced and correct clones were stored as bacterial stocks.

2.2.2.4 Site directed mutagenesis of Gadd45g dimerization mutants

For the generation of the Gadd45g dimerization mutants Gadd45g L80E and Gadd45g T79E two DNA primers containing point mutations in the sequence of murine Gadd45g were generated, which introduced amino acid changes of leucine at position 80 and threonine at position 79 to glutamate, respectively. For side directed mutagenesis 1µM of the forward and 1µM of the mutagenesis reverse primers were mixed with 50 ng

pRRL.PPT.SFFV.mGadd45g.IRES.VENUSnucmem.wPre in an 20µl sample containing 4µl Hotstar HiFi Polymerase buffer und 1 µl Hotstar HiFi-Polymerase (2.5 units).

Table 14: Program for side directed mutagenesis PCR

Temperature	95°C	94°C	68°C	72°C	72°C
Time	5 min	20 sec	30 sec	1 min	10 min
Program	Hold	30 cycles			hold
Reaction	initialisation	Denaturation	annealing	elongation	extension

For the side directed mutagenesis the PCR protocol as described in Table 14 was used.

The PCR products were separated by agarose gel electrophoresis and purified as described in chapter 2.2.1.6. The purified PCR products were cloned into the pGEM-T easy expansion vector (Promega) as described already in chapter 2.2.2.3. The sequence was validated by DNA sequencing 2.2.1.8. The mutated sequence and the lentiviral expression vector pRRL.PPT.SFFV.mGadd45g.IRES.VENUSnucmem.wPre were digested with Eco47III and SpeI and ligated together as described above to exchange the wild type sequences with the mutated fragment.

2.2.2.5 Generation of lentiviral expression vectors for constitutive active MAP3K4

The constitutive active variance of MAP3K4 manly consists of the kinase domain and the MKK binding side, it lacks the N-terminal auto-inhibitory domain and the Gadd45 binding site (Mita et al. 2002). Constitutive active MAP3K4 was cloned from full-length murine MAP3K4 by PCR adding a start codon (ATG) 5´ in front of the murine *Map3k4* ORF coding for 1312asp to the C-terminus (286aa). As a full-length MAP3K4 template the 20 ng plasmid pCMV5 MEKK4alpha WT (mouse) (Addgene vector #12187) was used (Gerwins et al. 1997) together with 2µl of 10µM gene specific primers following the Hotstar HiFi Polymerase protocol described in section 2.2.2.3. The PCR product was column purified and directly digested with the restriction enzymes BamHI and SpeI as was the lentiviral vector pRRL.PPT.SFFV.MCS.IRES.VENUSnucmem. Both vector and PCR fragment were purified by gel electrophoresis and ligated with T4 ligase.

2.2.2.6 Generation of lentiviral expression vector for constitutive active MKK6

Human constitutive active MKK6 (caMKK6) was subcloned from pCDNA3-Flag MKK6(glu) (Addgene vector #13518) into the MCS of the lentiviral vector (Raingeaud et al. 1996). The MKK6(glu) ORF was isolated from the pCDNA3 vector by digesting with the restriction enzymes NheI and XbaI, producing the compatible 5' overhang CTAG. To linearize the lentiviral vector pRRL.PPT.SFFV.MCS.IRES.VENUSnucmem.wPre was digested with the restriction XbaI. Both fragments were purified by agarose gel electrophoresis and gel

extraction (2.2.1.5, 2.2.1.6). The fragments were ligated and transformed into chemical competent Top10 bacteria. The correct insertion of the MKK6(glu) ORF was validated by sequencing of the isolated plasmid DNA.

2.2.3 Gene expression by quantitative RT-PCR

Quantitative gene expression analysis from 2000 cells or less was performed using the TaqMan® Gene Expression Cells-to-CT™ Kit (Life Technologies) following the manufacturers manual. Cells were harvested by centrifugation for 5-10 min at 290 x g or directly FACS sorted into Lysis solution. Reverse transcription was performed in a thermal cycler at 37°C for 60 min and a RT inactivation at 95°C for 5 min following the manufacturer's protocol.

2.2.3.1 Pre-amplification of specific genes by multiplex PCR

In order to analyze the expression profile or several genes from only few cells a multiplex pre-amplification step was integrated following the manufacturer´s instructions TaqMan® PreAmp Cells-to-CT™ Kit (Life Technologies). Therefore, a pooled assay master mix of 64 TaqMan® gene expression assays were mixed together with 1x TE buffer to a final concentration of 0.2x per assay (Table 10). A pre-amplification of 14 cycles was performed in a thermal cycler using the manufacturer's protocol. The amplified cDNA was diluted into 1xTE-buffer to a final 1 ml and aliquots of 50µl were stored at -20°C.

2.2.3.2 TaqMan® quantitative real time PCR

The quantitative real time PCR with TaqMan® is based 5'-exonuclease activity of Taq DNA polymerase cleaving a hydrolysing DNA-probe during hybridization to the complementary target sequence The DNA probe contains also a fluorophore and a quenching molecule, which are released upon cleavage of the DNA-probe. The resulting fluorescence signal is measured during the exponential stages of the PCR to quantify the accumulation of the product.

For quantification 1 µl of one gene expression assay was mixed into a well of a 96-well PCR-plate (4titude) according to the manufacturer's protocol with either cDNA or pre-amplified cDNA in 20 µl samples. Real-time PCR was performed on a LightCycler^{TM} 430 quantitative PCR machine (Roche). Data analysis was performed using the ΔΔCT-method (Schefe et al., 2006). For multiplex gene expression analysis the geometric mean of six housekeeping genes (*Hprt, B-Act, Pum1, Gusb, B2m, Gapdh*) was used to normalize the data using MS Excel 2007.

2.3 Cell culture

2.3.1 Cultivation of adherent cell lines

The cell lines HEK 293T (human embryonic kidney) and NIH 3T3 (murine embryonic fibroblasts) were cultivated in DMEM medium supplemented with 10 % foetal bovine serum (FBS), 20 mM HEPES and 3.5 mM L-glutamine in a standard cell culture incubator with 5.5 % CO_2 and 95% humidity at 37°C. Every 2 – 3 days, when cells were confluent to 70 – 80 %, passaging was performed using Trypsin-EDTA to detach the cells from the culture flask. Splitting ratio was variable, depending on the particular cell density and the doubling time of the cell line. All manipulations of the cells were performed using S1 and S2 assigned sterile clean bench according to the declared safety working conditions.

2.3.2 Cultivation of suspension cell lines

Suspension cell lines HL-60, U937, 32D ITD and Kasumi were cultivated in RPMI 1640 medium supplemented with 10 % FBS, 2 % L-glutamine in a standard cell culture incubator with 5.5 % CO_2 at 37°C. For 32D cells additional 2 ng/ml IL3 was added to the medium. Every 3 – 4 days, cultures were passaged according to the particular cell density and the doubling time of the cell line.

2.3.3 Conventional cell counting

Cell density was determined by manual cell counting using a Neubauer chamber. Cells were diluted with PBS and mixed with trypan blue indicating dead cells. Next, 10 µl of the suspension were placed on a Neubauer chamber and cell density was calculated by multiplication of the average amount of cells within the four counted squares of the chamber with the dilution factor and the chamber factor (10^4). Total cell count was further calculated by multiplication with the volume of the cell suspension.

2.3.4 Cell counting using Terasaki culture plates

For very low cell numbers 60-well NUNCbrand Terasaki culture plates (Greiner Bio-One, Kremmünster, Austria) were used to determine cell count. Up to 10 µl of diluted or undiluted culture samples were seeded on the conical bottom of the plate by brief centrifugation for 5 min at 290 x g. All visible cells on the bottom of the well were counted and multiplied with the dilution factor and the total volume of culture sample to calculate total cell amount in the given sample. Addition of 1 µl Tryphan blue also allowed live/dead cell discrimination.

2.3.5 Preservation of cell lines and primary cells

Cell lines and primary cells can be long-term stored at -196°C in liquid nitrogen atmosphere. Therefore, cells were pelleted by centrifugation (5 min at 300 g) and resuspended in 10 % DMSO diluted in FBS with a density of $1x10^6$ - $1x10^7$ cells/ml. Subsequently, cells were placed into a freezing box filled with isopropanol to allow slow freezing at -80°C. The next day, frozen cells could be transferred to a liquid N_2 cooled tank.

2.3.6 Thawing of cell lines

Frozen cells were rapidly thawed in a water bath at 37°C and subsequently transferred into pre-warmed culture medium. After centrifugation (5 min at 290 g), cells were resuspended in fresh medium and seeded in a 10 cm culture dish.

2.3.7 Virus production

Virus production was routinely performed using the calcium-phosphate precipitation method for transfection of HEK 293T based on a split-genome four lentiviral vector system by Claudia Jourdan, technical assistant (Dull et al. 1998). At the first day for each virus four 10 cm culture plates were seeded with 5x 10^6 HEK 293T cells in 10 ml fresh DMEM complete medium. On the second day the lentiviral expression plasmid (coding for the ORF of the desired gene), together with 5 µg of the pRSV.HIV-REV (coding for lentiviral reverse transcriptase), 12 µg pMDLg.HIVGag-Pol.pRRE (coding for HIV or structural genes Gag and Pol) and 12 µg pMD2.G.VSV-G (coding for the VSV-G envelop protein pseudo typing the lentiviral particles) were diluted into 1.8 ml endotoxin-free water and 200 µl 2.5 mM calcium chloride was added. Under constant air bubbling with a 2 ml pipette 2 ml 2x HEPES buffer was added drop wise to allow calcium phosphate precipitation of DNA complexes. After brief vigorously shaking the samples were incubated at room temperature for 20 min. In the meantime the medium of the cultured HEK 293T cells was changed to 8 ml fresh DMEM complete before 1 ml of the DNA-calcium-phosphate samples were added. After 6 hour in a cell culture incubator with 5.5 % CO_2 at 37°C the medium was removed and exchanged by fresh DMEM complete. On the fourth day the medium containing viral particles was completely removed from the HEK 293T cells. Remaining cells were sediment by centrifugation at 290 x g. The supernatant was sterile filtered with a 0.022 µm filter and then transferred into 50 ml ultracentrifugation buckets and centrifuged for 1 hour at 50,000 x g and 4°C to concentrate the viral particles. The supernatant was completely removed and the viral particles were resuspended in 200 µl SFEM. The viral particles were stored in 15 µl aliquots at -80°C.

2.3.8 Viral titer calculation

To calculate the titer of viral particles in the viral stocks 3x 10^4 NIH 3T3 cells were seeded into a well of 24-well culture plate. On the next day the exact cell number was determined from two of the 24 wells by cell counting. One viral stock aliquot was thawed and three dilutions were made from10^{-1} to 10^{-3} in DMEM. Each 10 µl of the virus dilutions were added to three wells containing NIH 3T3 cells. After 48 hours incubation the cell were harvested with Trypsin EDTA and washed once with DMEM medium. After centrifugation the triplicates were combined and suspended in 300 µl FACS buffer. The percent of transduced cells was determined by fluorescent reporter expression via FACS. The viral titer was calculated as virus particle per ml virus stock using the transduction efficiency the cell number at time of infection and the dilution factor.

Equation 1: virus titer calculation

Virustiter (particle/ml) = transduction efficiency x cell number x dilution factor (1/ml)

2.4 Prospective isolation of hematopoietic progenitor cells by FACS

To analyze early hematopoietic fate decision in detail it is absolutely prerequisite to enrich defined hematopoietic stem and progenitor cells to a very high purity. As already described in the introduction the hematopoietic system is a highly hierarchical organized organ with the hematopoietic stem cells at the very top, which can generate all mature hematopoietic cells. Because the hematopoietic system is very well understood we can use prospective markers to identify and sort the different cell populations by FACS.

2.4.1 Isolation of BM cells (BMCs) and lineage depletion

To isolate hematopoietic progenitors for further studies routinely femurs, tibiae, os coxae and sternum of 10 to 16 week old mice of the C57BL/6 strain were isolated and cleaned from remaining tissue. The isolated bones were crush with mortar and pestle in 5 ml PBS containing 10% FBS. The supernatant was filtered through a 100 µm filter to extract BMCs. The extraction was repeated with a total volume of 50 ml PBS 10% FBS. The amount of collected BMCs were calculated by manual cell counting 2.3.3 and resuspended in at FACS buffer at a concentration of 10^8 BMCs/ ml. In average 2.5- 3x 10^8 BMCs per mouse could be isolated. To enrich for progenitor populations a cell depletion of lineage positive cells was performed using the EasySep™ Biotin Selection Kit (Stemcell Technologies, Vancouver, Canada). Therefore the BMCs were marked with antibodies against mature blood cells labeled with biotin at a concentration of 0.1 to 0.2 µg/ ml each (CD3, CD11b, CD19, CD41, B220, Gr-1 and Ter119). The cells were washed and lineage depletion was performed with a fifth of

the recommended biotin selection cocktail according to the manufacturer's protocol. This procedure allows eliminating 90% of the mature cells from the cells which have to be sorted; the recovered lineage depleted cells are roughly 10% of the total bone marrow cells and enriched in the progenitor fractions, which are negative for differentiation markers.

2.4.2 Ficoll enrichment of BM mono nuclear cells

Femurs, tibiae and os coxae were isolated from each mouse separately for single mouse analysis and cleaned from remaining tissue. BMCs were extracted from bones in 5 ml sterile PBS containing 2% FBS with a 5 ml syringe plus blunt needle. If whole BMCs were needed for subsequent experiments the cells were counted, washed at least twice with PBS and an appropriate aliquot of cells were resuspended in FACS buffer and placed on ice for further process. To enrich for bone marrow mononuclear cells (BMMNCs) the remaining cells resuspended in 5 ml PBS solution were carefully placed on top of 5 ml Histopaque® 1083 (Sigmas) and isolated by ficoll density gradient centrifugation according to the manufacturer´s instructions. The interphase was collected and washed twice with PBS before the cells were counted. For progenitor analysis the BMMNCs were stained with biotinylated antibodies against mature blood cells (CD3, CD11b, CD19, CD41, B220, Gr-1 and Ter119).

2.4.3 Isolation of hematopoietic cells from spleen

The spleen was isolated and weighted on a small scale balance Sartorious Ac210s (Sartorius AG). Then it was cut with laboratory scissors and minced through a 100 µm cell strainer. Extracted cells were washed twice with PBS and resuspended in 100 µl FACS buffer. $2x10^6$ cells were stained with antibodies against hematopoietic mature cell markers.

2.4.4 Staining and sorting of murine hematopoietic progenitor populations

The lineage depleted cell fraction was counted and resuspended in 50 µl FACS buffer. In a next step lineage depleted fraction was stained with fluorescence labeled antibodies against progenitor markers (CD34, SCA-1, CD117, CD48, CD150, CD16/32), streptavidin against biotinylated lineage antibodies and the fixable viability dye eFluor® 506 (eBioscinece) following the staining protocol for 10^7 lineage depleted cells Table 15; the cells were incubated light shut for 30 min on ice before the cells were washed once with 15 ml PBS removing excessive antibodies. Single staining of each used fluorescent antibody was performed on lineage positive cells allowing compensation of the different fluorescent dyes. The cells were transferred to sterile polypropylene 5 ml FACS through a 70 µm cell filter to separate the cells and avoid cell clogging during cell sorting.

Table 15: Fluorescent antibody staining of lineage depleted progenitors for cell sorting

antibody	CD34	SCA-1	CD117	CD48	CD150	CD16/32	Streptavidin
clone	Ram34	D7	2B8	HM48.1	TC15-12F12.2	2.4G2	
fluorophore	eF660	PerCP-Cy5.5	PE-Cy7	FITC	PE	BD V450	APC-eF780
volume	total 25 µl	2 µl	3 µl	2 µl	3 µl	3 µl	2 µl

FACS Aria I and Aria III (Becton Dickinson) were used to prospectively sort for distinct cell types with specific marker combinations. The FACS Aria sorters were controlled with the software interphase of FACS Diva 7 (BD). Data analysis was performed with FACS Diva and FlowJo (Treestar, Inc.) software. A representative FACS Blot for the sort of distinctive hematopoietic progenitor populations is shown in Figure 6. Lineage negative cells were gated out of the viable mononuclear cells. From those cells the SCA-1, CD117 double positive (LSK; lineage-, SCA-1+, CD117+) hematopoietic stem progenitor cells (HSPCs) were subgated, which are roughly 0.5% of the progenitor enriched bone marrow population. This fraction contains the multipotent progenitors (MPPs; LSK, CD34+, CD150-) of about 0.4% and the long-term hematopoietic stem cells (LT-HSCs; LSK, CD34low, CD150+, CD48-), which are less than 0.05% of the progenitor enriched bone marrow cells. Taking into account that only a tenth of the total bone marrow cells is analyzed by FASCs calculated the LT-HSC to be one in 20.000 bone marrow cells. Additionally already restricted megakaryocyte-erythrocyte progenitors (MEPs; lineage-, SCA1-, CD117+, CD34-, CD150+) or granulocyte-macrophage progenitors (GMPs; lineage-, SCA-1-, CD117+, CD34+, CD150-, CD16/32+),which can only produce cells of their specific lineages, can be sorted at the same time. The sorted progenitor populations were usually collected into sterile polypropylene FACS-tubes containing 1 ml SFEM supplemented with 100 ng/ml SCF and TPO. After sorting the cells were counted with Terasaki plates (section 2.3.4) and kept on ice for further experiments.

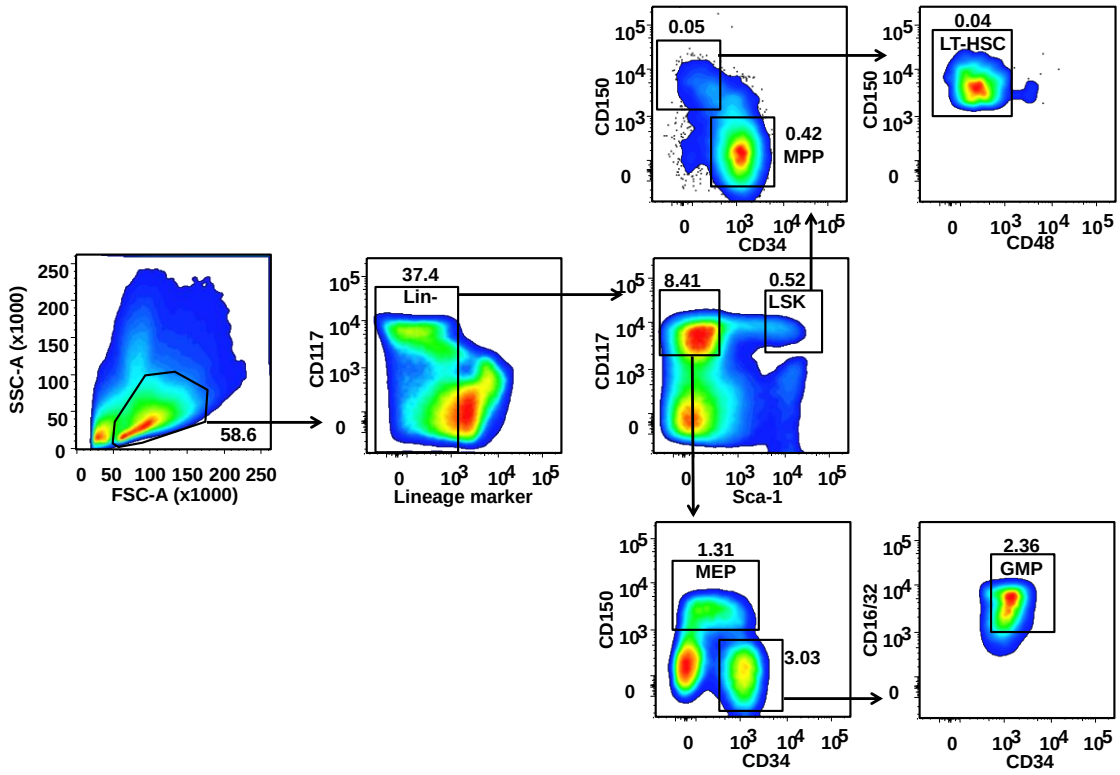

Figure 6: Sorting of hematopoietic stem and progenitor populations.

Representative sorting scheme of hematopoietic stem and progenitor populations from adult mouse bone marrow by FACS with indicated percentages, staining and sorting as described in Methods section. Sorting purity was always above 95%, as determined by FASC reanalysis of at least on progenitor fraction. Different progenitor cell populations were prospectively isolated using the following surface marker combinations: LSK (lin- SCA-1+ CD117+); LT-HSCs (lin- SCA-1+ CD117+ CD150+ CD34- CD48-); MPPs (lin- SCA-1+ CD117+ CD150- CD34+); GMPs (lin- SCA-1+ CD117+ CD150- CD34+ CD16/32+); MEPs (lin- SCA-1+ CD117+ CD150+ CD34low)

2.5 Primary hematopoietic cell assays

2.5.1 Hematopoietic cytokine stimulation of hematopoietic progenitors

For stimulation of hematopoietic progenitors with different cytokines LT-HSCs or MPPs were sorted into SFEM only containing SCF and starved for 2h at 37°C and 5%CO_2 before adding indicated cytokines (Peprotech) at 100ng/ ml for 1h stimulation in the presence of 10µg/ ml Cycloheximide (Chx, Invitrogen). Cells were washed 2 times and subjected to cDNA synthesis as described in section 2.2.2.2. Real-time PCR with TaqMan-assays (*Gadd45a*, *Gadd45b*, *Gadd45g*, Hprt, B2m) was performed on a LightCycler™ 430 (Roche) as described above section 2.2.3.2. The expression was normalized to *B2m* and shown relative to the unstimulated control.

2.5.2 In vitro differentiation assay of primary hematopoietic cells

Freshly sorted LT-HSCs and MPPs were counted and 100 cells were seeded into 96-well culture plates in 100 µl SFEM (Stemcell Technologies) supplemented with 100 ng/ml SCF and TPO (Peprotech). Cells were transduced with lentiviral particles using a multiplicity of infection (MOI) of 100 and cultured at 5% CO2 and 37°C for 3 to 10 days. For blockage of the kinase p38 the following inhibitors were added at indicated concentrations: BIRB796 (0.4 µM), SB203580 (10 µM), VX-702 (1 µM), LY2228820 (0.1 µM, all from Selleck), and for kinase JNK the inhibitors SP600125 (12.5 µM), JNK-IN-8 (1 µM, from Merck) were used. For differentiation promoting conditions SFEM was supplemented with 100 ng/ml SCF and TPO plus 20 ng/ml IL3 and IL6. All individual conditions were plated routinely as triplicates. At the indicated days of analysis the cells were harvested and an aliquot was used for cell counting on Terasaki plates described in section 2.3.4. Cells were stained with 100 to 40 ng (each 0.2 µl) fluorescence labeled antibodies against hematopoietic surface markers (CD48, CD117, CD16/32, CD11b and Gr1) to determine differentiation status. Fixable viability dye (eFluor506 and eFluor780, eBioscience) was added for live dead discrimination. Cells were washed once in PBS and resuspended in 200 µl FACS buffer. The flow cytometric data of the cells were collected on FACS CantoII or FACS FortessaII (BD) and analyzed using the FACS Diva (BD) or FlowJo (Treestar, Inc.) software.

2.5.3 Colony formation assay

For colony formation assays, 300 freshly sorted LT-HSCs, GMPs, or 900 MEPs were lentivirally transduced with an MOI of 100 in SFEM only containing 100 ng/ ml SCF. After a 24 hour incubation for the transduction, the cells were mixed into 3 ml freshly thawed methylcellulose medium (M3434, Stemcell Technologies) and vortexed. For MEPs 50 ng/ml TPO was added into the M3434 medium. 1.1 ml of the mixture was seeded into a 3 cm culture dish (Thermo Fischer Scientific). After 9 to 12 days incubation at 37°C with 5.5% CO_2 in cell incubator the cells are scored microscopically for successful transduction, colony formation and lineage distribution. To analyze the remaining colony forming potential in a sample serial re-plating of the derived cells were performed. Therefore the complete cells were washed off the culture plate with 5 ml PBS and washed at least three times to remove remaining methylcellulose. After cell counting the appropriate cell number was again diluted in 3 ml methylcellulose medium as described above.

2.5.4 Time-lapse imaging

For time-lapse imaging, 24-well culture plates (NUNCbrand, Greiner Bio-One) were equipped with silicon stem cell inserts (IBIDI), which prevent the migration of the cells out of the camera field. The silicon inserts consisted of 4 separated inner wells (positions) and 25 – 100 freshly sorted LT-HSCs were seeded per position. The cells were sedimented by brief centrifugation for 7 min at 290 x g. Virus particles were added with a MOI of 100 in 200 µl SFEM plus 100 ng/ ml SCF and TPO. FITC-conjugated anti-CD16/32 was added to the medium at 50 ng/ ml. After 3 hour incubation in the cell culture incubator (37°C, 5% CO_2) to saturate the culture plate with CO_2 the plates were gas-tight sealed with adhesive tape and placed inside a CellObserver 430 (Zeiss) microscope. This custom made microscope is equipped with remote-controllable, movable plate stage, a black housing and a temperature module to maintain optimum temperature (37°C) for cell culture. Phase contrast images were acquired every 2-3 min using a 10x phase contrast objective (Zeiss), and an AxioCamHRm camera (at 1388x1040 pixel resolution) with a self-written VBA module remote controlling Zeiss AxioVision 4.8 software. Fluorescence was detected every 1 hour with HXP illumination (Osram) and the filter sets for FITC (EGFP; F45-002) and tdTOMATO (F4-004, AHF Analyzetechnik).

2.5.4.1 Single cell tracking with Timm´s Tracking Tool

Tracking of individual cells and their progeny was performed manually with the help a self-written computer program (Timm´s Tracking Tool; TTT) developed by Timm Schroeder (Rieger, et al., 2009) which can determine and record multiple parameters of individual cells at each time point as well as their complete affiliations. The pictures from the time-lapse imaging are loaded into TTT. Single cells were observed and tracked manually over time by marking the cell, while skipping through single pictures. Cell fates such as cell division, cell death, lost or fluorescent reporter expression observed while tracking are added into the TTT interface and thereby saved into the pedigree. Cell tracking was performed for individual HSCs until the fate of all progeny in the fifth cell generation was determined. The generation time of an individual cell was defined as the time span from cytokinesis of its mother cell division to its own division. Dead cells were easily depicted by their shrunk, non-refracting appearance with immobility. Transgene expression was determined by nuclear membrane tdTOMATO fluorescence and expression of surface CD16/32 indicated the GMP-like stage of development.

2.5.5 Phosphoflow cytometry

FACS-sorted MPPs were transduced with indicated lentiviral particles (MOI 100) and cultured for 5 days in SFEM supplemented with 100 ng/ ml SCF and TPO. On the fifth day cells were fixed with 500 µl Fix Buffer I (BD) at 37°C for 20 min. Cells were washed twice with PBS containing 10% FBS, the supernatant was completely removed and the cells were then permeabilized by drop wise addition of 500 µl ice-cold Perm Buffer III (BD) under constant shaking. After 30 min incubation on ice the permeabilized cells were washed once and subsequently stained with phosphor-specific antibodies P-p38-PE and P-MK2-Alexa Fluor 647 (BD) to analyze the phosphorylation status of the cells by FACS. As positive control 5 day cultured MPPs were stimulated with 10 µg/ ml Anisomcyin (Sigma) for 30 min prior to fixation. A second sample was kept unstimulated to determine background signaling.

2.5.6 In vitro cell proliferation assay

10000 FACS-purified MEPs or GMPs seeded into 96-well culture plate were lentivirally transduced (MOI 50) and cultured for 5 days in SFEM supplemented with indicated cytokines. Cell expansion was determined visually by microscopy and cell counting of single wells (section 2.3.3). Viable cells were determined with the ViaLight Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza) according to the manufacturer´s instructions. This assay measures the amount of ATP in cell lysates of a culture sample via bioluminescent reaction; since all living cells require ATP to carry out their biochemical processes, the amount of ATP correlates with the number of viable cells within the cell lysate. Luminescence was measured with a Mithras LB940 luminometer (Berthold Technologies).

2.6 Animal experiments

2.6.1 Animal husbandry conditions

Mice were kept and bred according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) in the animal husbandry facility of Georg-Speyer-Haus (Frankfurt, Germany). All used mouse strains C57BL/6J, B6.SJL and *Gadd45g*$^{-/-}$ (Gadd45g^{tm1Flv} (Lu, et al., 2001)) were purchased from Charles River, Sulzfeld, Germany. Gadd45g^{tm1Flv} mice were bread in heterozygous Gadd45g$^{tm1Flv/wt}$ pairings giving birth to both homozygous Gadd45g$^{tm1Flv/tm1Flv}$ and wild type littermates for direct comparison. The animals were kept in individually ventilated cages (IVC) at a maximum of 7 animals per cage on a standard rodent diet (ssniff, Soest, Germany) and mains water at constant 22°C and with an 12 hour light-dark interval. General health status of all animals was monitored daily

by the scientist, the animal caretakers or the veterinarian. All mouse in vivo experiments were approved by the relevant local authorities (Regierungspräsidium Darmstadt).

2.6.2 Blood collection from mice

To obtain blood from mice by tail vein incision, mice were pre-warmed by a red light lamp for 10 – 15 min and subsequently placed in a mousetrap. Next, the tail vein was punctured with a scarp 0.6 mm needle and about 25 – 60 µl blood was collected per mouse using EDTA-coated microvettes. Erythrocytes were lysed using the Pharm lyse™ puffer (BD bioscience) according to the manufacturer's protocol. Thereafter, the remaining white blood cells were stained with fluorescence labeled antibodies against CD45.1, CD45.2, CD3, B220, Ter119, and CD11b / Gr1 (Table 12) for donor engraftment and lineage distribution and a Fixable Viability Dye (eBioscience) for dead/live cell exclusion. After washing with 10 ml PBS the cells were resuspended in 200 µl FACS buffer and subjected to FACS analysis. Lentivirally transduced hematopoietic cells were detected by their VENUS or tdTOMATO expression.

2.6.3 Competitive repopulation assay

To analyze the self-renewal capacity of different LT-HSCs competitive repopulating assays were performed. Mice from the mouse strain B6.SJL expressing the pan-hematopoietic marker variance CD45.1 were used as recipients to discriminate donor hematopoietic cells from C57.BL/6 or Gadd45g^{tm1Flv} mice expressing variance CD45.2. Recipient B6.SJL mice were lethally irradiated with 9 Gy using a Biobeam 2000 (Gamma-Service Medical GmbH, Leipzig, Germany) with a Cs137 gamma irradiation source 2 to 4 hours prior to transplantation. For GADD45G overexpression 350 FACS-sorted LT-HSCs from C57.BL/6 mice were lentivirally transduced with a MOI of 100 of either GADD45G or empty vector 24 hours prior transplantation. Transduction efficiency of the transplanted LT-HSCs was determined with a remaining cell aliquot after three additional days in culture by FACS. Lentivirally transduced cells were harvested and an equivalent of 350 LT-HSCs was mixed with $2x10^5$ ficoll-enriched BM mono nuclear competitor recipient cells from B6.SJL mice. The transplant mixture was tail vein injected into lethally irradiated B6.SJL (CD45.1$^+$) recipient mice. Freshly sorted 350 FACS-sorted LT-HSCs from aged matched *Gadd45g*$^{-/-}$ and *Gadd45g*$^{+/+}$ littermates, were tail vein injected into lethally irradiated B6.SJL (CD45.1$^+$) recipients together with $2x10^5$ ficoll-enriched BM competitor recipient cells.

Multi-lineage reconstitution was determined every 4 to 6 weeks post transplantation in peripheral blood as described in section 2.6.2. Primary recipients were sacrificed 21 weeks after transplantation, and $5x10^5$ ficoll-enriched BMCs (section 2.4.2) per mouse were

transplanted into a lethally irradiated secondary B6.SJL recipient mice treated as described above. For BM reconstitution analyses, ficoll gradient-enriched BMCs were pre-stained with biotin labeled antibodies of mature cell markers as described in section 2.4.2 followed by staining with fluorescence labeled antibodies CD45.1, CD45.2, CD34, SCA-1, CD117, CD48, CD150, CD16/32, streptavidin and Fixable Viability Dye (eBioscience) for progenitor population analysis. Reconstitution of mature blood cells was performed on total BMCs from primary transplant stain with fluorescence labeled antibodies against CD45.1, CD45.2, CD3, B220, Ter119, and CD11b / Gr1. Used FACS antibodies are listed in Table 12. Analysis of reconstitution was analyzed on a three- of four-laser FACS Aria or FortessaII. Secondary recipients were monitored and analyzed in the same manner as described for the primary transplant.

2.6.4 Short-term transplantation

Similar to competitive repopulation assay 10000 LSK cells from C57.BL/6 mice (CD45.2+) were lentivirally transduced 24 hour prior transplantation (section 2.6.3). Transduction efficiency of the transplanted LSKs was determined with a remaining aliquot of the transduced LSKs three days after transduction by FACS. After cultivation for 24 hours transduced cells were tail vein injected into lethally irradiated B6.SJL (CD45.1+) recipients together with $2x10^5$ BM competitor cells from B6.SJL. Mice were sacrificed 11 days after transplantation and the engraftment and distribution of stem and progenitor cell populations was analyzed by FACS as described in previous section 2.6.3.

2.6.5 Proliferation of shortly transplanted lentivirally transduced cells by BrdU incorporation

For the determination of proliferation in transplanted cells 8000 LSK cells from B6.SJL ($CD45.1^+$) were lentivirally transduced with GADD45G expressing vector. After cultivation for 24 hours the cells were injected into tail vein of lethally irradiated C57.BL/6 ($CD45.2^+$) recipient cells in presence of $2x10^5$ ficoll-enriched BM competitor cells from C57.BL/6 mice**.** To analyze the proliferation rate of transplanted cells Bromo-deoxyuridine (BrdU) uptake in donor and recipient hematopoietic cells was analyzed 7 and 14 days after transplantation. Therefore 1.5 mg BrdU (BD) was injected intraperitoneally 4 hours before the mice were sacrificed. Dead cells were determined by Fixable Viability Dye (eBioscience) staining and BrdU incorporation in living cells was determined according to the manufacturer´s instructions using the proliferation assay BD Pharmingen™ APC BrdU Flow Kit (BD Bioscience) with a PE-labeled anti-BrdU antibody (eBioscience) together with florescent-

antibody staining for CD45.1 and CD45.2 by FACS. Lentivirally transduced hematopoietic cells were detected by their VENUS fluorescence.

2.7 Statistical analyses

FACS analysis was performed using FACS Diva (BD) or FlowJo (Treestar, Inc.) software. The distributions of various populations from FACS analysis and combination of repeated experiments were calculated with MS office 2010 (Microsoft). Statistical analyses were performed with the program Prism 5 and 6 (GraphPad Prism Software Inc.) routinely calculating students T-Test with statistical significance levels *: $P<0.05$; **: $P< 0.01$; ***: $P< 0.001$

3. Results

3.1 Cytokines stimulate Gadd45g expression in hematopoietic stem cells and multipotent progenitors

While cytokines can induce expression of GADD45 proteins in differentiated hematopoietic cells (Beadling et al. 1993; Zhang et al. 1999b; Gupta et al. 2006b; Ju et al. 2009; Chi et al. 2004), it was not known whether *Gadd45* expression is also up-regulated in LT-HSCs or MPPs upon cytokine exposure. Sorted murine LT-HSCs were stimulated with TPO or IL3 after starvation, and the expression levels of all *Gadd45* genes were quantified via qPCR. While *Gadd45g* was strongly up-regulated by TPO stimulation, IL3 only had a minor effect. *Gadd45a* and *b* were unaffected by either TPO or IL3 stimulation (Figure 7**A**).

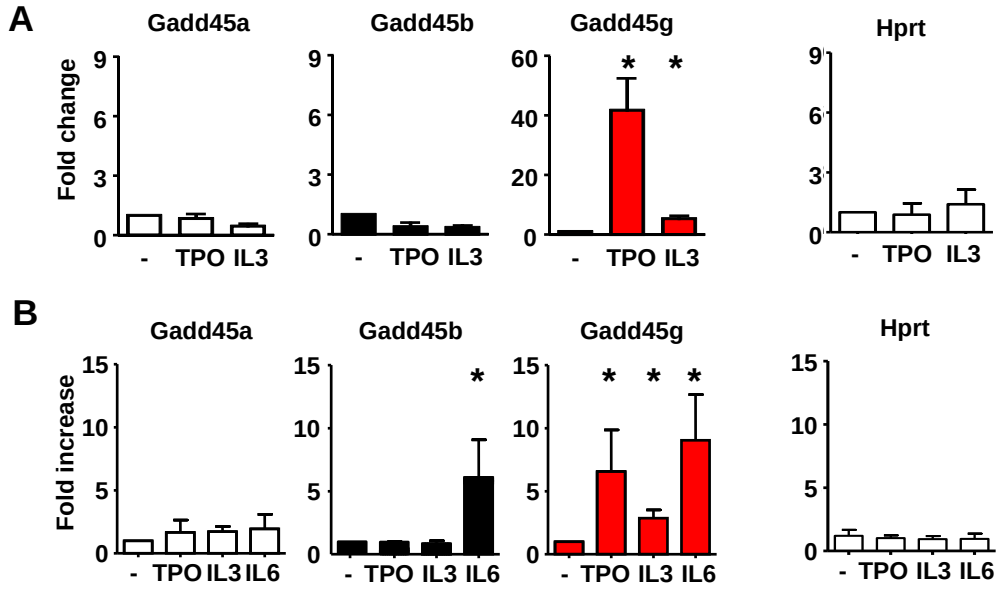

Figure 7: Hematopoietic cytokines stimulated the expression *Gadd45g* in HSPCs.

A) Quantitative RT-PCR of *Gadd45* genes in LT-HSCs stimulated with indicated cytokines for 1 hour. N= 3 experiments. Relative expression normalized to *B2m*. B) Quantitative RT-PCR of *Gadd45* genes in MPPs stimulated with indicated cytokines for 1 hour. N= 4 independent experiments. Relative expression normalized to *B2m*. *Hprt* served as second housekeeping gene, expression levels were unchanged by cytokine stimulation. The mean +/- SD is displayed. Statistical significance was calculated with student T-Test* P<0.05

Next it was assessed whether the expression of the *Gadd45* family members was induced by cytokines in MPPs, since they strongly express the respective receptors. Whereas *Gadd45a* was not regulated by any of the cytokines, *Gadd45b* was up-regulated only upon IL6 stimulation (Figure 7B). *Gadd45g* was strongly induced by TPO, IL3 and IL6 stimulation. However, the basal expression of *Gadd45g* was weak (Figure 7B). Since IL3 and IL6 are used in differentiation-promoting culture conditions in vitro (Ema et al. 2000), we hypothesized

that the activation of *Gadd45g* by differentiation promoting cytokines may link *Gadd45g* with the induction of differentiation in HSPCs.

3.2 GADD45G immediately induces the differentiation in HSCs and accelerates myelomonocytic differentiation

The stimulation of *Gadd45g* expression in LT-HSCs and MPPs by hematopoietic cytokines points to a vital role of GADD45G in early hematopoietic progenitors. To simulate the induction of GADD45G by cytokine stimulation in LT-HSCs and to separate the GADD45G function from additional effects induced by these cytokines, an ectopic lentiviral expression system was used (section 2.2.2.3). GADD45G was lentivirally expressed in freshly sorted murine LT-HSCs and the cells were cultured for several days in vitro. The transduced cells were distinguished by fluorescent protein (tdTOMATO+ or VENUS+) expression encoded from a bicistronic transcript via an internal ribosome entry site (GADD45G-IRES-FP). The cell count, percent of transduced cells and differentiation status of LT-HSCs were determined after 3, 5, 8 and 10 days by FACS (Figure 8A). Antibodies against differentiation stage specific surface markers were used to monitor the differentiation of transduced LT-HSCs over time. The most undifferentiated cells were depicted as $CD117^+$, $CD48^-$, $CD16/32^-$, $CD11b^-$, and $Ly\text{-}6G^-$ (referred to as immature progenitor cells). Cells already committed to granulocyte-macrophage lineage expressed CD48 and CD16/32 and were called GMP-like cells ($CD117^+$, $CD48^+$, $CD16/32^+$, $CD11b^-$). Further differentiation into myeloid cells was accompanied by up-regulation of CD11b and loss of CD117 ($CD117^-$, $CD16/32^+$, $CD11b^+$, referred to as mature myeloid cells). Finally, mature granulocytes could be identified by the additional expression of Ly-6G ($CD117^-$, $CD16/32^+$, $CD11b^+$, $Ly\text{-}6G^+$). Myeloid differentiation is visualized by simplified FACS blots starting form double negative immature cells through CD16/32 single positive GM-committed cells to double positive mature myeloid cells (Figure 8C). This differentiation is highly accelerated in GADD45G-expressing LT-HSCs as shown in the FACS blots of CD16/32 vs CD11b at different time points (Figure 8C).

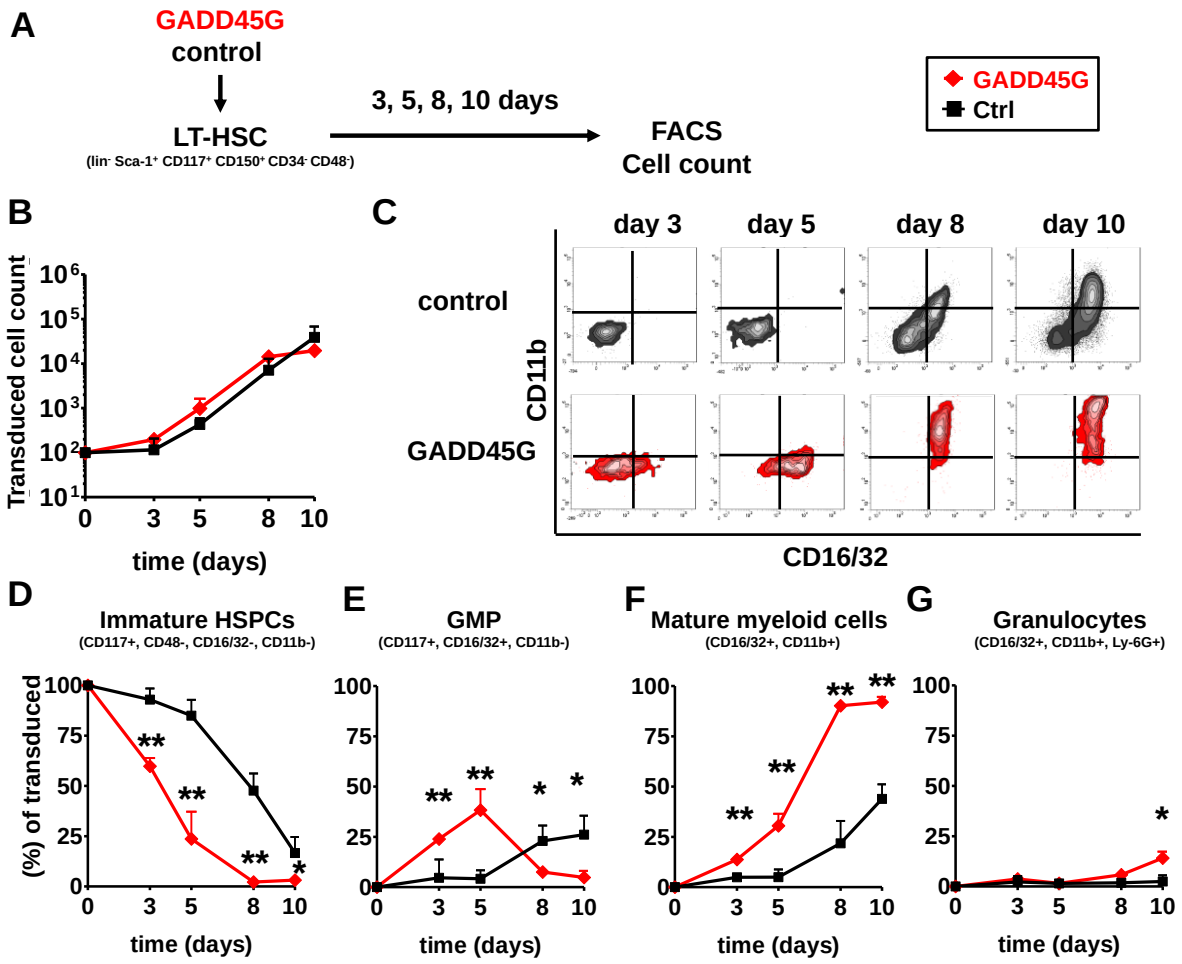

Figure 8: Enhanced differentiation of GADD45G-expressing LT-HSCs in vitro

A) Experimental scheme of the in vitro differentiation assay. B) Cell expansion of GADD45G- and control-transduced cells. Only transduced cells were counted. C) Representative FACS blots of cultured LT-HSC, gated for GADD45G- and control-transduced cells (tdTOMATO+). D-G) Quantification and statistical analyses of FACS results for each time point in liquid culture. Transduced cells (tdTOMATO+ or VENUS+) were gated for immature HSPCs, GMP-like cells, mature myeloid cells and granulocytes. N=3 independent experiments. The mean +/- SD is displayed. * P<0.05; ** P<0.01.

Contrary to the published function of GADD45G in growth arrest of cancer cell lines, forced GADD45G expression in murine LT-HSCs did not inhibit the proliferation of HSCs and their progeny at the population level (Figure 8B). The hematopoietic cells derived from the transduced LT-HSCs expanded more than 1000-fold over the first 8 days, displaying the high proliferative potential of LT-HSCs and their progeny. After 8 days, the GADD45G-expressing cells expanded slower compared to the control cells. This can be explained by the fact that HSCs expressing GADD45G undergo accelerated differentiation and stop proliferating once they have fully differentiated (Figure 8C). At day 3 of culture, only 5% of the control vector transduced LT-HSCs expressed the GMP like marker pattern, while already 25% of the GADD45G-expressing LT-HSCs reached the GMP-like stage of differentiation

(Figure 8E). Along the same line, the percentage of immature progenitor ($CD117^+$, $CD48^-$, $CD16/32^-$) cells dropped drastically upon GADD45G induction while over 90% of the control cells still resided in the very immature cell fraction at day 3 (Figure 8D). The immature cell population virtually disappeared under GADD45G expression at day 8, whereas it still comprised 48% of the cells in the control. On day 8, the majority of GADD45G-expressing cells had differentiated into mature cells of the granulocytic and monocytic lineage ($CD16/32^+$, $CD11b^+$) (Figure 8F). The control cells could reach that level of differentiation after 14 days (data not shown). Also the generation of mature granulocytes was significantly enhanced in the GADD45G population (Figure 8G). The FACS results were also confirmed by morphological signs of differentiation using giemsa staining (data not shown).

Taken together, the entire differentiation process in HSCs expressing GADD45G was accelerated by 4 to 5 days compared to control HSCs. Expression of GADD45G in LT-HSCs rapidly induces differentiation and accelerates the myelomonocytic lineage development.

3.2.1 GADD45G dimerization is essential for GADD45G induced differentiation

The crystal structure of GADD45G revealed a homo-dimerization interface (Schrag et al. 2008), in which two essential amino acids, T79 and L80, could be identified. Mutation of either one of these amino acids to glutamate (named T79E and L80E, respectively) resulted in dimerization-deficient non-functional proteins (Schrag et al. 2008). It was of interest to test whether this dimerization of GADD45G was necessary for the accelerated differentiation induction function in LT-HSCs. Two mutant murine GADD45G T79E and L80E constructs were generated by side directed mutagenesis of the GADD45G-IRES-VENUSnucmem vector (performed by Pangrazio de Giacomo during his diploma thesis, (de Giacomo 2012)). HEK293T cells transduced with both mutant constructs expressed the GADD45G protein. The mutant T79E and L80E constructs were then lentivirally transduced in LT-HSCs and their differentiation kinetics were analyzed as described in section 3.2.

The LT-HSCs transduced with dimerization mutants T79E and L80E proliferated and differentiated similar to the control vector transduced cells (Figure 9A; Figure 9B). Both populations lost the immature cells in a similar rate as the control construct while GADD45G-expressing LT-HSCs differentiated rapidly (Figure 9C). The generation and differentiation of the GMP-like cells with T79E and L80E constructs followed similar kinetics as with the control cells (Figure 9D). Wild type GADD45G-expression showed the expected accelerated differentiation in a 10 day culture. T79E cells gave rise to higher mature granulocyte and macrophages numbers (30%) compared to the control and L80E-transduced cells (19%) at

day 10 (Figure 9E). This was concordant with previously published data that the L80E mutation was more efficient in abolishing dimerization (Schrag et al. 2008).

To conclude, GADD45G dimerization is essential for the enhanced and accelerated differentiation of LT-HSCs into myelomonocytic cells. The GADD45G L80E mutant was used as an additional negative control in several assays.

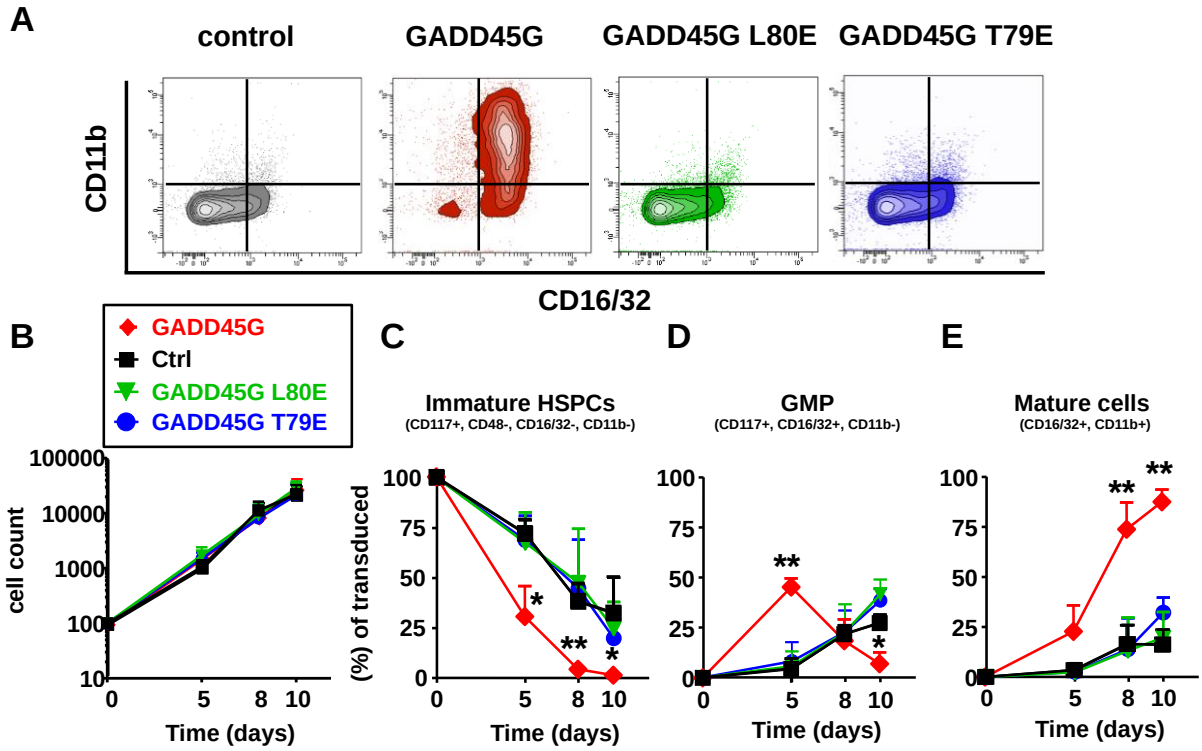

Figure 9: GADD45G dimerization was necessary for differentiation induction

A) Representative FACS blots of cultured LT-HSC after 8 days, gated for viable, transduced cells (VENUS+) B) Cell expansion of GADD45G-, mutant GADD45G- and control vector-transduced cells C-E) Quantification and statistical analyses of FACS results for each time point in liquid culture. Transduced cells (VENUS+) were gated for immature HSPCs, GMP-like cells, mature granulocytes and macrophages. N=3 independent experiments. The mean +/- SD is displayed. * P<0.05; ** P<0.01.

3.2.2 GADD45 alpha and beta also accelerate differentiation of LT-HSCs

The three GADD45 family members exhibit different functions in a cell type specific manner (Liebermann, Hoffman 2008). Since only GADD45G was stimulated in LT-HSCs by cytokines the three GADD45 members may play different roles in early hematopoiesis. FACS sorted LT-HSCs were transduced with lentiviruses coding for GADD45A, B or G (section 2.2.2.3). The amount of immature cells, GMP-like cells and mature cells of the granulocytic and macrophage lineage arising from the transduced cells were compared. This project was

performed together with Pangrazio Samuele De Giacomo during his diploma thesis (de Giacomo 2012).

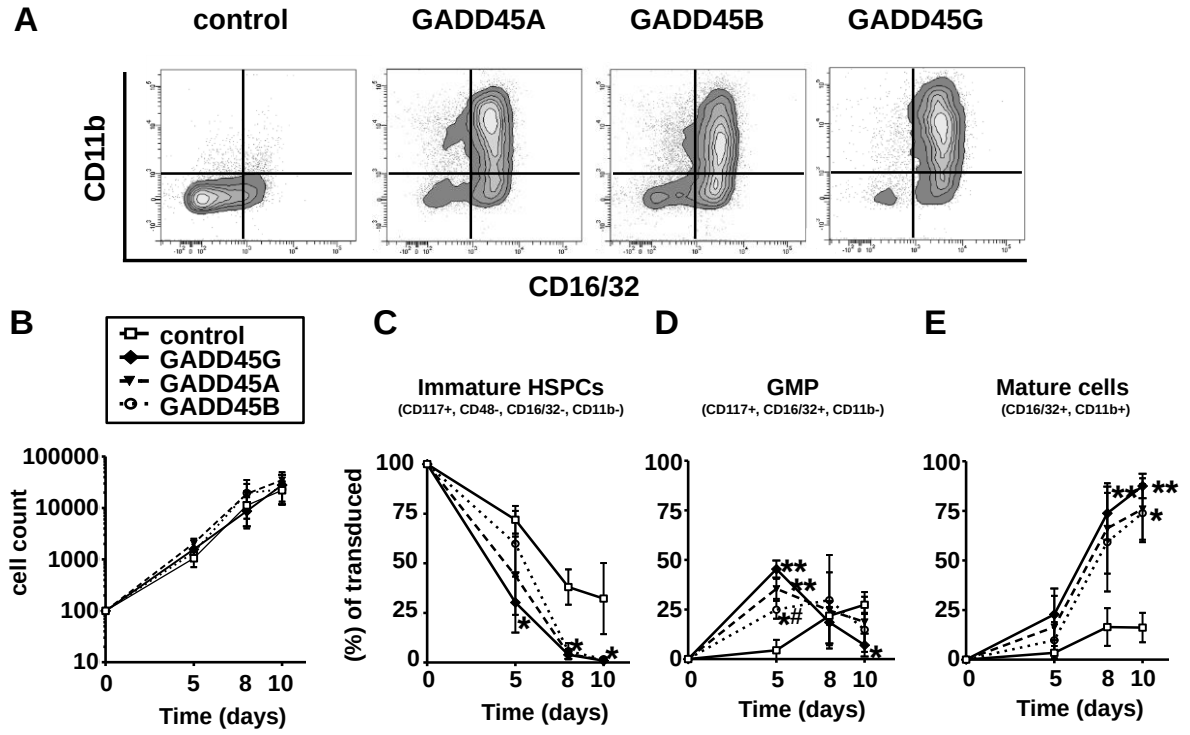

Figure 10: GADD45A and GADD45B also induced and accelerated differentiation in LT-HSC in vitro

A) Representative FACS blots of cultured LT-HSC after 8 days, gated for transduced cells (VENUS+) B) Cell expansion of GADD45family members and control transduced cells C-E) Statistical analyses of FACS results for each time point of liquid culture. Transduced cells (VENUS+) were gated for immature HSPCs, GMP-like cells, mature granulocytes and macrophages. N=3 independent experiments. The mean +/- SD is displayed. Significance to control * P<0.05, ** P<0.01 or to GADD45G # P<0.05 was calculated by T-Test.

All three proteins accelerated the differentiation of transduced LT-HSCs into cells of the granulocyte-macrophage lineage, as seen by the high percentage of mature cells ($CD16/32^+$, $CD11b^+$) after 8 days of culture (Figure 10A). Furthermore, none of the three proteins inhibited the proliferation of the LT-HSCs and their progeny (Figure 10B), as could have been expected from published data using cells lines (Zhan et al. 1994; Kearsey et al. 1995; Zhan et al. 1999; Vairapandi et al. 2002). In general, GADD45A, B and G promoted the differentiation of LT-HSCs. While more than 30% of the control cells remained at the immature stage, less than 10% of the GADD45-transduced cells retained that phenotype after 8 days (Figure 10C). However, it seems that GADD45G initiated differentiation the fastest with only 30% immature cells at day 5, compared to 41% and 69% with GADD45A and GADD45B, respectively. A GADD45 family member specific difference was most obvious in the rate at which the GMP-like cells were generated. GADD45G was the strongest inducer of

differentiation, forcing 50% of the cells into the GMP-like stage at day 5, followed by a fast decline of this population as the cells further differentiate (Figure 10D). GADD45A-transduced cells initially showed similar kinetics with 39% GMP-like cell at day 5, but with a slower decline at day 8 and 10. The rate of GMP development of the GADD45B-expressing cells (24%) was in between GADD45G- (50%) and control-transduced LT-HSCs (5%). GADD45B-expressing cells were significantly slower in progressing to the GMP-like stage, reaching their peak (32%) only at day 8. The generation of mature granulocytes and macrophages was also accelerated by all GADD45 proteins although the fastest progression was seen in the GADD45G-transduced cells, which also accumulated higher numbers of mature cells at day 10 (Figure 10E). In conclusion, all three GADD45 members induce and accelerate the differentiation in LT-HSCs, although at different kinetics.

3.2.3 Enhanced differentiation reduce colony forming potential

To confirm the differentiation induction potential of GADD45G in LT-HSCs via an unbiased, marker-unrelated functional test, GADD45G-expressing LT-HSCs were plated for colony formation into methylcellulose (Methocult M3434) followed by re-plating after 10 days for a secondary colony formation. This assay determines progenitor cell numbers with their ability to form colonies in methylcellulose-based medium. Mature cells on the other hand are terminally differentiated and cannot produce colonies.

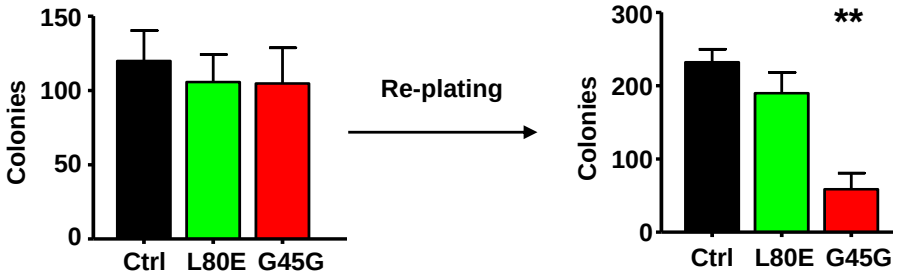

Figure 11: GADD45G reduced the number of colony-forming cells in serial re-plating assays

Colony formation assay of 200 LT-HSCs transduced with control or GADD45Gfollowed by re-plating of 2000 cells after 10 days under permissive cytokine conditions (Methocult M3434). Only VENUS+ colonies were counted. N=3 independent experiments. The mean +/- SD is displayed. ** P<0.01.

The GADD45G- or L80E-expressing LT-HSCs generated equal numbers and size of colonies comparable to control transduced LT-HSCs after 10 days. 2000 re-plated control- and L80E-expressing cells produced 220 or 196 colonies respectively, while the GADD45G cells produced only 54 colonies (Figure 11). This 4-fold decrease in colony formation functionally confirmed that GADD45G-expression accelerated the differentiation of LT-HSCs and thereby reduced the number of progenitors with colony forming capacity after 10 days.

Furthermore, the unaffected colony formation potential of L80E-expressing cells emphases that the dimerization of GADD45 proteins is crucial for their differentiation inducing function.

3.3 GADD45G switches HSCs from their self-renewal into differentiation in vivo

It has yet to be determined whether GADD45G expressing LT-HSCs maintained their self-renewal program under optimal in vivo conditions. The stem cell niche provides optimal conditions for long-term maintenance of HSCs. To assess if GADD45G switched the LT-HSCs from self-renewal into differentiation *in vivo*, LT-HSCs ($CD45.2^+$) freshly transduced with either GADD45G or empty vector were transplanted into lethally irradiated mice ($CD45.1^+$) and the engraftment was monitored over 21 weeks in peripheral blood and in BM (Figure 12A). Both GADD45G and control vector transduced donor LT-HSCs ($VENUS^+$, $CD45.2^+$) could engraft into recipients and donor cells were detected in peripheral blood 14 days after transplantation (Figure 12B). Engraftment levels of GADD45G- and control-transduced donor cells were 7.79 ± 1.44 % and 10.46 ± 1.91 % after 14 days, respectively. While the peripheral donor engraftment increased over 4 weeks in mice transplanted with control-transduced LT-HSCs, the peripheral engraftment from GADD45G-expressing LT-HSCs declined to only 2 %. After 18 weeks of transplantation, GADD45G-expressing mature cells were below the 1% threshold of positive engraftment and only in two mice from the GADD45G-transplanted cohort a slight engraftment of 1.8 and 5.2% remained. While after 14 days control-transduced cells in peripheral blood almost completely consisted of fast developing myeloid cells, already 13.17 ± 8.98 % of the GADD45G-expressing mature cells were B cells at this early time point (Figure 12D), indicating faster differentiation. Further after 21 weeks the engraftment was very low and only the long term persisting B cells survived in peripheral blood at substantial numbers.

BM engraftment of the transplanted cells was analyzed after 21 weeks. While all control transplanted mice showed VENUS+ CD45.2+ engraftment in the BM (20 to 74%), only two mice transplanted with the GADD45G cells showed low engraftment of 6.22 and 2.78 %, respectively ((Figure 12B)**.**

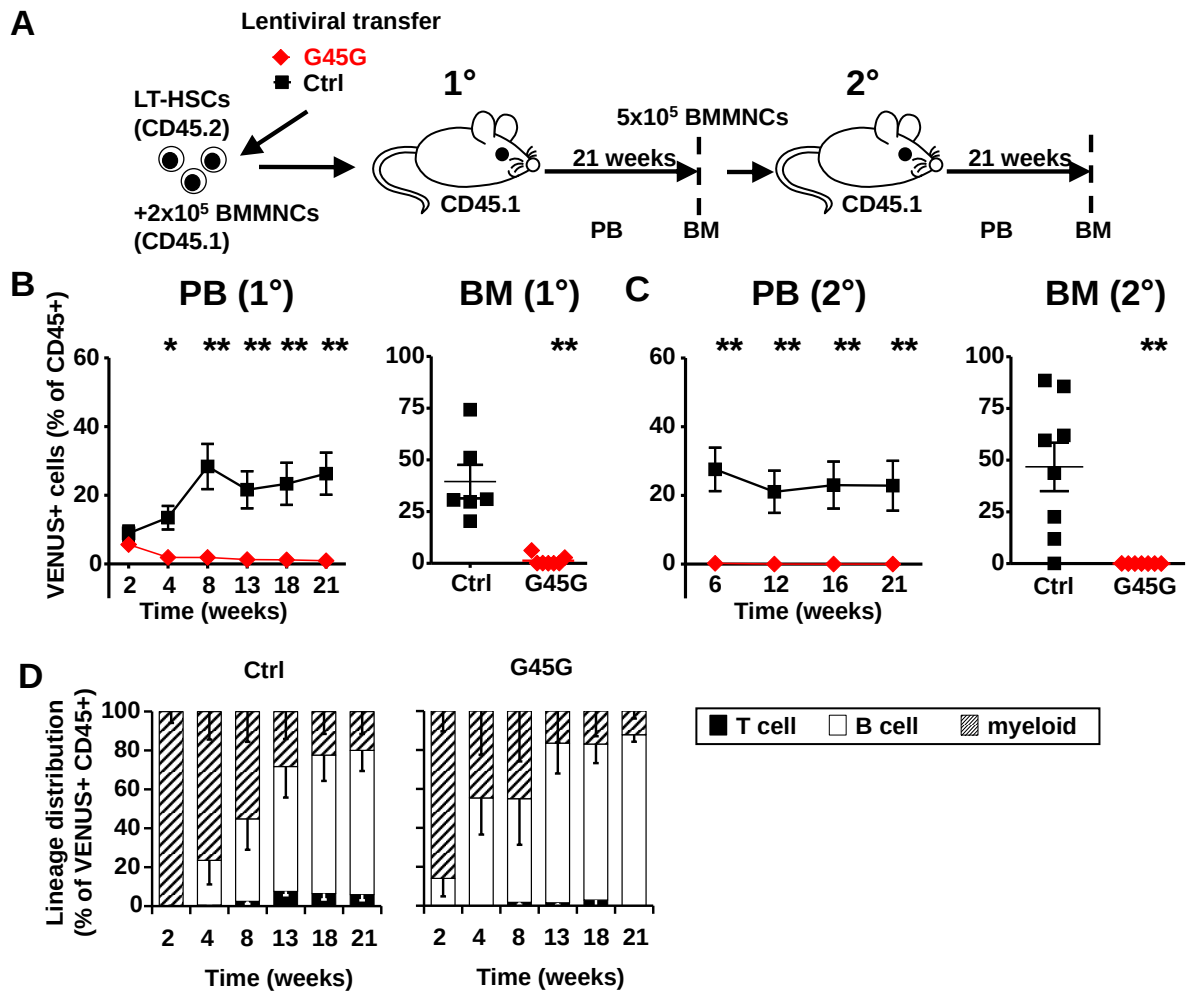

Figure 12: GADD45G induced differentiation of LT-HSCs in vivo

A) Experimental scheme of the serial transplantation experiment. Bone marrow mononuclear cells (BMMNCs). B) Transduced donor cell reconstitution (VENUS+) was determined via FACS in peripheral blood (PB) and in bone marrow (BM) of primary recipients (1°) (6 mice per condition). C) Transduced donor cell reconstitution (VENUS+) was determined via FACS in PB and BM of and secondary (2°) recipients (8 mice per condition). D) Multi-lineage distribution of successfully engrafted mice (engrafting threshold 1%) for control and GADD45G. The mean +/- SEM are displayed. * P<0.05; ** P<0.01.

To further challenge long-term self-renewal, BM cells from the primary transplants were transplanted into lethally irradiated secondary mice (Figure 12C). As expected from the BM analyses of primary recipients, no engraftment of GADD45G-expressing donor cells could be detected in peripheral blood or BM of secondary recipients. In contrast, cells from control-transduced donor HSCs from primary recipients engrafted well in both peripheral blood and BM (Figure 12C). This clearly illustrates that no GADD45G-expressing LT-HSCs endured in the BM of primary transplants. These data further indicate that GADD45G induces rapid differentiation at the expense of LT-HSC maintenance and self-renewal in vivo, resulting in the failure of long-term engraftment.

3.3.1 GADD45G-expressing HSPCs home and proliferate in BM

Since GADD45G was proposed to control cell cycle arrest and apoptosis, one of these fates could be responsible for the loss of self-renewal upon GADD45G expression (Fan et al. 1999; Zhang et al. 2001). To examine whether GADD45G expression influenced the cell cycle or viability of the transplanted cells in vivo, LSKs transduced with GADD45G were transplanted competitively into lethally irradiated recipients. The viability and the proliferation of donor cells were determined 7 and 14 days after transplantation (Figure 13A). Cell proliferation was measured by in vivo DNA incorporation of BrdU, using a 4 hour BrdU pulse prior to BM extraction. DNA content was measured by 7AAD-staining. BMCs were first gated for donor and recipient derived cells by expression of CD45.2 or CD45.1, respectively (Figure 13B). CD45.1 cells were further gated for GADD45G-expressing ($VENUS^+$) or untransduced ($VENUS^-$) cells. Cell cycle distribution of each subset was measured by total DNA content and BrdU incorporation. Shortly after BM irradiation and transplantation the majority of cells were actively proliferating. After 7 days, 33 % of the GADD45G-transduced donor cells have entered S-Phase (BrdU+) during the 4 hour pulse (Figure 13C). The percentage of cells in S-phase was reduced to 20% 14 days after transplantation, indicating that the repopulation slowed down as expected. Importantly, there was no difference in BrdU incorporation or cell cycle distribution of GADD45G-transduced to untransduced donor cells or and recipient cells (Figure 13C).

Cell viability in BM was assessed measuring fixable viability dye uptake via FACS. The percentage of viable cells was rather low (57 to 77%) for all three populations after 7 days, but the BM cells recovered and expanded 14 days after transplantation with more than 85% viability (Figure 13D). There was no change in the percentage viability of GADD45G-expressing donor cells compared to either untransduced donor cell or recipient cells at both 7 and 14 days. These experiments demonstrated that GADD45G inhibited neither cell proliferation nor survival *in vivo*. These findings further strengthen our hypothesis that GADD45G allows the generation of mature progeny at the expense of LT-HSC maintenance, which eventually results in loss of self-renewal and engraftment *in vivo*.

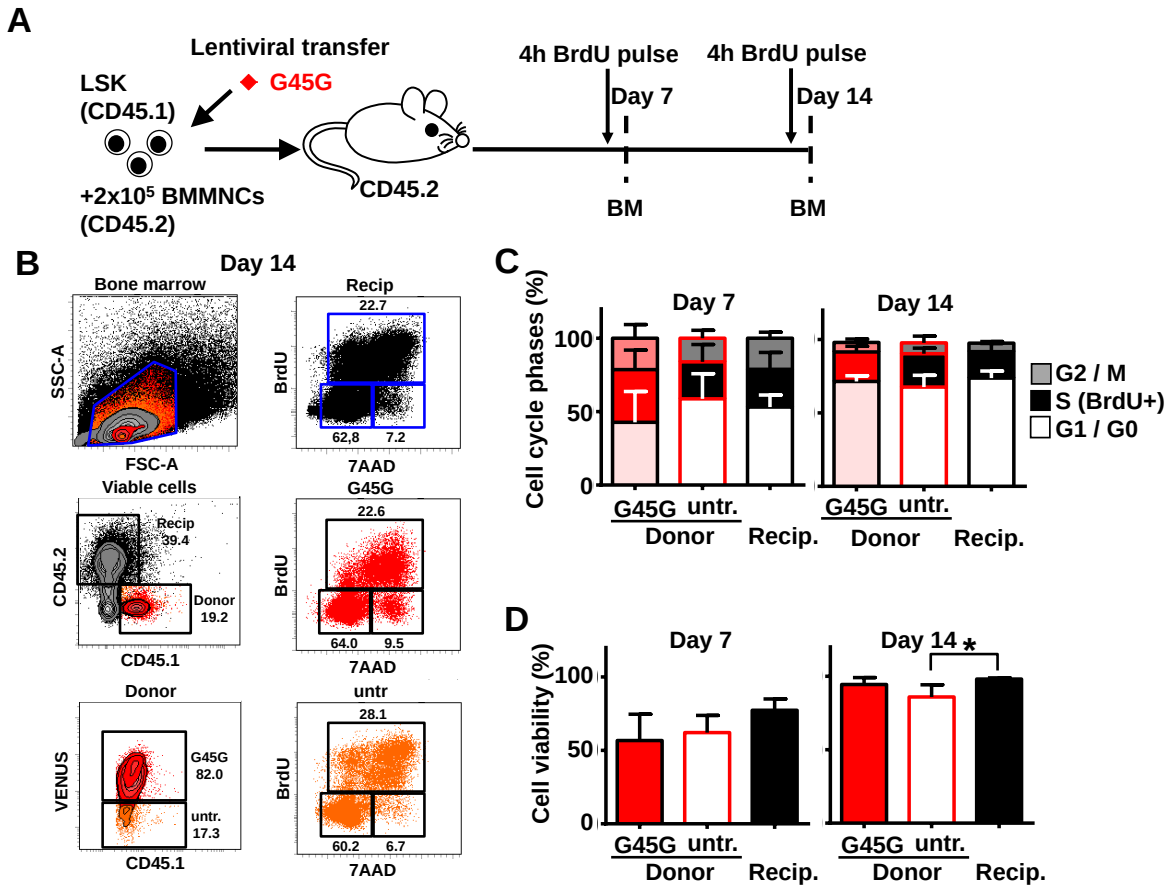

Figure 13: Normal proliferation and viability of GADD45G-expressing HSPCs in vivo

A) Experimental scheme of short term-transplantation with 4h BrdU-pulse prior to bone marrow extraction. B) Representative FACS blots of BrdU DNA incorporation of a 4h pulse and DNA content in bone marrow 14 days after transplantation of GADD45G transduced and untransduced donor cells as well as recipient cells. Gating for single BMCs to recipient (CD45.2+) or donor derived (CD45.1+) and further GADD45G expressing (VENUS+) cells is indicated in the left panels. Subsequent cell cycle analysis of each subset was performed by DNA staining with 7AAD and BrdU incorporation as shown in the right panels. Numbers next to the gates represent the percentages of the corresponding parent population. C) Cell cycle distribution via BrdU DNA incorporation in GADD45G transduced and untransduced donor cells as well as recipient cells 7 and 14 days after transplantation. D) Cell viability of GADD45G-expressing and untransduced donor cells as well as recipient cells 7 and 14 days after transplantation of transduced LSK in BM determined via FACS (N= 4 mice per time point). The mean +/- SD is displayed. * $P<0.05$.

3.4 Influence of GADD45G loss on hematopoiesis

Since the expression of GADD45G result in a promoted differentiation, the next question was whether the absence of GADD45G may enhance self-renewal and delay differentiation in LT-HSCs. In the knock-out mouse model B6.129S1-Gadd45g^{tm1Flv} J the *Gadd45g* gene was germline deleted by gene targeting into exon 3. The mice where viable and showed no aberrant organ function (Lu et al. 2001). They were described to have an inefficient TH1 effector cell activation upon IFNG or CD3 stimulation, due to an impaired p38 and JNK phosphorylation (Lu et al. 2001). But a thorough analysis has not been shown in detail. Therefore the first step was to perform a complete analysis of hematopoietic organs to evaluate hematopoiesis in steady state conditions.

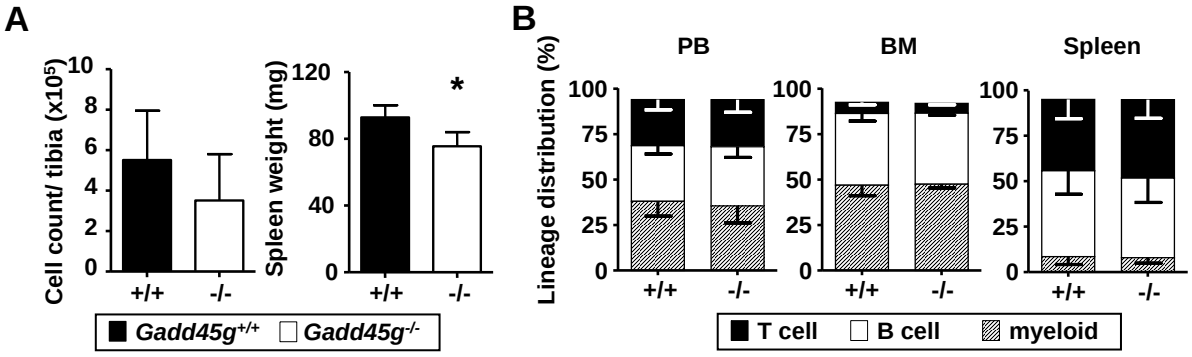

Figure 14: Mature blood cell production was unaffected in the absence of *Gadd45g*.

A) Cell count of total bone marrow cells of one tibia (N=5 mice per genotype) and the spleen weight (N=9 mice per genotype) of *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ mice. B) Lineage distribution of mature blood cells in peripheral blood (PB), bone marrow (BM) and spleen determined via FACS using antibodies against CD3 (T cells), B220 (B cells) and CD11b and Gr1(myeloid cells) (N= 9 mice per genotype). The mean +/- SD is displayed. * P<0.05.

There was no significant difference in the cellularity of bone marrow cells from wild type or knock-out mice, but the spleen weight of the *Gadd45g*$^{-/-}$ mice was significantly reduced (Figure 14A). This may be due to an insufficient cytokine response of hematopoietic progenitors that lead to overall lower production of mature cells. To test if any of the lineages were more favored than the others; the percentage of myeloid, B and T cells was analyzed in peripheral blood, bone marrow and spleen. In agreement with previously published data (Lu et al. 2001), no change in mature blood cell distribution was seen in any of the compartments (Figure 14B). In peripheral blood, 40% of the nucleated blood cells comprised of myeloid granulocytes and macrophages. The remaining 60% were nearly equal levels of lymphoid B and T cells. In BM, most of the mature cells are macrophages, which play an important role in HSC niche maintenance while only very few T cells could be detected in bone marrow. In

contrast, the spleen was rich in lymphoid B and T cells as the spleen, along with the lymph nodes, is the primary organ of lymphocytes.

To test whether *Gadd45g*$^{-/-}$ mice had altered progenitor populations due to insufficient cytokine signaling, a complete analysis of BM progenitors form *Gadd45g* deficient and wild type mice were performed. Representative FACS blots of progenitors population in the BM of *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ mice are displayed in (Figure 15A and B), respectively.

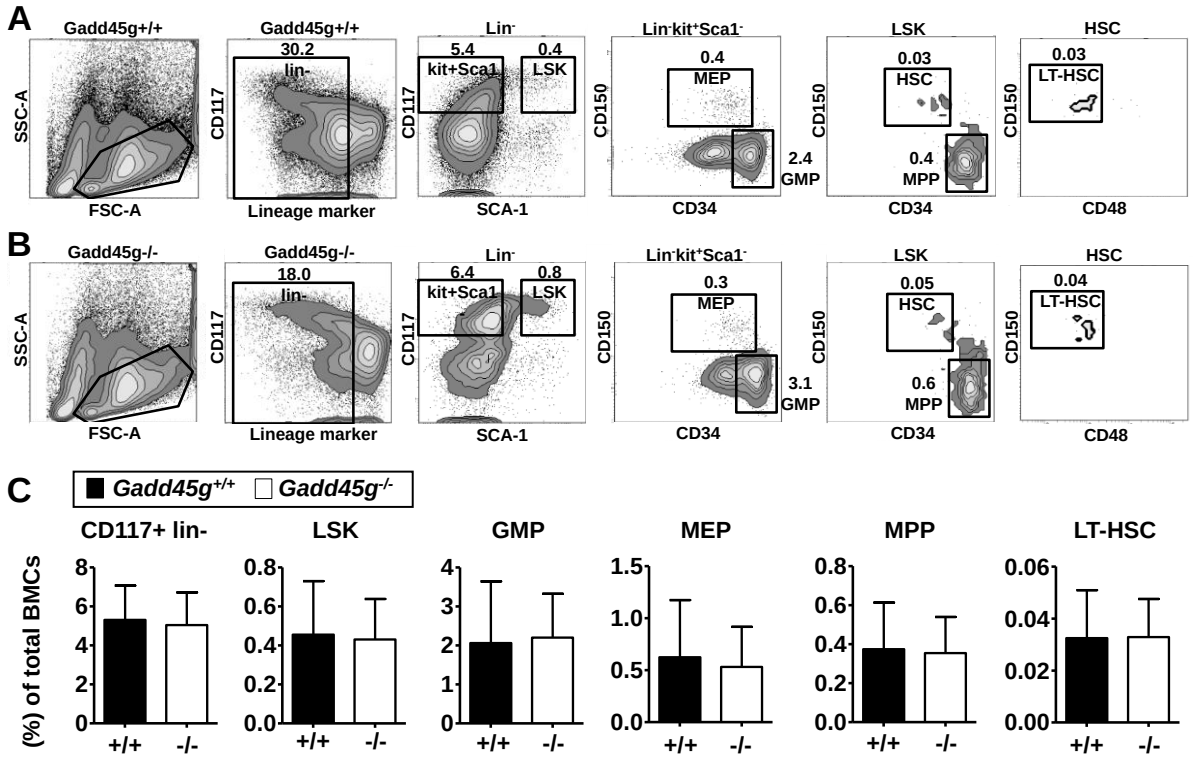

Figure 15: HSPC composition was normal during steady-state hematopoiesis in the absence of *Gadd45g*

A) Representative FACS analysis of bone marrow progenitors of *Gadd45g*$^{+/+}$ mice. B) Representative FACS analysis of bone marrow progenitors of *Gadd45g*$^{-/-}$ mice. Parent population is labeled on top of each FACS blot; percentages are calculated to the total of progenitor enriched bone marrow fraction subjected to FASC analysis. C) Calculation of stem and progenitor populations in BM of *Gadd45g*$^{+/+}$ and Gadd45g$^{-/-}$ mice (N= 9). The mean +/- SD is displayed.

No difference could be detected in the percentage of the different progenitor populations in the bone marrow of the knock-out mice compared to wild type littermates under steady state (Figure 15C). The complete myeloid restricted LSK progenitor fraction which contains the GMPs and MEPs made up 5% of the progenitor enriched fraction. GMPs and MEPs comprised 2 and 0.5 % of the enriched fraction respectively. The LSK compartment comprised roughly 0.4 % of the lineage negative enriched fraction. Given that the lineage negative enriched fraction accounts for only 10% of the total bone marrow cells, four in 10000 total BMCs can be included in the LSK fraction. The LSK fraction typically

consisted of 80% MPPs, which comprises less than 0.4% of the progenitor enriched fraction. The frequency of LT-HSCs was only 3 cells of 10000 progenitor enriched cells; the total amount of LT-HSCs in the BM was therefore less than 3 cells per 100,000 bone marrow cells. Since loss of GADD45G did not affect the steady state hematopoiesis, the influences of GADD45G on hematopoiesis under stress was investigated.

3.4.1 Slower expansion of *Gadd45g*$^{-/-}$ LT-HSCs in vitro

As previously shown, *Gadd45g* is induced by the cytokines TPO, IL3 and IL6. Furthermore, forced expression of GADD45G in LT-HSCs led to accelerated differentiation into the macrophage and granulocytic lineages. But it was unclear, if the LT-HSCs and progenitors from the *Gadd45g*$^{-/-}$ mice (a) have a dampen response to these cytokines and (b) may differentiate slower than their WT counterparts.

To test if the loss of GADD45G has a direct effect on cytokine stimulation, LT-HSCs from *Gadd45g*$^{-/-}$ and $^{+/+}$ mice were isolated and cultivated under various cytokine conditions for 5 days. While there was no difference in the cell expansion under minimal cytokine conditions with only SCF, the *Gadd45g*$^{-/-}$ LT-HSCs proliferated slower compared to the WT LT-HSCs under SCF and TPO (Figure 16A). Addition of IL3 and IL6 to the cytokine conditions of SCF / TPO boosted the expansion of both *Gadd45g*$^{-/-}$ and WT LT-HSCs by 8-fold. However, the number of *Gadd45g*$^{-/-}$ cells was significantly lower than of *Gadd45g*$^{+/+}$ cells (Figure 16A).

The differentiation kinetics in *Gadd45g*$^{-/-}$ cells was also slowed down during culture. The percentage of emerging GMPs from *Gadd45g*$^{-/-}$ LT-HSCs were significantly reduced under minimal and full cytokine conditions and mature cells were absent after 5 days of minimal cytokine culture (Figure 16B). It is noteworthy that the addition of TPO was sufficient to promote proliferation and to initiate differentiation at least of a small subset of WT LT-HSCs within 5 days of culture, whereas LT-HSCs stimulated only with SCF hardly proliferated and 95% of them remained undifferentiated. The addition of IL3 and IL6 to the cytokine cocktail tremendously boosted the differentiation of LT-HSCs to a similar extent as ectopic GADD45G expression. However, unlike GADD45G expression, IL3 and IL6 stimulation additionally enhanced proliferation of hematopoietic cells.

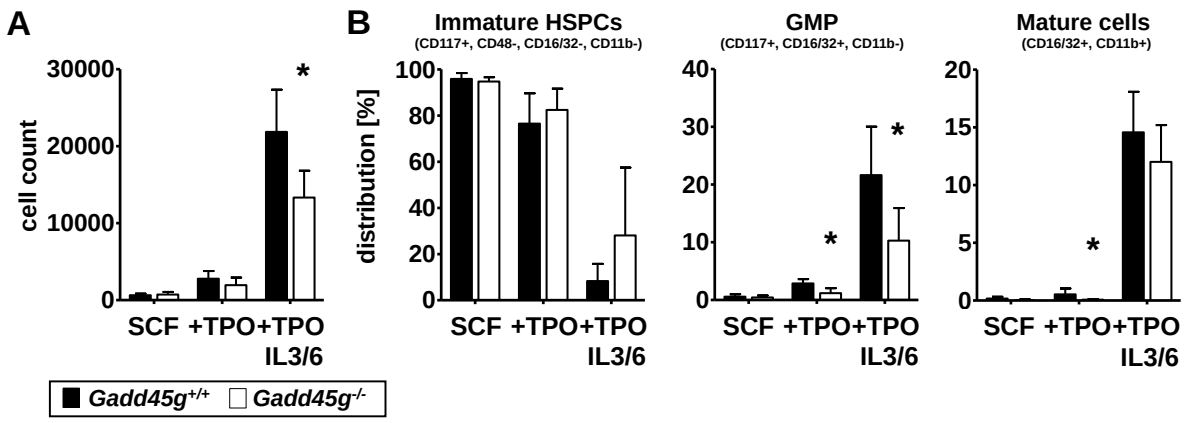

Figure 16: Reduced cytokine response of *Gadd45g*$^{-/-}$ LT-HSCs resulted in diminished cell expansion and delayed differentiation

A) Cell expansion of *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ LT-HSCs after 5 days in various cytokine conditions. B) Quantification and statistical analyses of FACS results. *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ cells were gated for immature HSPCs, GMP-like cells, mature granulocytes and macrophages. N= 5 independent experiments. The mean +/- SD is displayed. * P<0.05

Based on these results, GADD45G has a vital role in the cytokine response of LT-HSCs and their progeny, since the *Gadd45g*$^{-/-}$ LT-HSC responded less vigorously to the addition of differentiation promoting cytokines. However, , it can also be concluded that GADD45G is not the only pathway of IL3- and IL6-mediated differentiation induction. Disruption of GADD45G signaling therefore may be used in future to modulate differentiation induced by cytokines and to uncouple proliferation signals form differentiation signals.

Since a delayed differentiation was seen in *Gadd45g*$^{-/-}$ LT-HSCs after cytokine stimulation, it was next determined whether the loss of GADD45G would enhance the in vitro expansion of BM progenitors by delaying their differentiation during serial colony formation assays. Every 9th day, 20000 BMCs from *Gadd45g*$^{-/-}$ and *Gadd45g*$^{+/+}$ mice were re-plated into methylcellulose-based medium and the number and size of the colonies were assessed. There was no difference seen neither in the colony counts nor in the lineage potential of *Gadd45g*$^{+/+}$ and $^{-/-}$ cells in the first and second re-plating (Figure 17A). However, in the third re-plating, *Gadd45g*$^{-/-}$ cells gave rise to significantly higher numbers of colonies compared to the *Gadd45g*$^{+/+}$ cells. The average size of the colonies derived from *Gadd45g*$^{-/-}$ BMCs was also greater in the second and third re-plating (Figure 17B).

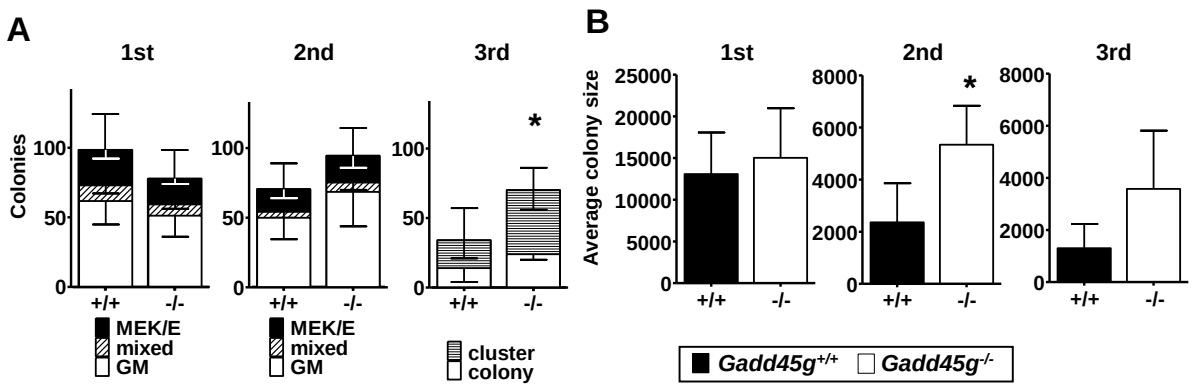

Figure 17: Enhanced numbers of colony forming cells after serial re-plating of *Gadd45g*$^{-/-}$ BMCs

A) Colony numbers and lineage potential of *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ BMCs in 1st, 2nd and 3rd re-plating. 20000 BMCs were plated for 1st and 20000 cells were re-plated after 9 days for 2nd and 3rd re-plating. Coloneis were scored as more than 30 cells, lesser cells numbers were counted as clusters. Error bars above the bar represent SD of total colonies. Error bars inside each bar represent variance in respective lineage. B) Average colony size after 1st, 2nd and 3rd re-plating. Average colony size was calculated by total cell count divided by number of colonies per plate. N= 6 experiments. The mean +/- SD is displayed. * P<0.05

These results indicate that the *Gadd45g*$^{-/-}$ BMCs remain a higher proportion of immature cell with enhanced colony forming potential after in vitro differentiation compared to *Gadd45g*$^{+/+}$ cells. These data are also consistent with studies on *Gadd45a*- and *b*-deficient mice (Gupta et al. 2006b). BMCs from both *Gadd45a*- and *Gadd45b*-deficient mice produced fewer colonies in the first plating, but exhibited a higher colony forming potential in the subsequent re-plating. In conclusion, the GADD45 proteins promote the differentiation of hematopoietic progenitors and the loss of any of them is sufficient to delay differentiation and prolong the proliferative capacity of hematopoietic progenitors. However, the regulation of Gadd45a and b expression during hematopoiesis requires further investigation.

3.4.2 Enhanced self-renewal of HSCs of *Gadd45g*$^{-/-}$ mice

Since *Gadd45g*$^{-/-}$ LT-HSCs showed a delay in differentiation and an enhanced colony re-plating capacity in vitro, the next obvious question was whether the absence of GADD45G could also result in enhanced self-renewal and delayed differentiation of LT-HSCs in vivo. Therefore, LT-HSCs from *Gadd45g*$^{-/-}$ and $^{+/+}$ mice were transplanted competitively into lethally irradiated recipient mice followed by a re-transplantation of the primary recipients derived BMCs into lethally irradiated secondary recipients (Figure 18A). LT-HSCs of both genotypes engrafted equally well into recipient mice, as peripheral blood of primary recipients were successfully reconstituted and donor cell chimerism steadily increased over the duration

of the primary transplant (Figure 18B). Multi-lineage contribution of donor cells could be detected in peripheral blood of all recipients 21 weeks after transplantation (Figure 18D). After 21 weeks, the mice were sacrificed and the BM engraftment was analyzed. Around 80% donor engraftment was achieved with LT-HSCs from both Gadd45g-deficient and WT mice, indicating that there was no difference between the repopulation capacities of both cells in primary transplants. Therefore, the self-renewal capacity of LT-HSCs was further challenged through secondary transplantation. BMCs ($2.5*10^5$) from primary transplants were competitively transplanted into secondary recipients at an equal ratio to the competitor BMCs. *Gadd45g*$^{-/-}$ BMCs were superior in reconstituting secondary recipients, assessed in the peripheral blood and the BM (Figure 18C). Multi-lineage contribution of donor cells from primary transplants could be detected in peripheral blood of all secondary recipients (Figure 18E). Taken together, LT-HSCs from both Gadd45g-deficient and WT mice could reconstitute primary recipients equally well but *Gadd45g*$^{-/-}$ BMCs were superior in repopulating secondary recipients. Therefore, the absence of GADD45G increases the stem-ness of LT-HSCs, most likely by enhancing their self-renewal potential in vivo.

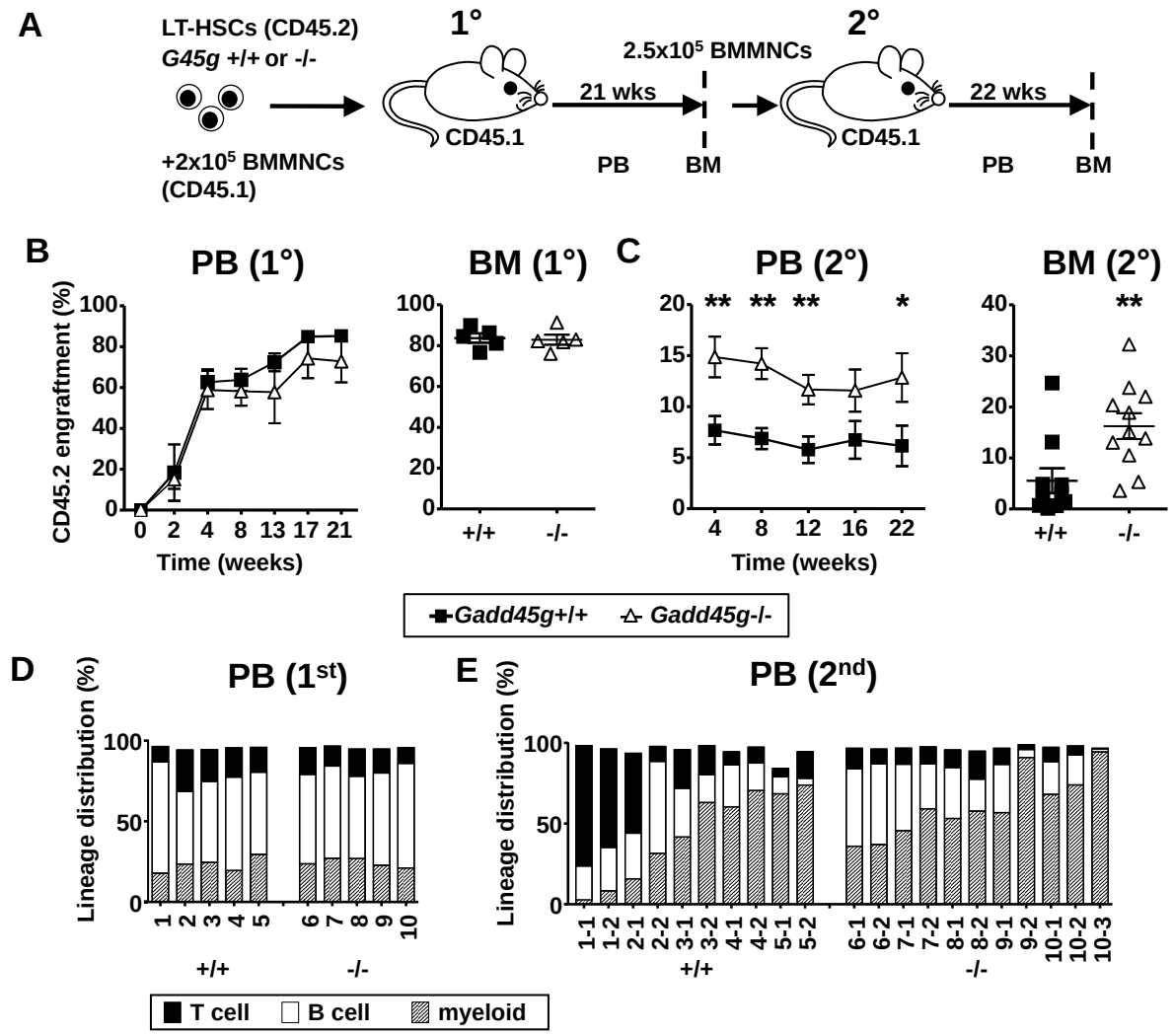

Figure 18: Enhanced self-renewal of *Gadd45*$^{-/-}$ LT-HSCs assessed by serial transplantations

A) Experimental scheme for serial transplantation. BMMNCs, bone marrow mononuclear cells. B) LT-HSCs from adult *Gadd45g*$^{-/-}$ and *Gadd45g*$^{+/+}$ littermates were transplanted into lethally irradiated recipients (5 mice per genotype), and donor cell reconstitution was measured via FACS in peripheral blood (PB) and in bone marrow (BM) of primary (1°) recipients. C) Donor cell reconstitution was determined via FACS in PB and in BM of secondary (2°) recipients (10 and 11 mice per genotype for secondary transplantation, respectively). Data are represented as the mean +/- SEM. * P<0.05, ** P<0.01. D) Donor cell distribution in PB via FACS using antibodies against CD3, B220 and myeloid markers CD11b and Gr1 after 21 weeks of primary transplantation. (H) Donor cell distribution in PB via FACS using CD3, B220 and myeloid markers CD11b and Gr1 after 22 weeks of secondary transplantation.

To examine whether this higher engraftment in secondary recipients was due to a higher number of LT-HSCs in the BMCs of *Gadd45g*$^{-/-}$ primary recipients, the proportion of lineage committed progenitor populations was also analyzed in the BM of primary and secondary recipients. Representative FACS blots show similar distribution of donor derived-HSPC populations in BM of primary transplanted mice (Figure 19A). Cells were first gated

for donor (CD45.2+) or recipient derived cells (CD45.1+) followed by the progenitor populations (GMPs, MEPs, LSK and HSCs) using the described marker combinations (see chapter 2.4). No significant difference in the percentage of the analyzed HSPC populations could be detected between *Gadd45g*$^{-/-}$ and *Gadd45g*$^{+/+}$ primary recipients (Figure 19B). Interestingly, except of one WT recipient with high levels of HSCs, the HSCs population was greater in *Gadd45g*$^{-/-}$ recipients than in their WT counterparts, whereas the LSK level was similar in both recipient groups. It can be surmised that the larger HSC population may be responsible for the twofold higher reconstitution in the secondary recipients. In the BM of secondary recipient of both groups, similar levels of all HSPC populations were detected (Figure 19C).

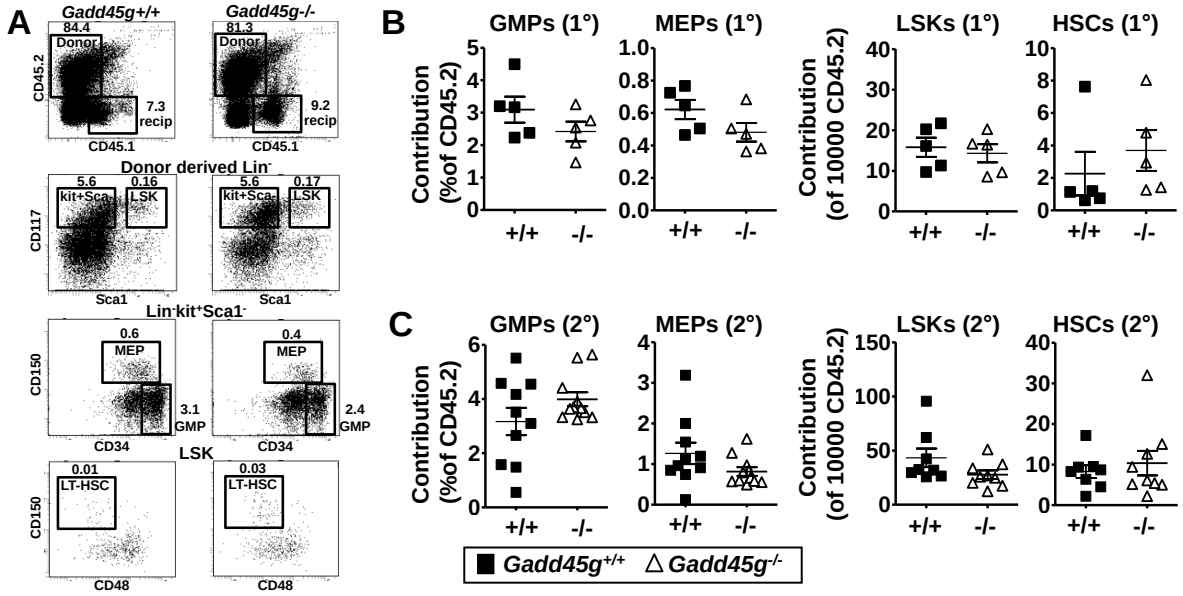

Figure 19: Donor HSPC contribution of *Gadd45g-/-* LT-HSCs in primary and secondary mice

A) Representative FACS blots for gated HSPCs in bone marrow cells of primary recipients of LT-HSC from *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ mice. Cells were first gated for donor (CD45.2^{+}) or recipient (CD45.1^{+}) derived cells. Donor CD45.2 cells were further gated for different HSPC populations. B) Quantification of FACS results of GMP, MEP, LSK and HSC contribution in BM of primary recipients. C) Quantification of FACS results of GMP, MEP, LSK and HSC contribution in BM of secondary recipients. Data represent individual values per mouse and the mean +/- SEM are shown as bar.

3.5 GADD45G promotes myelomonocytic differentiation at the expense of megakaryocytic-erythroid lineage fate

Expression of GADD45G induced an enhanced and accelerated differentiation of LT-HSCs into mature granulocytes and macrophages which comprised nearly 95% of all cells after 10

days in liquid culture. This robust development into myelomonocytic cells suggested that GADD45G may support the development of the granulocytic and monocytic lineages over other lineages. To test this hypothesis, megakaryocyte development was analyzed in liquid culture of GADD45G-transduced LT-HSCs.

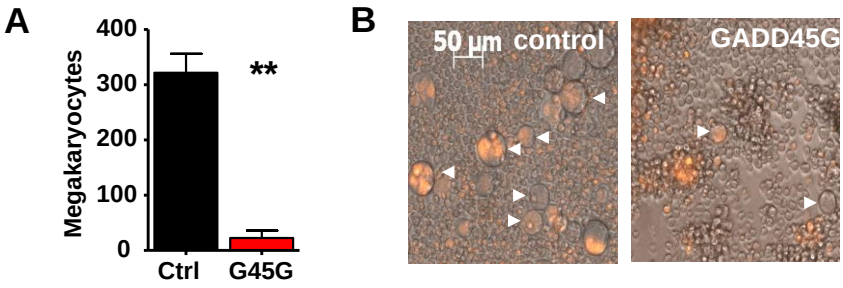

Figure 20: Reduced development of megakaryocytes in GADD45G-expressing LT-HSCs in culture
A) Quantification of tdTOMATO^{+} megakaryocytes after a 7 day culture of 100 control vector and GADD45G-transduced LT-HSCs. The transduction efficiency was equal in both groups. N=3 experiments. B) Representative microscopic images show reduced number of megakaryocytes in GADD45G-transduced sample. The mean +/- SD is displayed. ** P<0.01.

Megakaryocytes typically arise after several days of culture from LT-HSCs and can be easily depicted microscopically due to their large size (cell diameter of ~ 50 µm) and polyploid nuclei (Figure 20B). Cells expressing GADD45G showed a severe reduction in the development of megakaryocytes (Figure 20A). More than 300 megakaryocytes developed from 100 control LT-HSCs on day 7, but only 22 megakaryocytes developed from LT-HSCs expressing GADD45G.

The loss of megakaryocyte development and the robust differentiation into GM lineage suggested that GADD45G may induce a lineage bias in LT-HSCs. Colony formation assays of sorted LT-HSCs were conducted to test whether GADD45G or the other GADD45 members introduce a differentiation bias for a particular lineage in LT-HSCs. Colony assays provide information about colony size, rate of transduction and clonal ability to differentiate into mature myeloid lineages. LT-HSCs were transduced with a GADD45 family member, GADD45G L80E mutant, and the empty vector control. The cells were plated 24h after transduction into methylcellulose (supplemented with permissive myeloid cytokines SCF, IL3, IL6, EPO and transferrin to allow all lineages to develop) and evaluated the colonies microscopically after 10 days. Only colonies expressing VENUS in more than 50% cells were scored as transduced colonies. The different myeloid colonies were classified on the basis of the colony and cell morphologies.

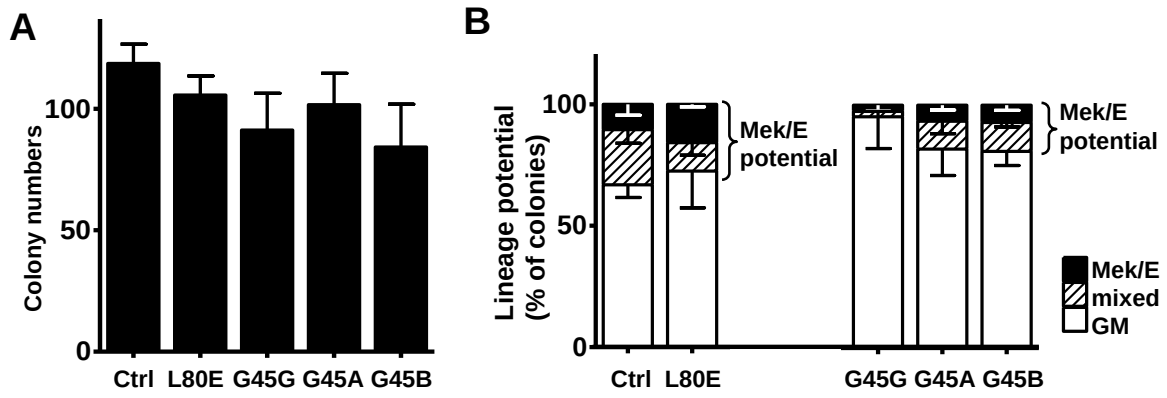

Figure 21: Absence of Mek/E development in LT-HSCs expressing GADD45G, but not in LT-HSCs expressing the other family members GADD45A and B

A) Colony formation assay of 200 LT-HSCs transduced with control vector, family members GADD45A, B, G and GADD45G L80E under permissive cytokine conditions. Only transduced (VENUS+) colonies were counted. N=3 experiments. B) Lineage potential of 200 transduced LT-HSCs, colonies with Mek/E lineage contribution are highlighted in brackets. The mean +/- SD is displayed.

The number and size of the transduced colonies were similar for all tested constructs (Figure 21A and data not shown). However, LT-HSCs transduced with GADD45G showed an increase in the colonies of myelomonocytic lineages with a concomitant decrease in the number of megakaryocytic-erythroid (Mek/E) lineage colonies. Only 6.2 ± 2.5% of colonies with Mek/E potential were scored from GADD45 LT-HSCs as opposed to more than 30% from control and L80E mutant LT-HSCs (Figure 21B). Strikingly, the Mek/E colony output of GADD45A- and GADD45B- expressing LT-HSCs were only slightly reduced (approx. 20% MeK/E containing colonies). This clearly underscores that the three GADD45 family members have similar, but not overlapping functions in early hematopoietic fate choices. All Gadd45 family members induced the differentiation in LT-HSCs, however, while GADD45G promoted myelomonocytic differentiation at the expense of the Mek/E lineage, Mek/E development was unaffected in the presence of GADD45A and GADD45B.

3.5.1 GADD45G prevents MEP formation in vivo

From the data above the question arose, whether the myelomonocytic lineage bias of GADD45G-expressing LT-HSCs was also operative in vivo. Therefore, sorted LSK cells were transduced with GADD45G or control vector and transplanted in lethally irradiated recipients after 24 hours. Since the engraftment of GADD45G-expressing cells was still detectable after two weeks of transplantation, homing of GADD45G- or control-transduced HSPCs to the BM and their differentiation into committed progenitors was assessed 11 days after transplantation (Figure 22A).

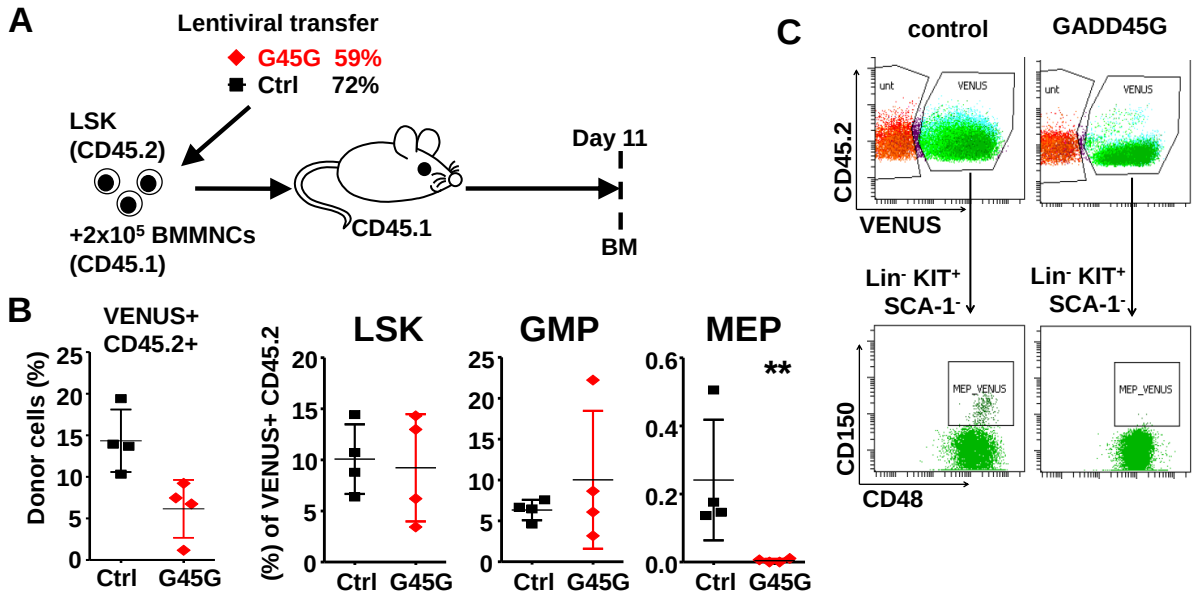

Figure 22: GADD45G prevented MEP formation in short-term transplantation of HSPCs in vivo

A) Experimental scheme of short-term repopulation of GADD45G- or control vector-transduced LSK for progenitor development of in bone marrow. B) Donor cell engraftment and HSPC distribution of transduced LSKs 11 days after transplantation. C) Representative FACS blots highlight the absence of MEPs in GADD45G positive donor cells. The mean +/- SD is displayed. ** P<0.01.

LSK cells transduced with either GADD45G or control vector homed and engrafted in the recipient BM. No difference could be detected in the LSK or GMP compartments of both recipient groups (Figure 22B). As hypothesized, GADD45G-expressing donor MEPs were almost absent, thus underlining a lineage bias of GADD45G-expressing HSPCs (Figure 22B,C). GADD45G therefore contradicted the generation of MEPs not only in vitro, but also in vivo. Since GADD45G expression could seemingly override the optimal multi-lineage development conditions of the BM, it was possible, that GADD45G instruct multipotent cells to differentiate preferentially into granulocyte-macrophage and lymphoid cells.

3.6 Single cell tracking revealed fast onset of GM commitment

The GADD45-mediated bias in the choice of LT-HSC to differentiate into the Mek/E lineage was analyzed continuously using a sophisticated video-microscopy-based cell tracking technique. This approach enabled to assess the differentiation kinetics induced by GADD45G in LT-HSCs and their progeny at the single cell level and a high temporal resolution. Freshly sorted LT-HSCs were plated into 24-well plates equipped with silicon culture inserts, which prevent the migration of the cells out of the camera field. Lentiviruses coding for GADD45G or a control were added to the LT-HSCs and the time-lapse imaging was subsequently started allowing the real time observation of virus transduction (see section 2.5.4). After observing the cells for 7 days the image data was analyzed by tracking individual cells using a self-written computer program (Timm´s Tracking Tool; TTT) which can determine and record multiple parameters of individual cells at each time point as well as their complete affiliations. These parameters were stored in large cell pedigrees, with the LT-HSC at the apex (section 2.5.4.1). Single LT-HSCs and all their progeny were tracked until the fifth generation; the mother generation was designated as G1. A representative pedigree with all cell fates and parameters is shown in Figure 23B. The generation time of an individual cell was defined as the time span from cytokinesis of its mother cell to its own division. Dead cells were easily detected by their shrunken, non-refracting appearance and immobility. Transgene expression was monitored by expression of the fluorescent reporter tdTOMATO. Differentiation into GM committed cells was evaluated by the surface expression of CD16/32 using a fluorescence labeled antibody. The “time delay” of CD16/32 marker expression was measured as the duration between the first onset of transgene expression in the ancestor cell and the appearance of CD16/32 on the surface of the cell. The development of single LT-HSCs into GM committed cells after GADD45G expression is shown in a short movie. Only the GADD45G expressing daughter cells (fluorescence images of nuclear tdTOMATO in middle column) differentiate into GMPs with CD16/32 surface expression (right column) emphasizing the strong differentiation induction of GADD45G. Untransduced cells remain undifferentiated until the end of the movie after 4 days and 16 hours. Every 4th phase contrast image from the time-lapse imaging and every fluorescent picture were used to assemble the movie. The movie is also available under:

http://www.cell.com/cms/attachment/2019805484/2039755836/mmc2.mp4

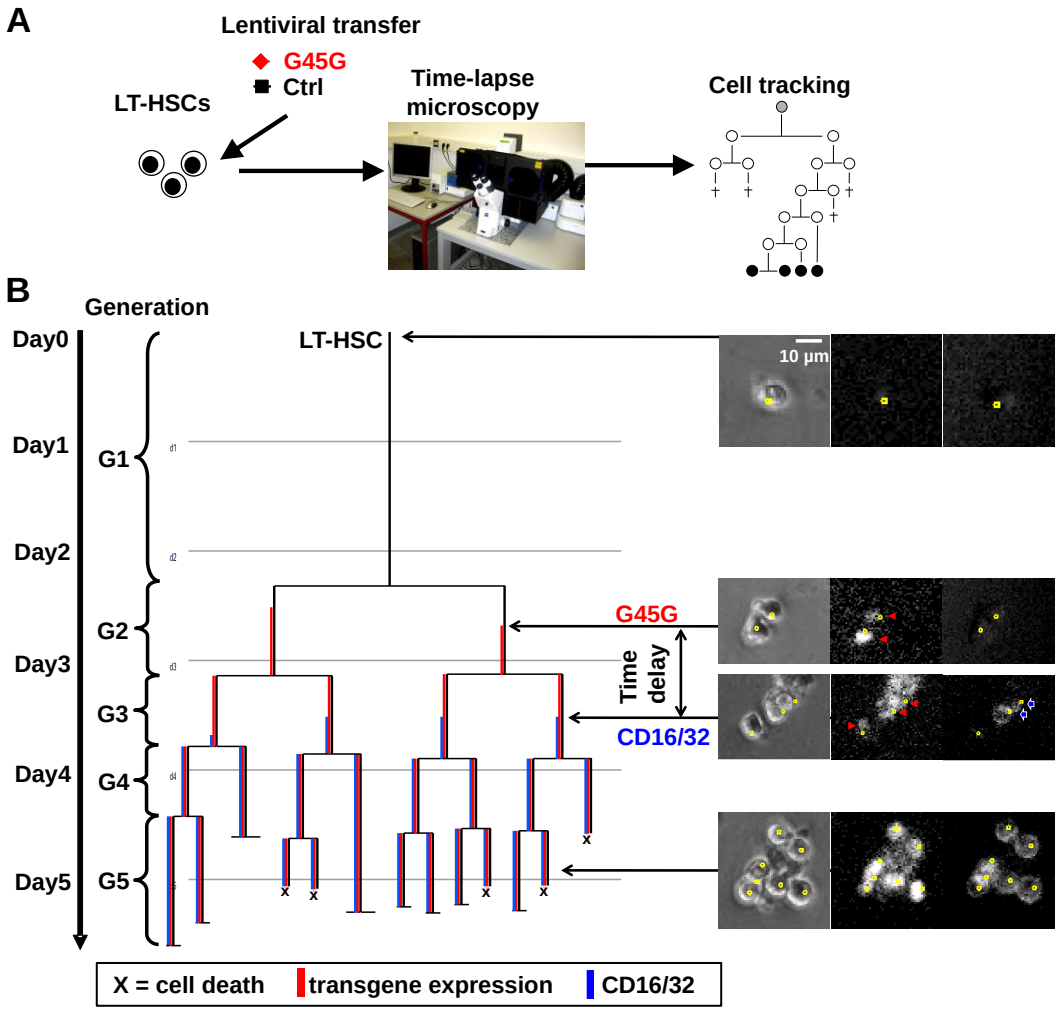

Figure 23: Time-lapse microscopy-based single cell tracking of individual LT-HSC transduced with GADD45G

A) Experimental scheme of video-microscopy-based cell tracking. B) Example pedigree of an individual LT-HSC and its progeny up to the 5th generation over 5 days. Successful transduction can be determined by tdTOMATO expression (red arrow head), GM commitment by antibody staining of CD16/32 on the surface of the cell (blue arrow). The time of GM commitment after tdTOMATO expression can be determined in real time. Example pictures of the indicated time points corresponding to the pedigree are displayed on the right.

A total of 54 control and 52 GADD45G-expressing single LT-HSCs and their progeny were tracked. Growth arrest of GADD45G-expressing LT-HSCs and their progeny was not detectable till the fifth generation, and similar generation times were measured for GADD45G- and control vector-transduced cells of generation 2 to 5 (Figure 24A). The onset of GM commitment in GADD45G-expressing cells was very rapid, with 11% of the 2nd and 3rd generation progeny showing already surface CD16/32 expression (GMP-like cells). The control cells started expressing CD16/32 only in the 7th and 8th generations. At this time point, all GADD45G-expressing cells have already reached the stage of GM commitment (Figure 24B). This accelerated differentiation could also be depicted through the “time delay” of GM

commitment after transgene expression, which was 84 hours for control transduced cells and only 36 hours in GADD45G cells (Figure 24C). These results further support the findings that GADD45G functions as a rapid inducer of LT-HSC differentiation.

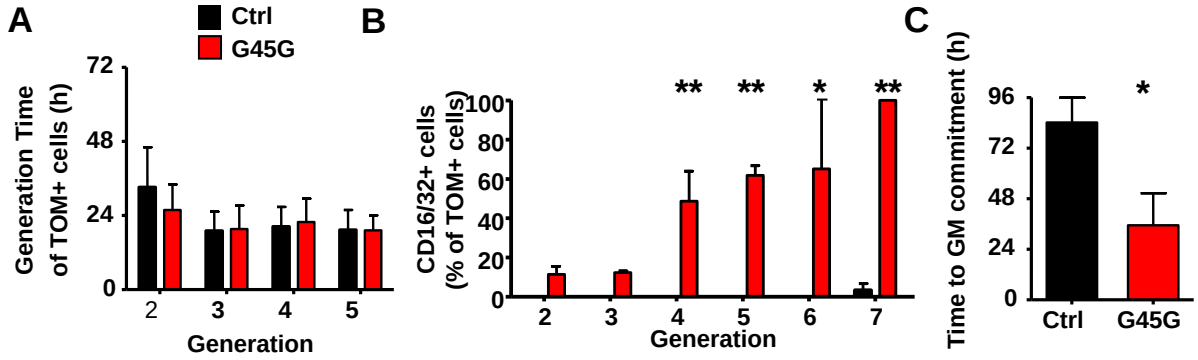

Figure 24: Rapid onset of GM commitment after GADD45G expression in LT-HSCs

Statistical analyses of cell tracking data of control- or GADD45G-transduced LT-HSCs. A) Generation times of transduced LT-HSCs and their progeny (TOMATO+). B) Percentage of CD16/32 expressing cells in each cell generation. Cell data extracted from 54 control- and 52 GADD45G-expressing pedigrees C) Interval of transgene expression (TOMATO+) and onset of CD16/32 expression (GMP-like stage). Cell data extracted from 29 (G45G) and 8 pedigrees (control), representing 226 cells for G45G and 46 for control becoming CD16/32 positive. The mean +/- SD is displayed. * $P<0.05$, ** $P<0.01$.

3.6.1 GADD45G selects against megakaryocyte-erythroid differentiation

The cell tracking technology was also utilized to elucidate the mechanism of the GADD45G-mediated lineage choice in differentiating LT-HSCs. There are two hypotheses to explain the lineage bias against Mek/E cell fate. First, GADD45G may instruct a GM lineage program in multipotent cells, forcing the cell to develop into myelomonocytic cells (lineage instruction). Second, GADD45 may inhibit the proliferation, maturation and survival of Mek/E lineage-committed progenitors leading to a selective elimination of these lineages (lineage selection). Both scenarios were tested first by continuously tracking LT-HSCs and their progeny, which differentiated into the Mek/E lineage. Megakaryocyte development was scored by the occurrence of endomitosis (incomplete cytokinesis that gives rise to cells with large polyploid nuclei) and/or the large cell diameters of more than 20µm (Figure 25A). Endomitosis can be easily detected using the nuclear membrane bound tdTOMATO as viral transduction reporter. While 22 % of the control LT-HSCs developed into megakaryocytes, only 5% of GADD45G expressing-LT-HSCs contained megakaryocytic progeny (Figure 25B), confirming previous colony-formation assay data (Figure 21).

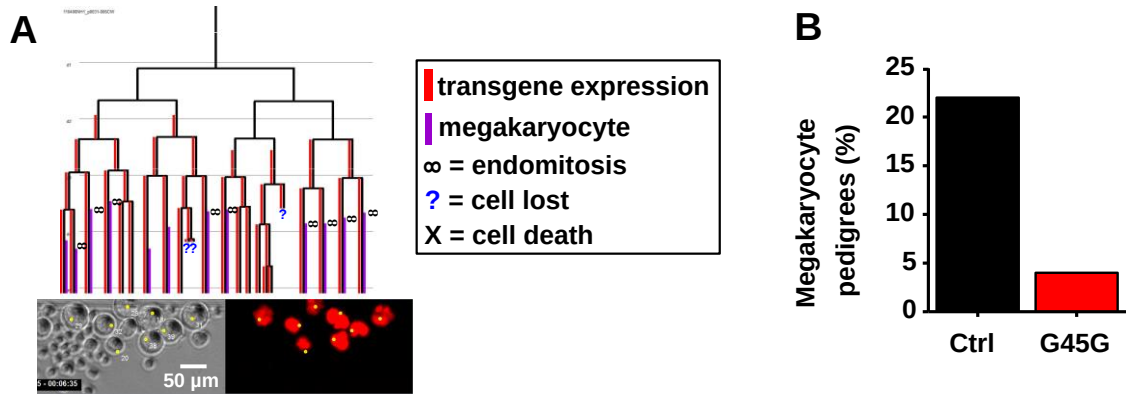

Figure 25: GADD45G expression diminished megakaryocyte development.

A) Typical megakaryocytic pedigree with representative picture. B) Percentage of pedigrees with megakaryocyte development extracted from 54 control- and 52 GADD45G-expressing pedigrees.

To dissect instructive versus selective lineage choice of LT-HSCs expressing GADD45G we analyzed whether the absence of megakaryocytes was due to a disproportionate increase in GMPs (instruction) or due to an increase in death of non-GM committed cells (selection). To illustrate this point, two typical pedigrees of control- and GADD45G-transduced LT-HSCs are shown in Figure 26. The control transduced LT-HSCs divided in the first 48 hours and their daughter cells in the 2nd generation started to express tdTOMATO. Very few cell deaths were tracked over the five generations and CD16/32 was also not detected in this time frame (Figure 26A). The GADD45G-transduced LT-HSCs and their progeny had similar generation times but expressed CD16/32 much earlier (Figure 26B) as shown in previous results (Figure 24). The most striking feature in the GADD45G-expressing pedigrees was the high rate of cell death in cells before the onset of CD16/32 (time point of GMP commitment, Figure 26B).

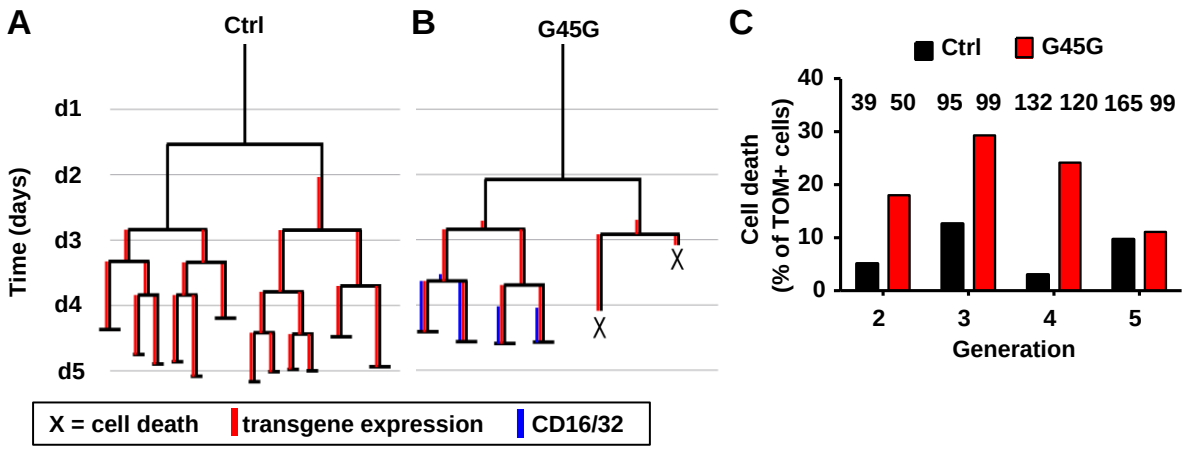

Figure 26: Enhanced cell death indicated that GADD45G expression selected against megakaryocyte development.

A) Example pedigree of a control vector-transduced LT-HSC and its progeny with no cell death or GM commitment until the 5th generation. B) Example pedigree of a GADD45G-transduced LT-HSC and its progeny with accelerated GM commitment and elevated cell death of potentially Mek/E-committed cells. C) Quantification of cell death events in each cell generation, before cells have differentiated into GMP-like cells (CD16/32+). Analyzed cell numbers are indicated above graph.

This higher rate of cell death in the non-GM fraction, which can be presumed to be an uncommitted or Mek/E committed (MEP) fraction, was consistently seen in the GADD45G-expressing LT-HSCs through subsequent generations (Figure 26C). This clearly suggests that GADD45G expression selectively eliminates the Mek/E lineage or in other words, GADD45G has a selective rather than an instructive role in lineage choice.

3.6.2 MEP development is not compatible with the GADD45G-mediated differentiation program

To corroborate that the GADD45G-mediated program is not compatible with Mek/E-committed cell fate, the consequences of GADD45G expression was investigated in prospectively isolated MEPs. Therefore MEPs and GMPs were transduced with either control, GADD45G or the L80E mutant lentiviral vector and then seeded in methylcellulose-based medium for colony-formation. The expression of GADD45G, but not the L80E mutant drastically reduced the colony output of MEPs in comparison to control-transduced MEPs (Figure 27A). The colony forming potential of GMPs was unaffected, with similar colony counts in all three conditions. Noteworthy, the colonies of GADD45G-, but not L80E-expressing GMPs were further differentiated into mature macrophages. Importantly, no signs of trans-differentiation of GADD45G-expressing MEPs into other lineages were observed. As already presented in chapter 5.3.3 HSCs expressing GADD45G exhibited a severe loss of

megakaryocyte- erythrocyte development, which did not appear with the dimerization mutant L80E.

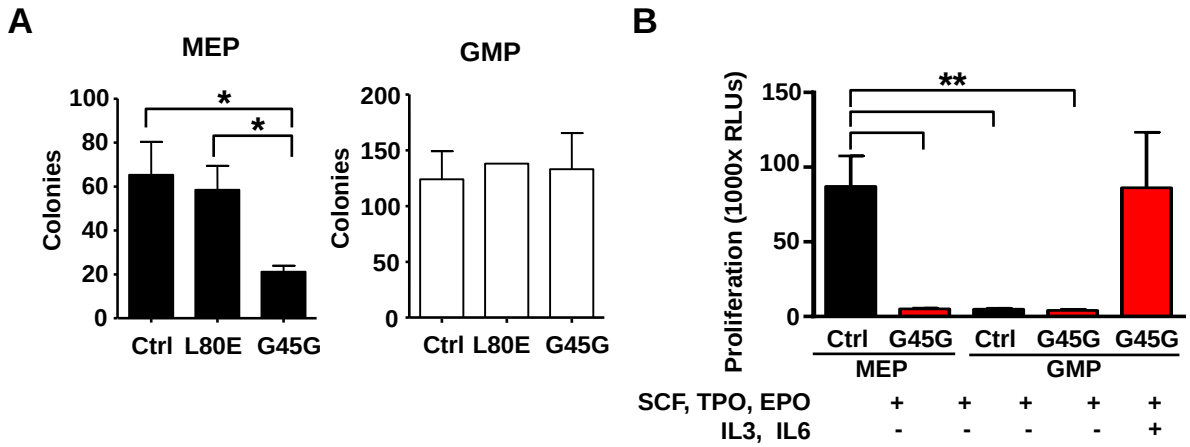

Figure 27: MEP development was not compatible with the GADD45-mediated differentiation program. A) Colony formation assay of 200 GMPs or 400 MEPs transduced with GADD45G, L80E or control vector under permissive cytokine conditions. N=3 experiments. B) FACS sorted MEPs and GMPs lentivirally transduced with GADD45G or control vector were cultured for 5 days in indicated cytokine conditions, and viable cell numbers were determined by a proliferation assay kit (RLU, relative light units), N=5 biological replicates. The mean +/- SD is displayed. * P<0.05, ** P<0.01.

Another line of evidence was provided when the proliferation and survival of MEPs and GMPs were assessed. MEPs and GMPs were transduced with either GADD45G or control vector and cultured in Mek/E-promoting medium (SCF, TPO, EPO) for 4 days. Living cells were scored using an ATP-based bioluminescent detection proliferation assay (ViaLight Plus Cell Proliferation and Cytotoxicity BioAssay Kit; Lonza). The results were also confirmed by microscopic cell count of individual samples.

While control-transduced MEPs normally proliferated and differentiated in culture, GADD45G-expressing MEPs could not expand under Mek/E-promoting conditions and largely died (Figure 27B). GMPs expressing either control or GADD45G vector did not proliferate under Mek/E-promoting conditions, because their survival requires other cytokines. When the medium was changed to GM-promoting conditions by adding IL3 and IL6, GMPs expressing GADD45G could perform their normal in-vitro proliferation.

In summary, this set of experiments conclusively proves that the ectopic expression of GADD45G promotes the differentiation of GMPs, but blocks the differentiation and maturation of Mek/E-committed cells, therefore selecting against Mek/E development.

3.7 GADD45G mediates differentiation via the MAP3K4 – p38 pathway

GADD45G orchestrates its diverse functions by the interaction with a variety of other factors (Moskalev, et al., 2012). The previously described experiments showed that GADD45G did not cause a cell cycle arrest in HSPCs, largely excluding cdc2/cyclinB1 or p21 as possible interactors for GADD45G-mediated differentiation. We rationally selected the stress-related kinases p38 MAPK and JNK as possible interactors with GADD45G, as they have already been connected with cell differentiation (Ventura et al. 2007; Zhu et al. 2009; Salerno et al. 2012; Geest, Coffer 2009). Dimeric GADD45G can physically interact with mitogen-activated protein kinase kinase kinase 4 (MAP3K4) (Mita et al. 2002). GADD45G binds to MAP3K4 near the auto-inhibitory domain, which results in a conformational change of MAP3K4 releasing the auto-inhibitory domain and activating MAP3K4. Activated MAP3K4 phosphorylates MKK3/ MKK6, which in turn leads to p38 MAPK or JNK phosphorylation, which then activates downstream effectors. Furthermore, GADD45G may directly bind p38 to activate it directly (Bulavin et al. 2003; Zhu et al. 2009).

To assess the involvement of the p38 pathway in the GADD45G-mediated differentiation program, LT-HSCs transduced with GADD45G were cultured in the presence of four different specific p38 inhibitors, each exhibiting distinct pharmacokinetics (Baudet et al. 2012). The concentrations used for these inhibitors restricted their selectivity to the p38A isoform (Saklatvala 2004). The expansion and differentiation of the cells was monitored by FACS after 5 days of culture. In concordance with previous results, 58% and 65% of DMSO treated control and L80E expressing LT-HSCs, respectively, still remained immature, whereas only 26% of the GADD45G-expressing cells belonged to immature cells after 5 days (Figure 28A). This rapid differentiation induction mediated by GADD45G was completely blocked upon addition of any p38 inhibitors, and the cells behaved similar to the control conditions. Consequently, GADD45G-induced differentiation was completely abrogated by p38 inhibition. While 17% of the GADD45G expressing cells showed a mature phenotype after 5 days in culture without inhibitors, addition of any one of the four inhibitors reduced mature cell count to less than 3%, similar to control or L80E mutant expressing cells. The inhibition of p38 in control and L80E expressing LT-HSCs had no effect on their differentiation.

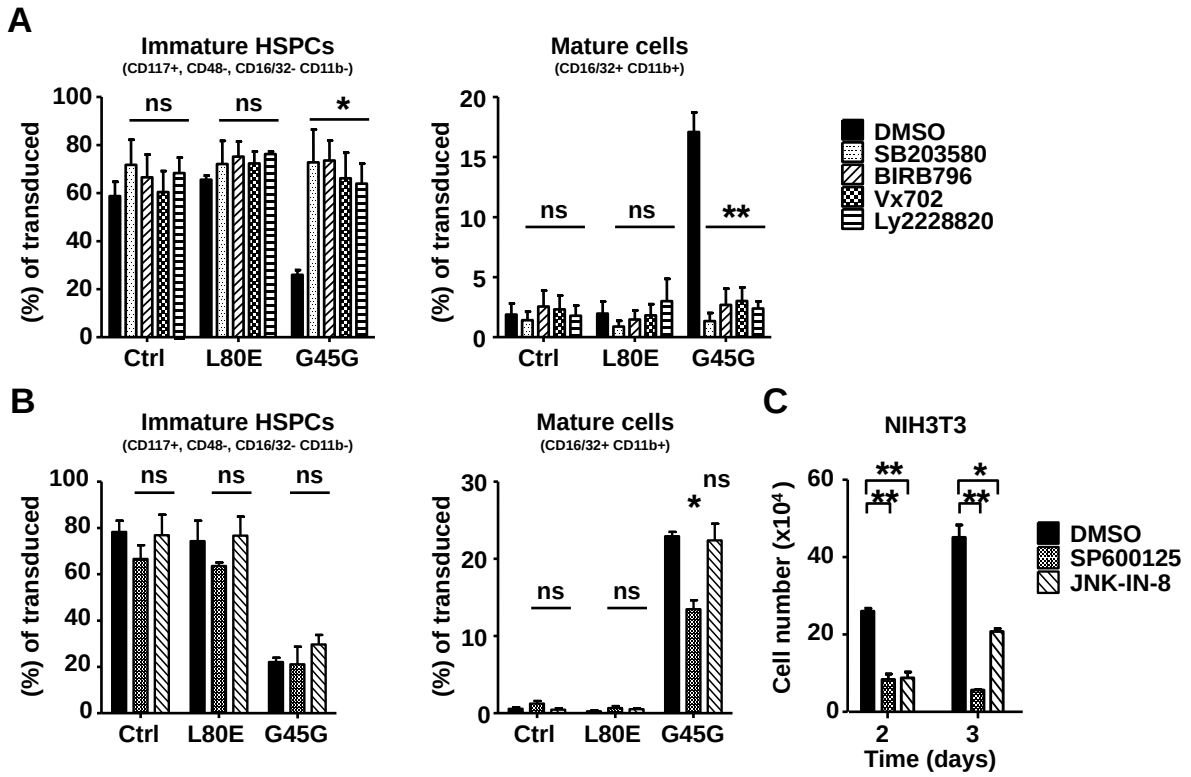

Figure 28: GADD45G required MAPK p38, but not JNK activation, for differentiation induction.

A) 5-day culture of transduced LT-HSCs in the presence of four different p38 inhibitors. FACS gated for immature HSPCs and mature cells. B) 5-day culture of transduced LT-HSCs in the presence of 2 JNK inhibitors. FACS gated for immature HSPCs and mature cells. C) Positive control for JNK inhibitor activity by reduced NIH 3T3 expansion. N=3 independent experiments. Data are represented as the mean +/- SD. * P<0.05, ** P<0.01, ns= not significant

Since the MAP3K4 pathway can also phosphorylate and activate JNK, GADD45G-mediated differentiation was also analyzed in the presence of JNK inhibitors (Zhang et al. 2012). SP600125 only partially blocked GADD45G-induced differentiation and JNK-IN-8 did not inhibit GADD45G-induced differentiation (Figure 28B), suggesting that GADD45G selectively activates p38 and not JNK. JNK inhibitor activity was functionally tested by growth inhibition in NIH/3T3 cells using at the same concentration as in the differentiation experiment. Both JNK inhibitors significantly reduced expansion NIH/3T3 cells proving the efficacy of the used inhibitor concentrations (Figure 28C).

To establish that GADD45G directly phosphorylates and activates p38, the phosphorylation status of p38 and one of its common downstream targets, MAPK-activated kinase 2 (MK2), was tested in GADD45G-expressing MPPs by intracellular phosphoflow cytometry. As a positive control, un-transduced MPPs were stimulated for 30 min with 10 µg/ml anisomycin that increased the MFI of phosphorylated p38 and MK2 by 300% (Figure 29A and B).

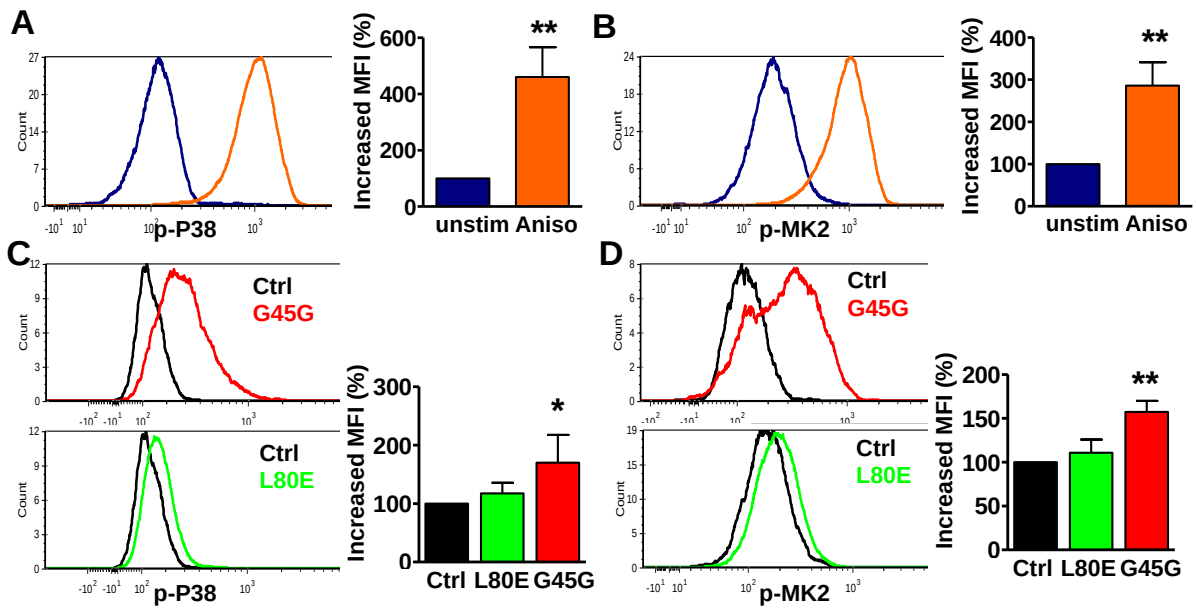

Figure 29: GADD45G caused phosphorylation of p38 and MK-2 in MPPs.

Histogram and quantification of MAPK p38 A) and down-stream kinase MK2 B) phosphorylation in MPPs after stimulation with 30 min anisomycin. FACS histogram and quantification of phosphorylated p38 C) and phosphorylated MK2 D) in 5-day-cultured MPPs after GADD45G, L80E or control vector transduction assessed via intracellular phosphoflow cytometry. The mean +/- SD of N= 4 independent experiments are calculated. * P<0.05, ** P<0.01

GADD45G-expression resulted in a stron g shift in p38 and MK2 phosphorylation relative to control MPPs, with an MFI increase by 60% (Figure 29C and 29D respectively). The dimerization mutant GADD45G L80E on the other hand was not able to activate p38 and MK2. These results clearly indicated that p38 was directly activated by GADD45G.

It was next hypothesized that activation of the p38 pathway would be sufficient to induce LT-HSC differentiation independently of GADD45G expression. Therefore MAP3K4 and MKK6, both molecules downstream of GADD45G that activate p38, were investigated for their potential to phenocopy GADD45G-mediated differentiation induction of LT-HSCs and MPPs. To specifically activate p38, constitutively active forms of MAP3K4 and MKK6, namely caMA3K4 and caMKK6, were cloned and lentivirally transduced into LT-HSCs.

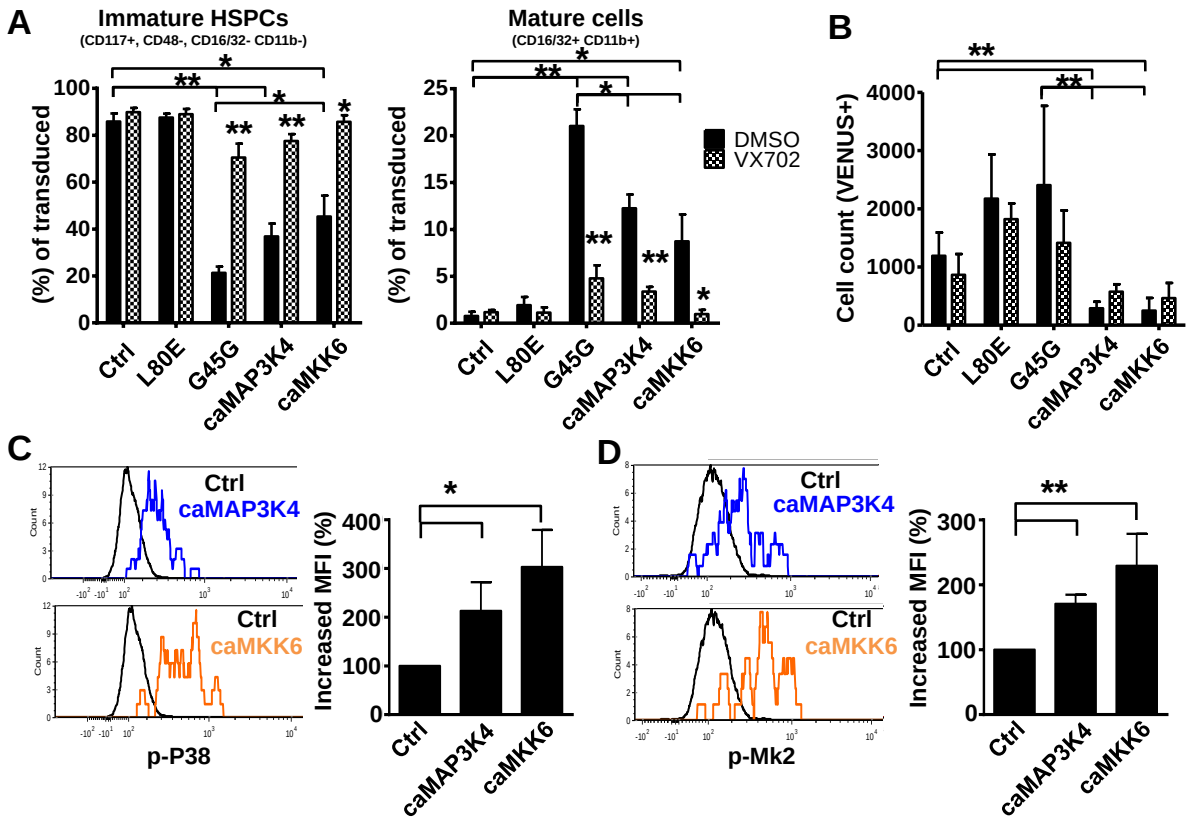

Figure 30: Constitutive active kinases downstream of GADD45G phenocopied GADD45G-induced differentiation of LT-HSCs.

A) 5 day culture of transduced LT-HSCs in absence or presence of the p38 inhibitor Vx702. FACS for immature HSPCs and mature granulocytes and macrophages. B) Cell count of transduced cells (VENUS$^+$) after 5 days in culture. N=5 independent experiments. C) FACS histogram and quantification of phosphorylated p38 and D) phosphorylated MK2 in 5 day-cultured MPPs after transduction of constitutively active MAP3K4 (blue line), constitutively active MKK6 (orange line) or control vector (black line) assessed via phosphoflow cytometry. N=3 experiments. ca, constitutively active. Data are represented as the mean +/- SD. * P<0.05, ** P<0.01.

Expression of caMAP3K4 and caMKK6 induced and accelerated the differentiation of LT-HSCs into mature myeloid cells (Figure 30A). This enhanced differentiation could be blocked by p38 inhibition. Both constitutively active kinases resulted in a lower percentage of mature cells compared to GADD45G. Strikingly, the number of caMAP3K4- or caMKK6-transduced cells was drastically reduced after 5 days (Figure 30B). This may be explained by the activation of JNK by these kinases which results in higher apoptosis rates. Notably, the level of phosphorylated p38 and MK2 was even enhanced in caMAP3K4 and caMKK6-expressing MPPs compared to GADD45G-expressing MPPs (Figure 30C and D). This was not an unexpected result as the level of phosphorylation and therefore activation of p38 would increase with the proximity of the kinase to p38 in the signaling pathway.

In conclusion, both constitutively active MAP3K4 and MKK6 were able to phenocopy GADD45G expression in LT-HSCs and MPPs, suggesting that p38 activation is the major signaling pathway mediating the GADD45G-induced differentiation program.

3.7.1 GADD45G caused induction of genes related with differentiation

In order to identify the genes which are activated by GADD45G, qPCR assays were performed on freshly transduced LT-HSCs. LT-HSCs transduced with either GADD45G or control were cultured for only 60 hours and then sorted for immature cells which express the transgene (VENUS^{+}) and were negative for the differentiation marker CD48. Expression of selected genes was measured via multiplex qPCR (TaqMan® PreAmp Cells-to-CT™ Kit; Life Technologies). The expression levels were normalized to a panel of six housekeeping genes *GusB1, Gapdh, Hprt, ActB, Pum1 and B2m*. Finally, the fold changes in expression levels of the genes between GADD45G- and control vector -expressing LT-HSCs were calculated.

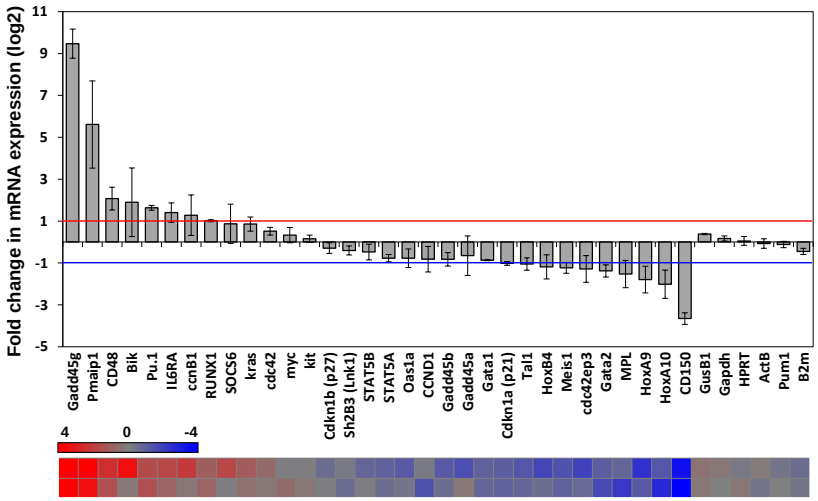

Figure 31: GADD45G induced genes involved in differentiation and inhibited self-renewal genes.

Change in gene expression in LT-HSCs transduced with GADD45G compared to control-transduced LT-HSCs. LT-HSC were transduced with GADD45G or control vector and cultured for 3 days. Cells were sorted for VENUS+ CD48- cells (transduced immature cells) and subjected to mRNA extraction. Selected gene expression was measured by multiplex qPCR. Housekeeping genes used for normalization are displayed on the right. N=2 experiments.

GADD45 transduced of LT-HSCs showed a 500 fold increase in the *Gadd45g* mRNA levels compared to control LT-HSCs (Figure 31). Notably, *Gadd45a* and *Gadd45b* expression was largely unchanged upon GADD45G expression. *Stat5a* and *Stat5b*, the main transcription factors involved in cytokine-induced *Gadd45g* expression were mostly unaffected by

GADD45G overexpression. Two genes of the pro-apoptotic Bcl2 family, *Pmaip1* and *Bik1,* were highly elevated, possibly accounting for the antagonistic Mek/E lineage bias of GADD45G. Markedly, *CD48,* a surface maker for hematopoietic progenitors and a marker for loss of stemness of LT-HSCs, was one of the most prominent genes up-regulated upon GADD45G expression. Several other genes linked to granulocyte-macrophage differentiation such as *IL6Ra*, as well as the transcription factors *Pu.1* and *Runx1* (Nerlov, Graf 1998; North et al. 2004) were elevated by GADD45G expression. On the other hand, *Gata1* and *Tal1,* transcription factors activating megakaryocyte and erythrocyte differentiation (Orkin, Zon 2008) were down-regulated, as were MEP surface markers like *Mpl* and *CD150*. Furthermore, *Gata2,* the *Hox* genes *HoxB4*, *A9* and *A10* as well as *Meis1*, which are all associated with stemness and self-renewal in HSCs, were also down-regulated after GADD45G expression. These data clearly shows at a molecular level, that GADD45G-expressing LT-HSCs are induced to differentiate and have lost their self-renewal program. Furthermore, this gene expression analysis strongly indicates that GADD45G expression provides a gene network in HSPCs that favors myelomonocytic over megakaryocyte/erythrocyte lineage choice and self-renewal. However, a direct and instructive regulation of these genes by the GADD45G-MAP3K4-p38 pathway to induce differentiation can yet not be postulated and requires further elucidation. It may be possible that these gene expression changes only mirror the functional consequences of induced differentiation upon GADD45G expression in LT-HSCs.

3.8 GADD45G reduces proliferation of leukemic cell lines

In many hematopoietic malignancies, uncontrolled proliferation of progenitor cells causes a severe dysfunction of the hematopoietic system. In myeloid leukemias such as acute promyeloid leukemia (APL) and acute myeloid leukemia (AML), the differentiation block in the leukemic cells leads to their unlimited expansion, which eventually results in a displacement of normal hematopoietic cells. Anti- APL therapies with all-trans-retinoic acid (ATRA) (Nowak et al. 2009) and Arsenic trioxide (Estey et al. 2006) function by inducing differentiation in the leukemic cells.

GADD45G as a DNA-damage response and tumor suppressor gene, is epigenetically silenced in many malignancies (Bahar et al. 2004; Ying et al. 2005; Zhang et al. 2010; Guo et al. 2013a; Guo et al. 2013b). Differentiation induction and apoptosis in cancer cells through re-expression of GADD45G has been shown in several studies (Ying et al. 2005; Perugini et al. 2009). Re-expression of GADD45G in the myeloblastic leukemic cell line M1 and in two

acute lymphoblastic leukemia (ALL) cell lines T-cell MOLT-4 and pre-B-cell Reh resulted in growth arrest and induction of apoptosis (Zhang et al. 2001; Scuto et al. 2008). To test the effect of GADD45G on AML growth, GADD45G was over-expressed in three established AML cell lines - HL-60, U937 and Kasumi1. Cell expansion was analyzed after 2 and 8 days of transduction via manually counting the cells and assessing the transduction efficacy via FACS.

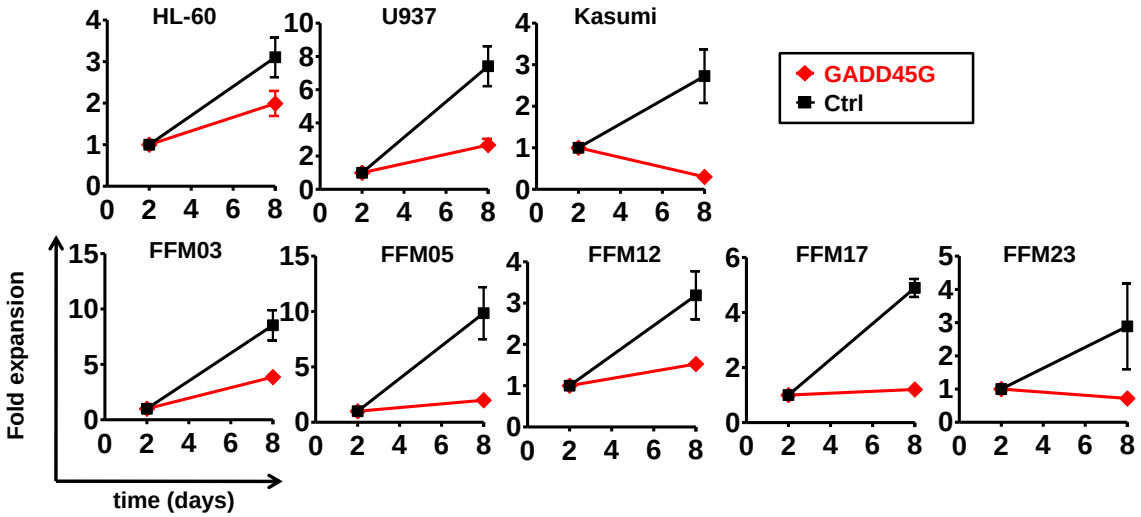

Figure 32: GADD45G expression impaired the expansion of leukemic cells.

5000 cells of the indicated acute myeloid leukemia cell lines and primary patient-derived cells were plated and transduced with either control vector or GADD45G expression vector. Cell count of transduced cells was determined at day 2 and day 8 after transduction. Transduced cell counts were normalized to day 2. N=2 experiments.

All leukemic cell lines were severely affected by the expression of GADD45G (Figure 32). Transduced HL-60 and U937 cells only marginally expanded from day 2 to day 8. Kasumi cells transduced with GADD45G underwent rapid decrease in numbers due to induction of apoptosis. We also tested five primary AML-patient derived cell lines that were kindly provided by Gesine Bug from the Hematology Department of the University Hospital, Frankfurt. GADD45G expression affectively reduced the expansion of all primary leukemic cells (Figure 32 lower panel). In the AML samples FFM05 and FFM17, cell growth was completely blocked by GADD45G expression and in FFM23 GADD45G resulted in a net loss of transduced cells at day 8 compared to day 2.

To test whether the observed growth inhibition was due to GADD45G-induced cell death or due to induced differentiation, the phenotype of the cells was investigated with a live/dead cell exclusion and with an antibody staining for myelomonocytic differentiation markers CD14 and CD11b via FACS analyses 8 days after GADD45G transduction.

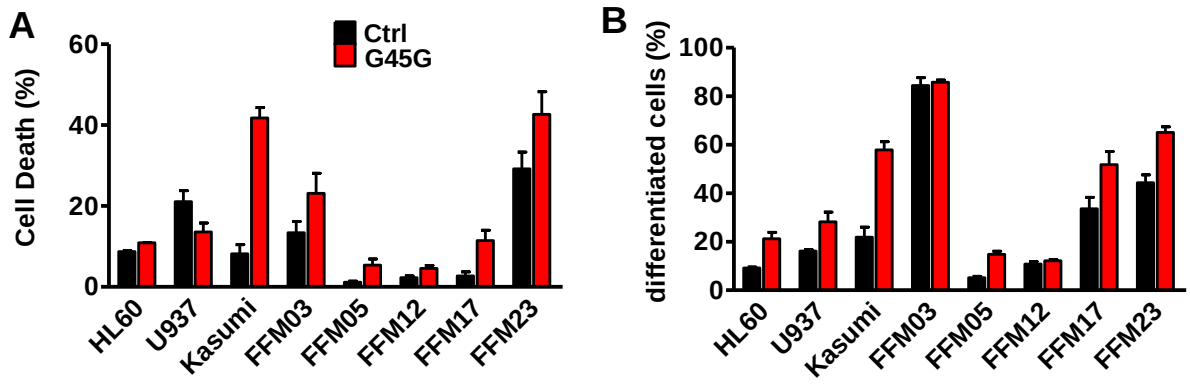

Figure 33: GADD45G induced cell death and differentiation of AML cells.

A) Cell death of transduced AML cells calculated by staining of apoptotic and necrotic cells with fixable viability dye at day 8 of control vector or GADD45G vector transduction. B) Percentage of differentiated cells was assessed by CD11b and CD14 surface expression of transduced cells at day 8.

In all samples, except for HL-60 and FFM12, an increased cell death could be observed (Figure 33A). Furthermore, the majority of cells induced the expression of the two differentiation markers CD14 and CD11b, indicating that the leukemic cells start to differentiate once GADD45G is expressed. Only two primary leukemic cell lines FFM03 and FFM12 did not show an enhanced differentiation upon GADD45G re-expression. However, 80% of the cells of patient FFM03 already expressed CD14 and CD11b at basal conditions (Figure 33B). In conclusion it could be shown in this preliminary experiment that forced expression of GADD45G results in efficient inhibition of leukemic cell growth accompanied by increased cell death and terminal differentiation. This makes the signaling pathway mediated by GADD45G an interesting target strategy for AML therapy. The pathway may be utilized to overcome the differentiation block in AML to prevent uncontrolled cell expansion.

4. Discussion

4.1 The function of GADD45G as a toggle switch in cytokine-mediated differentiation induction

In this study, the physiological and pathological function of GADD45G expression in HSCs was elucidated on normal and stress-mediated hematopoiesis. GADD45G strongly induced and accelerated the differentiation of LT-HSCs in vivo and in vitro. Once GADD45G was up-regulated in LT-HSCs, the differentiation occurred rapidly within 36 hours, and the cells were unable to self-renew anymore. Differentiation in HSCs is promoted by differentiation-inducing cytokines, such as IL3 and IL6 (Ema et al. 2000). However, the molecular integration of these extrinsic cytokine signals into a differentiation program remained largely elusive. Here we show that GADD45G is a molecular linker of cytokine-induced differentiation induction in HSCs.

As presented in this study, TPO, IL3 and IL6 were able to induce *Gadd45g* in LT-HSCs and MPPs, while only IL6 was able to induce *Gadd45b* expression in MPPs. IL3 and IL6 are known differentiation promoting cytokines (Peters et al. 1996; Ohmori et al. 2009) and the addition of one or both leads to enhanced differentiation of cultured cells (Ema et al. 2000). The effects of over-expressing GADD45G on LT-HSC differentiation were similar to those elicited by IL3 and IL6. All the three tested cytokines activate STAT signaling. TPO and IL3 mostly activate STAT5A/B signaling via JAK2 phosphorylation (Hoffmeyer et al. 2001), while IL6 mainly activates STAT3 signaling, which is connected to differentiation induction of hematopoietic progenitors (Kato et al. 2005). STAT5 enhances proliferation of hematopoietic progenitors by activating the NF-κB signaling pathway (Nosaka et al. 1999; Kato et al. 2005). Only promoters of *Gadd45b* and *Gadd45g,* but not *Gadd45a* contain putative STAT binding sides which differ in their structures (Balliet et al. 2003). These differences in the promoter structures and therefore STAT affinities may explain why only IL6 was able to induce *Gadd45b*, while *Gadd45g* was stimulated by all cytokines. The weak effect of IL3 and the absence of any effect of IL6 on LT-HSCs can likely be explained by the absent surface expression of their corresponding receptors CD123 and CD126, respectively (McKinstry et al. 1997; Morita et al. 2010). Both cytokine receptors are expressed only at very low levels starting in activated LT-HSCs and early MPPs (Takizawa et al. 2012) and are up-regulated during GM commitment (Gazit et al. 2013). Stimulation of MPPs and GMPs with IL3 and IL6 boost their proliferation, but at the same time accelerate their differentiation

(Yonemura et al. 1996; Ema et al. 2000), highlighting the differentiation promoting function of IL3 and IL6 in hematopoiesis.

The question remains as if there are factors that suppress GADD45G-induced differentiation in LT-HSCs to allow self-renewal under homeostasis. Both GADD45G mRNA and protein are only expressed at low levels in steady-state, but can be rapidly triggered by cytokine stimulation via the transcription factors STAT5A/B in LT-HSCs (our study) and in lineage restricted hematopoietic cells (Hoffmeyer, et al., 2001). The mRNA of *Gadd45g* is very instable with a half-life of less than one hour in murine embryonic stem cells (Sharova et al. 2009). Therefore, it is likely that some factors are involved in mRNA and protein destabilisation, especially in the niche, that keeps GADD45G protein levels below a threshold to prevent differentiation. The instability of the *Gadd45g* mRNA and its product further support the notion that GADD45G acts as a toggle switch in LT-HSCs that is kept in an "off" state during LT-HSC maintenance and self-renewal, and is triggered by extrinsic factors to become "on" for differentiation induction. Once triggered, GADD45G mediates its function to commit LT-HSCs into differentiation, and shortly after disappears again. A similar kinetics was measured by cytokine stimulation in HSC (Beerman et al. 2014) Figure 7).

Several studies have shown that TPO and its receptor MPL are important to maintain HSCs in a quiescent state *in vivo* (Yoshihara et al. 2007; de Graaf, Metcalf 2011; Qian et al. 2007). However, TPO is regularly used to expand murine and human HSPCs in short term cultures (Zhang, Lodish 2005; Boitano et al. 2010). How TPO controls both expansion and quiescence in HSCs is still unclear, although other factors in the niche *in vivo* might play an additive role which is missing *in vitro*. Only recently a novel mechanism of MPL receptor "inside-out" signaling upon TPO stimulation was described, which may induce HSC quiescence by integrin signaling in the BM niche (Umemoto et al. 2012). MPL stimulation results in a conformational change in the αvβ3-integrin into a high-affinity form. Binding of the integrins by ligands provided by the niche causes a phosphorylation of integrin-β3 that induces enhanced expression of stemness-related genes and decreased HSC *ex vivo* expansion with a higher long term repopulating capacity (Umemoto et al. 2012). This might explain the dual function of TPO, namely the induction of quiescence *in vivo*, and the promotion of HSC proliferation *in vitro* (Heike, Nakahata 2002; Sauvageau et al. 2004; Huynh et al. 2008; Zhang, Lodish 2008; Boitano et al. 2010). It might be important to test whether the integrin signaling would destabilise GADD45G *in vivo* and thereby prevent differentiation induction.

To further elucidate the molecular pathway by which GADD45G elicits accelerated differentiation, several known interaction partners of GADD45G were analysed. As shown via

single cell tracking, the cell cycle kinetics were not altered in GADD45G-transduced cells. Therefore, the focus was directed to the mitogen activated protein kinases (MAPK) p38 and JNK, that have been implicated in progenitor differentiation in haematopoiesis (Rausch, Marshall 1999; Geest, Coffer 2009), osteoblastogenesis (Hu et al. 2003) and colon regeneration (Houde et al. 2001). As presented in this thesis the inhibition of p38 but not JNK blocked GADD45G-induced differentiation. The molecular mechanism of GADD45G-mediated p38 activation is well established. (Takekawa, Saito 1998; Mita et al. 2002). GADD45G binds to the auto-inhibitory domain of MAP3K4, which gets activated and phosphorylates itself. The activated MAP3K4 can phosphorylate downstream kinases MKK3 and MKK6, which in turn phosphorylate MAPK p38 or JNKs. MAPK p38 or JNKs are effector kinases, which eventually can activate other kinases, cell cycle regulators or transcription factors. GADD45G may also activate p38 through direct binding (Bulavin et al. 2003; Zhu et al. 2009). Intriguingly, phosphorylated p38 activated GATA-3 in LT-HSCs (Frelin et al. 2013) which translocates into their nuclei resulting in the exit from the self-renewal program and in entering a differentiation commitment. This study has revealed a molecular link between cytokine-induced differentiation in LT-HSCs and the intracellular, p38-mediated GATA3 activation and the loss of self-renewal.

Preliminary gene expression analysis after GADD45G expression on LT-HSCs showed a down-regulation of self-renewal genes and an up-regulation of genes of the myeloid-monocytic lineage. Gene expression analysis of LT-HSCs in the presence or absence of p38 inhibitors will help to identify genes that are specifically and directly regulated by the GADD45G-p38 axis, and are immediate inducers of the differentiation commitment program in LT-HSCs.

4.2 Manipulation of the GADD45G/ MAP3K4/ p38 activation for enhanced LT-HSC self-renewal

This study has shown that *Gadd45g*$^{-/-}$ HSCs are superior in their self-renewal capacity. *In vitro*, *Gadd45g*$^{-/-}$ cells exhibit a higher and protracted colony formation potential and a slower expansion of HSCs after cytokine stimulation. The loss of GADD45G renders the hematopoietic cells partially unresponsive to cytokine signaling and delays the onset of differentiation. The *in vitro*-observation was confirmed by competitive serial transplantation, demonstrating that secondary transplantation of *Gadd45g*$^{-/-}$ HSCs exhibited an increased self-renewal and a better repopulation potential than their wild type controls. These findings

suggest that targeted inhibition of the GADD45G-mediated pathway could be feasible to prolong cultivation of HSCs in culture without differentiation induction.

As discussed before, p38 activation is pivotal in the pathway of differentiation induced by GADD45G as inhibition of p38 completely abolished the accelerated differentiation. P38 regulates a variety of cellular processes such as differentiation, cell cycle arrest/senescence and apoptosis in a cell type-specific manner (Platanias 2003; Zarubin, Han 2005; Ventura et al. 2007). It has been shown that activation of p38 by EPO, IL-3 and G-CSF stimulates hematopoietic progenitor proliferation and differentiation (Rausch, Marshall 1999; Geest, Coffer 2009). Furthermore, stress stimuli such as elevated ROS levels can activate p38 in HSCs eventually leading to HSC exhaustion (Ito et al. 2006). Prolonged treatment of the HSCs with antioxidants or p38 inhibitors restores HSC capacity. Knockdown of p38 in primary human HSPCs enriched the CD34+ fraction after 20 days of culture (Baudet et al. 2012). When treated with p38 inhibitors cultured CD34+ CB cells also reconstituted better in immune-deficient mice than the untreated CBCs. HSPCs from both murine BM and human cord blood activated p38 after several days in culture (Wang et al. 2011; Zou et al. 2012; Baudet et al. 2012). After inhibition of p38, significantly more LSK or CD133+CD38- HSPCs with higher colony or cobblestone area forming cell potential could be maintained in culture. The treated cells showed better engraftment into lethally irradiated mice compared to untreated cells indicating that p38 inhibition maintains HSCs *in vitro*. No signs of altered hematopoietic functions were detected upon p38 inhibition apart from decrease in ROS levels and an increase in CXCR4 expression (Wang et al. 2011; Zou et al. 2012). While these studies have shown that of p38 inactivation improves human and murine HSC maintenance, they have failed to analyse the direct effect of p38 inactivation on HSCs since they only used HSC enriched fractions instead of defined HSC populations. Single cell analysis of highly purified HSCs treated with p38 inhibitors can be performed to determine whether p38 inhibition directly promotes HSC expansion by blocking differentiation. Hematopoietic cytokines provide essential signals for various cell fates and functions, such as i.e. proliferation, survival, differentiation, and maturation (Rieger, Schroeder 2009). However, the molecular pathways that coordinate all these different functions from a single receptor in a cell type and differentiation stage-specific manner are still largely unclear. Uncoupling the proliferation-promoting pathway from the differentiation-promoting pathway would allow us to use these cytokines in HSC expansion cultures without inducing differentiation. With our findings that GADD45G is specifically activating p38 downstream of IL3 and IL6 signaling, we may have a change using p38 inhibitors in the presence of IL3 to fuel HSC proliferation while blocking

differentiation. Indeed, first single HSC tracking data revealed that the cell cycle is not changed by the addition of p38 inhibitors to IL3 containing cell cultures.

Most of the current HSC expansion methods still rely on ectopic expression of self-renewal factors in HSCs or co-culture of the cells with stromal elements (Kelly et al. 2009; Flores-Guzmán et al. 2013). These methods have many drawbacks which limit their clinical practice because of concerns of HSC transformation by gene transfection, high costs of the hematopoietic growth factors, and difficulty to standardize stromal elements to meet the FDA regulations. *Ex vivo* expansion of human HSCs with small molecule inhibitors may open new avenues for regenerative medicine as the number of functional stem cells is the bottle neck for successful therapy. The prolonged *ex vivo* culture of HSCs necessary to deliver the gene therapy constructs reduces the efficacy of these protocols as the HSCs start to differentiate soon after they are taken into culture. A culture system which prevents differentiation of HSCs would therefore allow the HSCs to be effectively targeted for gene delivery or gene correction before transplantation into the patient. To evaluate the potential of p38 inhibitors for applications in regenerative medicine, a detailed analysis of the expanded cells is necessary to determine the amount of remaining HSCs and their potential for long-term repopulation of the host.

4.3 GADD45G acts as a mediator of selective lineage choice by extrinsic control

All the three GADD45 proteins accelerated the differentiation of hematopoietic progenitors with GADD45G emerging as the strongest activator of differentiation. Notably, none of the GADD45 proteins evoked growth arrest or apoptosis in the early hematopoietic cells, as suggested by their broadly distributed function as growth arrest genes in other cellular systems (Liebermann 2013). Various studies support the notion that there might be considerable redundancy in function of individual Gadd45 family members, besides very specific functions controlled in a cell type-specific manner. The stimulation of myeloblastic leukemia cells M1 with GM-CSF or IL3 lead to terminal differentiation accompanied by rapid induction of *Gadd45b* and *g* expression (Zhang et al. 1999b). Interestingly, ectopic expression of either isoform also induced growth arrest and differentiation in those cells (Selvakumaran et al. 1994; Zhang et al. 2001), pointing to a function of all three GADD45 isoforms in hematopoietic maturation. No major perturbations in homeostatic hematopoiesis could be detected in mouse models with a knockout of any of the *Gadd45* genes (Gupta et al. 2006a; Liu et al. 2005; Hoffmeyer et al. 2001), suggesting that the GADD45 proteins may have redundant functions. Despite a general role of GADD45 family members in HSCs

differentiation induction, we noticed a fundamental difference in hematopoietic lineage choice using the different GADD45 family members. GADD45G-expressing LT-HSCs mainly gave rise to granulocytes and macrophages in myeloid differentiation cultures with an absence of Mek/E cell fate. In contrast, GADD45A- or GADD45B-transduced LT-HSCs still exhibited unchanged ME potential. Therefore GADD45G has an additional function in hematopoietic differentiation by promoting the GM lineage choice over ME lineage. Along this line, BM cells from *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice generated far fewer mature macrophages and granulocytes compared to WT cells upon cytokine stimulation (Gupta et al. 2006b).

The fact that only *Gadd45g* promoter has a C/EBP binding site pointed to an important role of GADD45G in lineage instruction (Balliet et al. 2003). The transcription factors C/EBP A and B are very potent trans-differentiation factors that can force nearly all hematopoietic cells into monocyte and macrophage development (Xie et al. 2004). Two models controling lineage choice have been postulated, lineage instruction and lineage selection (Metcalf 1998; Enver et al. 1998). In lineage instruction, all progenitor cells of a multipotent cell would be induced to develop into one lineage (in this case GM committed cells) by switching their internal programs specifically into this lineage (Metcalf 1998). In contrast, a selection process would eliminate cells that are committed to other lineages (in this case ME cells) by the inability to provide signals for survival and maturation (Enver et al. 1998). To discriminate between the two scenarios, development of LT-HSCs expressing GADD45G into GMPs was continuously tracked via video-microscopy and cell tracking. Cell death was up-regulated in GADD45G-transduced cells, that were not already restricted to GM lineage (as detected by expression of CD16/32), compared to control transduced cells. Since loss of megakaryocytes in culture occurred partly due to enhanced apoptosis of uncommitted progenitors, a selective process was likely. The importance of our single cell tracking technique was shown in cooperation with Benajmin Rengstl to unravel the origin of multi-nucleated Reed-Sternberg (RS) cells in hodgkin lymphoma (Rengstl et al. 2013). Giant multinucleated RS cells display the pathognomonic hallmark of Hodgkin lymphoma and contain high immunomodulatory potential inducing a tumor-promoting microenvironment. With the single cell tracking we could show, that RS cells derived from re-fusion of small mononucleated Hodgkin cells. In another project single cell tracking revealed, that transcriptional changes after FuBP1 knock-down upregulate p21 and apoptotic genes which cause increased apoptosis and prolonged generation times in HSCs and progenitors, which eventually lead to HSCs exhaustion and severe anemia (Rabenhorst, Thalheimer, Gerlach, *et al*., submitted manuscript).

There are at least two explanations as to why the development into megakaryocytes and erythrocytes is blocked. First, the differentiation of early progenitors is altered to produce other lineages hindering the production of MEPs and second, MEPs do arise but cannot differentiate into mature cells. To test if MEPs can arise in vivo from GADD45G-expressing HSPCs, LSK cells where transduced either with GADD45G or control and transplanted into lethally irradiated mice. HSPCs expressing GADD45G could home to and engraft in the bone marrow producing LSK cells and GMPs, but not CD150 positive MEPs. This suggested that GADD45G leads to a decision against megakaryocyte and erythroid development at an earlier progenitor level, presumably already at the LMPP / MEP bifurcation. MEPs, in contrast to GMPs, also exhibit a severe reduction in colony formation after GADD45G expression, further supporting a selective mechanism behind GADD45G induced lineage choice in HSCs. Significantly, no sign of trans-differentiation could be detected in MEP culture, which might occurred if an immature cell is forced to express an instructive factor like C/EBP. On the other hand, the rapid induction of differentiation and the commitment of nearly all cells to the GM lineage, suggests an instructive process initiated by GADD45G. The meta-stable progenitor stages seem unchanged, but are temporally shortened to a minimum to allow epigenetic and proteome rearrangements necessary for mature cell development. This finding opens a new perspective of our current understanding of sequential differentiation programs in hematopoiesis, as for the current school of thought is that differentiation proceeds through several progenitor stages and several cell divisions (Rothenberg 2007; Kueh et al. 2013).

Interestingly, *Gata1* and *Tal1,* both transcription factors important for megakaryocyte and erythrocyte differentiation (Orkin, Zon 2008), were down-regulated in LT-HSC upon GADD45G expression. Other prominent genes that were down-regulated were *Meis1*, *Gata2* and the *Hox* genes – transcription factors linked to HSC self- renewal and maintenance (Gazit et al. 2013). In contrast, the transcription factor *PU.1*, which drives differentiation of hematopoietic progenitors into granulocyte/ macrophage linages, was found to be one of the most strongly induced genes in LT-HSCs upon GADD45G expression (Nerlov, Graf 1998). Moreover, genes marking hematopoietic differentiation such as the surface molecules CD48 and IL6 receptor were also up-regulated (Peters et al. 1996; Kiel et al. 2005). These global expression data indicate that GADD45G expression alters the transcriptome of hematopoietic stem and progenitor cells favoring myelomonocytic differentiation over self-renewal or megakaryo-/ erythropoiesis. Therefore, GADD45G may also have an additional inductive function towards the GM lineages promoting a suppression of ME-supporting genes. Since the exact timing between viral transduction and expression of GADD45G remained elusive, it

is not possible to state if the differences in transcription described here are the cause or the consequence of GADD45G-mediated differentiation and lineage selection. Further experiments aiming to identify direct targets of GADD45G activation in LT-HSCs, GMPs and MEPs have to be performed to unravel the network by which the GADD45-mediated pathway exerts its functions. Inducible GADD45G lentiviral expression systems will be utilized to enable timed onsets of expression in HSCs.

Several studies have shown that p38 signaling can also induce apoptosis in different cell lines (Tront et al. 2006). The outcome of p38 signaling seems to depend on the intensity of the stimulus as well as the cell type (Takekawa et al. 2011; Liebermann et al. 2011). Activation of p38 in MEPs may lead to induction of apoptosis in a cell type specific manner. More importantly, p53 was identified as important regulator of polyploidization and platelet release during megakaryopoiesis by impeding cell cycling and promoting apoptosis (Fuhrken et al. 2008). During early megakaryopoiesis, the p53-mediated apoptosis is balanced with NF-κB mediated endomitosis and polyploidization, while at later stages NF-κB signaling reduces and *Gadd45a* is induced (Chen et al. 2007). GADD45A was associated with up-regulation of other pro-apoptotic genes including *Pmaip1* and *Bim* (Chen et al. 2007). Interestingly in early progenitors derived from GADD45G-expressing HSCs, pro-apoptotic Bcl2 family members *Pmaip1* and *Bik* were up-regulated. This up-regulation may lead to cell cycle arrest and induction of apoptosis, which would normally occur only in late megakaryocyte development. GADD45G may also induce apoptotic signaling by JNK pathway or induce cell cycle arrest through interaction with cdc2 and p21. Modulation of cell cycle arrest could prevent progression of ME progenitors to polyploid megakaryocytes. How GADD45G and not the other two members exert selection against ME lineage development is yet to be analysed. Therefore differential transcriptome analysis of HSCs, GMPs and MEPs expressing inducible GADD45 systems (TET-controlled lentiviral vectors) are planned.

4.4 GADD45G as potential gate keeper for tumorigenesis, stress-induced damage and aging

The GADD45 proteins are also known to function as stress sensors and regulators of DNA repair (Barreto et al. 2007; Liebermann 2013). Typically, stressed cells first undergo cell cycle arrest allowing repair of their damaged DNA, failing DNA repair they undergo apoptosis to prevent transformation and development of cancer (Liebermann, Hoffman 2008). Alternatively, the induction of terminal differentiation in LT-HSCs and hematopoietic

progenitors into cells with limited lifetime also provides a mechanism to preserve the integrity of the blood system. This safety mechanism of LT-HSCs was proposed as a function of the transcription factor BATF in aged HSCs to eliminate damaged HSCs from the system (Wang et al. 2012). In the same study, an enhanced expression of GADD45G was also seen in aged HSCs and depletion of GADD45G promoted self-renewal and accumulation of HSCs (Wang et al. 2012). Another recent study showed that all three GADD45 proteins are highly expressed in HSCs compared to later progenitors (Beerman et al. 2014), which is consistent with data from our lab (de Giacomo 2012). Moreover, they measured strong up-regulation of GADD45G after induction of cell cycle in quiescent HSCs along-with other factors involved in DNA damage response. Stressors like radiation or mutagens also induce GADD45 expression, as shown in several tissues and cell types (Liebermann et al. 2011). If the stress stimulus would be strong enough, this would trigger loss of HSC self-renewal and induction of differentiation to prevent damage accumulation, which eventually would lead to eradication of those cells form the stem cell pool. A very recent study on *Gadd45a*-deficient mice showed that loss of GADD45A promoted HSC survival upon ionizing radiation by preventing apoptosis but accumulation of DNA damage, which increased susceptibility of the mice to leukemia (Chen et al. 2014). This is consistent with our and others' findings that *Gadd45*-deficient hematopoietic cells have a defect in terminal differentiation (Hoffmeyer et al. 2001; Gupta et al. 2006b). As discussed earlier, p38 is a direct target of elevated ROS levels (another stress indicator) in HSCs and inhibition of p38 signaling was able to restore HSC function in stressed ROS high cells (Ito et al. 2006). This suggests that the GADD45G-p38 axis may also be important to eradicate stressed cells from the stem cells pool. It remains to be analysed whether GADD45G also plays a role in DNA damage repair and genome integrity in HSCs. HSCs from aged mice showed elevated levels of GADD45 proteins and accumulation of DNA damage (Beerman et al. 2014). Activation of HSCs resulted in a rapid induction of GADD45 proteins and increased DNA damage response. Concordantly, forcing HSCs into cell cycle is accompanied with increased DNA-damage (Walter et al. 2015). Even physiologic stress, such as infection or chronic blood loss, leads to accumulation of DNA damage and may be the cause of increased DNA damage in aged HSCs. In case of Fanconi anemia mouse model with insufficient DNA repair, repeated activation lead to HSCs exhaustion and BM failure. Similar stress studies with 5-FU (Beerman et al. 2014; Walter et al. 2015) on *Gadd45g*-deficient mice already revealed a faster recovery, associated with an increase in the proliferation rate (data in communication with Roberta Scognamiglio, HiStem, Heidelberg). Currently, *Gadd45g*$^{-/-}$ mice and aged match littermate are housed in our facility

till they are of the right age to be analysed. The composition of progenitors in the bone marrow and their capacity to form colonies, the extent of accumulated DNA damage and the ability of the DNA damaged HSCs to react on cytokine stimuli will be analysed.

Epigenetic inactivation of *Gadd45* genes has been observed in many malignancies including hematopoietic disorders (Liebermann, et al., 2011). *Gadd45g* was of special interest because its locus is epigenetically silenced in many tumors and hematopoietic malignancies (Bahar et al. 2004; Ying et al. 2005; Zhang et al. 2010; Guo et al. 2013a; Guo et al. 2013b). In this study, GADD45G expression in different leukemic cell lines lead to significant reduction in cell proliferation accompanied by differentiation marker expression and induction of apoptosis. *Gadd45g* down-regulation in leukemic cells may have a dual beneficial role for the latter (Casolari et al. 2012). Firstly, down-regulation of DNA damage response genes may cause the acquisition of several genomic alterations which allow tumor progression and secondly, prevent differentiation of the leukemic cells. Re-expression of GADD45G or activation of its down-stream pathways in leukemic cells may induce apoptosis in highly damaged cells or induce terminal-differentiation, the latter activating cell cycle arrest. Terminal differentiation may be especially helpful in targeting leukemic cells as it is now largely accepted that AML is a stem cell driven decease (Bonnet, Dick 1997; Shlush et al. 2014). Many of the genes found down-regulated by GADD45G expression in HSCs are thought to be important in leukemic stem cell progression (Horton, Huntly, Brian J P 2012). For example, Meis1 and HoxA9 are essential for leukemia self-renewal and leukemia survival (Wong et al. 2007; Faber et al. 2009). Further studies on AML samples from the UCT bio bank are planned to analyse expression of GADD45 proteins in AML. GADD45G-mediated signaling should be utilized therapeutically to switch mis-regulated self-renewal programs in cancer-initiating cells into differentiation. Promising experiments have been already performed in PH- ALL samples (Scuto et al. 2008). Reactivation of GADD45G expression using a histone deacetylase inhibitor, LBH589 lead to induction of apoptosis in ALL samples.

5. Summary

Hematopoietic stem cells (HSCs) have the unique abilities of life-long self-renewal and multi-lineage differentiation. They are routinely used in BM or stem cell transplantations to reconstitute the blood system of patients suffering from malignant or monogenic blood disorders. For an adequate production of each blood cell lineage in homeostasis and under stress conditions, the fate choice of HSCs to either self-renew or to differentiate must be strictly controlled. The incomplete understanding of the molecular mechanisms that control this balance makes it still impossible to maintain or expand undifferentiated HSCs in culture for advanced regenerative medical purposes.

The aim of this thesis was the identification and molecular characterisation of mechanisms that control the decision of HSCs to self-renew or to differentiate, and how they are connected to extrinsic cytokine signaling control. Prior to this thesis, a screening for genes upregulated under self-renewal promoting thrombopoietin (TPO) signaling via the transcription factors STAT5A/B in HSCs was conducted, and Growth arrest and DNA damage inducible 45 gamma (Gadd45g) was one of the regulated genes. GADD45G was described as stress sensor, DNA-damage response and tumor suppressor gene, that is epigenetically silenced in many solid tumors and leukemia. Furthermore, Gadd45g is upregulated in aged HSCs with impaired multi-lineage reconstitution abilities, and it is induced by differentiation promoting cytokines in GM-committed cells. However, the function of GADD45G in LT-HSCs was unknown. All these points warrant further investigation to unravel the function of GADD45G on early cell fate decisions of HSCs in hematopoiesis.

The expression of *Gadd45g* was stimulated by hematopoietic cytokines TPO, IL3 and IL6 both in HSCs and MPPs, making GADD45G in interesting target to focus on. To simulate the cytokine-induced expression GADD45G was lentivirally transduced in HSCs. Surprisingly, GADD45G did not induce cell cycle arrest or cell death in hematopoietic cells neither *in vitro* nor *in vivo,* as reported in many cell lines. Instead GADD45G revealed an enhanced and markedly accelerated differentiation of HSCs into mainly myelomonocytic cells, similar as observed for IL3 and IL6 containing cultures. Also in vivo, GADD45G rapidly initiates the differentiation program in HSCs at the expense of self-renewal and long-term engraftment, as shown by serial HSC transplantation experiments. Along the same line, HSCs from *Gadd45g*-knock out mice exhibited an increased self-renewal. *In vitro*, *Gadd45g*$^{-/-}$ progenitors showed higher and prolonged colony formation potential and slower expansion after cytokine stimulation. The loss of *Gadd45g* increased HSC self-renewal and improved repopulation in secondary recipients, determined by serial competitive transplantations. Taken

together, GADD45G could be identified as molecular link between differentiation-promoting cytokine signaling and rapid differentiation induction in murine LT-HSCs.

As presented in this thesis the differentiation induction of GADD45G was mediated by the activation of the cascade of MAP3K4 – MKK6 –p38 MAPK. Small molecule inhibition of p38, but not JNK, blocked the GADD45G-induced differentiation. GADD45G binds to MAP3K4 and releases its auto-inhibitory loop by a change in confirmation, initiating this cascade. Phosphoflow cytometry demonstrated the activation of p38 and a downstream kinase MK2 by GADD45G expression in MPPs. Furthermore, the expression of constitutive active MAP3K4 and MKK6 were able to phenocopy GADD45G-induced differentiation, which could be blocked by p38 inhibition.

The other two family members GADD45A and B also induced accelerated differentiation in LT-HSCs. Interestingly, only GADD45G suppressed the differentiation into megakaryocyte and erythrocyte (Mek/E) lineage cells suggesting a role of GADD45G in lineage choice. Long-term time-lapse microscopy-based cell tracking of single LT-HSCs and their progeny revealed that, once GADD45G is expressed, the development of LT-HSCs into granulocyte-macrophage-committed progeny occurred within 36 hours, and uncovered a selective lineage choice with a severe reduction in Mek/E cells. Furthermore, no megakaryocytic-erythroid progenitors (MEPs) could develop from HSPCs in BM 2 weeks after transplantation suggesting a very early selection against Mek/E cell fates. In line with these findings, GADD45G-transduced MEPs could not expand or form colonies *in vitro*, demonstrating that the differentiation program induced by GADD45G is not compatible with Mek/E lineage fate. Gene expression profiling of HSCs indicated that GADD45G promotes myelomonocytic differentiation programs over programs for self-renewal or megakaryo-/ erythropoiesis. The here identified differentiation induction potential of GADD45G is so strong that the expression of GADD45G in primary acute myeloid leukemia (AML) cells inhibited their expansion accompanied by enhanced differentiation and increased apoptosis.

The here presented work shows that IL3 and IL6 induce a differentiation program in HSCs via GADD45G and p38 closing the link of extrinsic cytokine signaling and differentiation induction. Since the loss of *Gadd45g* increased the self-renewal and slowed HSC differentiation, this may be utilized, i.e. by p38 inhibition, to *ex vivo* maintain and expand HSCs by preventing cytokine-induced differentiation. Furthermore, Re-expression of GADD45G may overcome the differentiation block in leukemia to eliminate these cells by driving them into terminal differentiation and apoptosis.

6. Zusammenfassung

Das hämatopoetische System stellt ein hoch regeneratives Organ dar. Täglich werden mehr als 2,5x 10^{11} reife Blutzellen pro Tag in unserem Körper produziert. Alle reifen Blutzellen werden aus einer kleinen Population von hämatopoetischen Stammzellen (*hematopoietic stem cells*; HSCs) über mehrere, sich stark vermehrende Vorläuferzellstufen gebildet. HSCs besitzen die einzigartige Eigenschaft, sowohl sich selbst ein Leben lang zu erneuern (Selbsterneuerung), als auch sich in alle hämatopoetischen Zelltypen zu differenzieren (Multipotenz). HSCs spielen bei Knochenmarks- und Stammzell-Transplantationen in der regenerativen Medizin eine essentielle Rolle, da sie die komplette Wiederherstellung des Blutsystems eines Patienten bei vererbten Blutkrankheiten und nach Tumortherapie ermöglichen. Um die korrekte Produktion der reifen Blutzellen unter Homöostase und unter Stressbedingungen zu gewährleisten, müssen die Prozesse, die die Selbsterneuerung und Differenzierung der HSCs steuern, streng reguliert werden. Allerdings sind die molekularen Prozesse, die diese Entscheidungen steuern, noch unzureichend verstanden. Trotz enormer Anstrengungen ist es bis heute nicht möglich, HSCs hinreichend zu expandieren, da die Zellen nach kurzer Zeit in Kultur ihre Selbsterneuerung verlieren und zu differenzieren beginnen. Die Nutzung der HSCs in der Grundlagenforschung und in der regenerativen Medizin wird dadurch stark beeinträchtigt.

Auf der Suche nach Molekülen, die die Balance zwischen Selbsterneuerung und Differenzierung in LT-HSCs regulieren, wurde in Vorarbeiten ein Screening durchgeführt. Dabei wurde das „*Growth arrest and DNA damage inducible gene 45 gamma*“ (*Gadd45g*) Gen mittels Thrombopoietin (TPO) Stimulation über die Transkriptionsfaktoren STAT5A/B spezifisch in HSCs hochreguliert. Die GADD45 Familienmitglieder sind Stress-Sensoren, die durch Interaktion mit Partnerproteinen mehrere fundamentale Prozesse wie Zellzyklus-Kontrolle, DNA-Schadensreparatur, Apoptose und Zellüberleben beeinflussen. Untersuchungen an Mäusen zeigten, dass die GADD45 Mitglieder an der Reifung und Aktivierung von T-Zellen, Granulozyten als auch Makrophagen beteiligt sind. *Gadd45g* ist zudem in HSCs von alternden Mäusen erhöht exprimiert, welche im Alter eine reduzierte Selbsterneuerung aufweisen.

Die vorliegende Arbeit untersucht die Funktion von GADD45G in der Hämatopoese, im Besonderen in der frühen Schicksalsentscheidung zwischen Selbsterneuerung und Differenzierung, sowie den damit verbundenen molekularen Mechanismus. In Expressions-analysen von HSCs und multipotenten Vorläufern (*multipotent progenitors;* MPPs), welche prospektiv durch FACS hochrein angereichert wurden, konnte gezeigt werden, dass von den

drei Familienmitgliedern nur *Gadd45g* durch TPO und zwei Differenzierung fördernde Zytokine Interleukin (IL) 3 und 6 stimuliert wurde, während *Gadd45a* unverändert blieb und *Gadd45b* nur durch IL6 in MPPs reguliert wurde. Dies ließ vermuten, dass *Gadd45*g für die Induktion der Differenzierung in hämatopoetischen Vorläuferzellen eine Rolle spielt. Um die Expression von *Gadd45g* in HSCs und MPPs zu simulieren und sie losgelöst von anderen Effekten der Zytokin-Stimulation zu untersuchen, wurden lentivirale Expressionsvektoren, die für *Gadd45g* und einen fluoreszierenden Reporter kodieren, verwendet.

HSCs und MPPs können durch eine Kombination von Fluoreszenz-gekoppelten Antikörpern gegen Oberflächenproteine und der Durchflusszytometrie unterschieden und zu hoher Reinheit, für weitere Analysen, angereichert werden. FACS-sortierte HSCs wurden mit GADD45G oder Leervektor transduziert und ihre Differenzierung in Zellkultur über 10 Tage beobachtet. Dabei zeigte sich, dass GADD45G keinen proliferativen Nachteil für die HSCs und ihre Nachkommen hatte. Allerdings wurde die Differenzierung der transduzierten HSCs in Richtung von Granulozytärer/Monozytärer (GM) Entwicklung deutlich verstärkt und beschleunigt. Diese beschleunigte Differenzierung konnte durch Verwendung von zwei Dimerisierungsmutanten GADD45G T79E und L80E verhindert werden, was darauf hindeutet dass GADD45G nur als Dimer die Differenzierung der HSCs induzieren kann. Um die beschleunigte Differenzierung durch GADD45G auch auf funktionellem Level zu bestätigen, wurden transduzierte HSCs wiederholt in Methylcellulose ausgesät und ihre Fähigkeit Kolonien zu bilden, analysiert. Die Expression von GADD45G reduzierte bereits die Bildung sekundärer Kolonien, die im Vergleich zu Kontrolle weiter ausdifferenziert waren, was auf eine beschleunigte Differenzierung und den Verlust der Vorläuferzellen zurückführen lässt.

Um zu untersuchen ob GADD45G auch fähig ist, das Selbsterneuerungsprogramm von HSCs unter optimalen Bedingungen *in vivo* zu überschreiben und die Differenzierung der HSCs auszulösen, wurden HSCs für 24h in Kultur transduziert und dann intravenös in letal bestrahlte Mäuse injiziert. Nach zwei Wochen konnten zwar GADD45G-transduzierte Zellen im peripheren Blut der Empfängertiere nachgewiesen werden, doch ging dieser Beitrag über die Zeit verloren. Nach 21 Wochen fanden sich nur noch im Knochenmark von zwei der Empfängertiere geringe Mengen an GADD45G-transduzierte Zellen, während alle Leervektor-transduzierten Kontrollen robust angewachsen sind (20 bis 70% Donor-Blutzellproduktion). Um die Selbsterneuerungsfähigkeit der Zellen weiter zu prüfen, wurde das Knochenmark der Empfängertiere in sekundäre letal bestrahlte Empfängermäuse injiziert. Wie zu erwarten war, konnten die Leervektor-transduzierten Knochenmarkszellen erfolgreich

die sekundären Empfänger bevölkern, jedoch konnte in keiner der Empfängertiere GADD45G–exprimierende Zellen nachgewiesen werden. GADD45G induzierte eine schnelle Differenzierung, was zum Verlust der Selbsterneuerung der HSCs und letztendlich zum Scheitern einer Langzeit-Erneuerung der Empfängermäuse führte. Diese Daten beschreiben GADD45G als molekulare Verknüpfung zwischen der Stimulation durch extrinsische Zytokine und der Induktion der Differenzierung in HSCs.

Im Gegensatz dazu wiesen HSCs von *Gadd45g*-defizienten Mäusen eine gesteigerte Selbsterneuerung auf. Dazu wurden Mäuse vom Stamm B6.129S1-Gadd45g^{tm1Flv} J verwendet, die eine Keimbahn-Deletion ab dem dritten Exon des *Gadd45g* Gens aufwiesen. *In vitro*, reagierten *Gadd45g*-defiziente HSCs schwächer auf die Stimulation mit Zytokinen, die Zellen expandierten langsamer und waren nach fünf Tagen weniger differenziert als Zellen von Wildtyp Mäusen. Des Weiteren bewahrten *Gadd45g*-defiziente Zellen ein höheres und verlängertes Kolonie-bildendes Potential, was auf mehr Vorläuferzellen mit verzögerter Differenzierung hindeutet. *Gadd45g*-defiziente HSCs erreichten nach Transplantation in letal bestrahlte Mäuse zunächst ähnlich gute Erfolge bei der Ansiedlung in den primären Empfängertieren wie Wildtyp HSCs. Doch zeigten die *Gadd45g*-defizienten HSCs in der sekundären Transplantation eine gesteigerte Selbsterneuerung mit deutlich besserer Langzeit-Wiederbesiedlung als die Zellen von Wildtyp Geschwistertieren. Durch den Verlust von GADD45G sind die HSCs teilweise unempfänglich für die Stimulation mit Zytokinen, wodurch die Differenzierung der Zellen verlangsamt ist.

Um diese gesteigerte Selbsterneuerung für eine verlängerte Kultivierung von HSCs ohne Differenzierung nutzen zu können, sollte der Signalweg, durch den GADD45G die Differenzierung induziert, analysiert werden. Unter den bekannten Interaktionspartnern von GADD45G schien der MAPK (*mitogen activated protein kinase*) Signalweg von Bedeutung. GADD45 bindet dabei an die auto-inhibitorische Domäne von MAP3K4, wodurch MAP3K4 aktiviert wird und sich selbst phosphoryliert. Aktiviertes MAP3K4 kann nun weitere Kinasen unterhalb von sich im Signalweg MKK3 und MKK6 phosphorylieren und aktivieren, die nun ihrerseits MAPK p38 oder JNK phosphorylieren. JNK und p38 sind bekannte Stress-Kinasen, die häufig mit der Induktion von Apoptose in Verbindung gebracht werden. In dieser Arbeit konnte gezeigt werden, dass GADD45G die Differenzierung in HSCs und anderen hämatopoetischen Vorläufern hauptsächlich über den MAPK p38 Signalweg, nicht aber über JNK, induziert. GADD45G führte zu einer erhöhten Phosphorylierung von p38 und seiner Effektorkinase MK2 in hämatopoetischen Vorläuferzellen, was durch phosphospezifische Durchflusszytometrie gezeigt wurde. Die Inhibition von p38, nicht aber die von JNK, durch

spezifische Inhibitoren blockierte die beschleunigte Differenzierung von HSCs durch GADD45G *in vitro*. Des Weiteren konnten die beiden konstitutiv aktiven Mutanten von MAP3K4 und MKK6, die unterhalb von GADD45G in diesem Signalweg stehen, die beschleunigte Differenzierung durch GADD45G teilweise kopieren und ebenfalls durch einen p38 Inhibitor blockiert werden. Diese Ergebnisse passen zu aktuellen Daten, wonach hämatopoetische Vorläuferzellen in Anwesenheit von p38 Inhibitoren zu einer besseren Rekonstitution des Knochenmarks von bestrahlten Mäusen führen. Weitergehende Versuche sollen nun bestätigen, dass die p38 Inhibitoren tatsächlich die Selbsterneuerung der HSCs verbessern und somit eine verlängerte Kultivierung der HSCs ohne Differenzierung erlauben.
Der Vergleich der drei Familienmitglieder GADD45A, B und G zeigte, dass alle drei eine beschleunigte Differenzierung auslösen, aber nur GADD45G unterdrückte dabei die Entstehung von Megakaryozyten und Erythrozyten (Mek/E). Mikroskopische Zeitrafferaufnahmen und Einzelzellverfolgung von Leervektor- oder GADD45G-transduzierten HSCs und ihrer Nachkommen zeigten, dass tatsächlich die meisten GADD45G-transduzierten Zellen innerhalb von wenigen Stunden einen GM-Linien-gebunden Zustand erreichen. Dabei waren die Generationszeiten jedoch unverändert. Die nicht Linien-gebunden Zellen hingegen wiesen einen erhöhten Zelltod auf, was für eine Selektion gegen Zellen spricht, die in die Mek/E-Linie differenzieren. Knochenmarksanalysen zwei Wochen nach Transplantation von hämatopoetischen Vorläufern zeigten, dass die GADD45G Expression auch die Entstehung von Mek/E Vorläuferzellen, sogenannten „*megakaryocyte erythrocyte progenitors*“ (MEPs) unterbindet. Wurden jedoch FACS-sortierte MEPs mit GADD45G transduziert, führte dies zu einer deutlich geringeren Expansion und Lebensfähigkeit der Zellen sowie einer reduzierten Koloniezahl im Vergleich zum Leervektor, während GADD45G kaum einen Effekt auf transduzierte GMPs hatte. Es konnte auch keine Transdifferenzierung der GADD45G-exprimierenden MEPs festgestellt werden. Dies bedeutet, dass Zellen, die in die MeK/E Linie differenzieren, nicht mit dem GADD45G-induzierten Differenzierungsprogramm zurechtkommen und selektiv absterben.
Interessanterweise zeigten Genexpressionsprofile von GADD45G-transduzierten HSCs, dass sowohl *Gata1* und *Tal1* zwei Transkriptionsfaktoren herunterreguliert wurden, die für die Differenzierung von Megakaryozyten und Erythrozyten relevant sind, als auch die Gene für zwei bekannte Oberflächenproteine *CD150* und *Mpl,* die auf Megakaryozyten exprimiert werden, ausgeschaltet wurden. GADD45G führte aber auch zum Verlust der Selbsterneuerung. So waren *Meis1*, *Gata2* und die *Hox* Gene *HoxB4, HoxA9 und HoxA10,* die alle zum Selbsterneuerungs-Netzwerk der HSCs gezählt werden durch GADD45G-

Expression herunterreguliert. Im Gegensatz dazu wurde die Expression des myeloide Transkriptionsfaktors PU.1, sowie die Differenzierungsmarker CD48 und IL6 Rezeptor durch GADD45G hochreguliert. Diese globalen Änderungen im Expressionsprofil von GADD45G-exprmierenden HSCs deuten eindeutig auf einen Verlust der Selbsterneuerung und eine Förderung der GM Differenzierung gegenüber den Mek/E Linien hin. In dieser Hinsicht wäre es interessant, direkte Zielgene der von GADD45G-induzierten Differenzierung in HSCs, GMPs, sowie MEPs mittels eines induzierbaren lentiviralen Expressionssystems zu untersuchen. GADD45G ist zudem in HSCs von alten Mäusen hochreguliert und wird mit geringer Selbsterneuerung der HSCs verknüpft. In einer Transkriptom-Analyse von hämatopoetischen Vorläuferzellen konnte gezeigt werden, dass stressinduzierten Zytokine in HSCs die Expression aller GADD45 Proteine zusammen mit anderen Proteinen der DNA Reparatur auslösen. Damit könnten die GADD45 Proteine auch als Wächter für die Integrität des Genoms dienen. Für *Gadd45a* konnte bereits gezeigt werden, dass sein Verlust zu einer Genom Instabilität und einer erhöhten Leukämiebildung in alternden Mäusen führt. Des Weiteren sind die *Gadd45* Gene, im Besonderen *Gadd45g* in verschieden Tumorentitäten aber auch einigen Leukämien epigenetisch ausgeschaltet. Dabei erfüllt diese Inaktivierung der *Gadd45* Gene zwei Funktionen. Zum einen erlaubt der Verlust von Wächterproteinen einen erhöhte genomische Instabilität und damit Tumorprogression. Zum anderen verhindert der Verlust die Differenzierung der leukämischen Zellen. Wie in dieser Arbeit gezeigt wurde, führt die Re-Expression von GADD45G zu einer stark verminderten Expansion von leukämischen Zellen durch erhöhten Zelltod und eine gesteigerte Differenzierung. Weitere Versuche mit primären AML Proben sind geplant, um die Expression der GADD45 Proteine in AML zu messen. Es soll untersucht werden, ob der GADD45G Signalweg therapeutisch genutzt werden kann um fehlregulierte Selbsterneuerung in Leukämie- initiierenden Zellen in Richtung Differenzierung getrieben werden kann.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass die Zytokine IL3 und IL6 über GADD45G und p38 die Differenzierung in HSCs induzieren. Damit konnte die Lücke der bekannten pro-differenzierenden Wirkung von IL3 und IL6 bis zur p38 Aktivierung und dem damit einhergehenden Verlust der Selbsterneuerung geschlossen werden. Der Verlust von GADD45G hingegen führt zu einer erhöhten Selbsterneuerung und verlangsamten HSC Differenzierung. Der hier dargestellte Signalweg, initiiert durch GADD45G, kann daher in Zukunft, zum Beispiel durch p38 Inhibition, genutzt werden, um Protokolle für die ex vivo Expansion von HSCs zu entwickeln und wäre damit für die regenerative Medizin von großem Interesse.

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8. Appendix

8.1 Abbreviations

1°	primary
2°	secondary
7AAD	7-aminoactinomycin D
AGM	Aorta-gonad-mesonephros region
AICD	Activation-induced cell death
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANG-1	Angiopoietin 1
ATRA	All transretinoic acid
B cell	Bursa-derived cell
B2m	β2 microglobulin
BM	Bone marrow
BMC	Bone marrow cell
BMMNC	Bone marrow mononuclear cell
BrdU	Bromodeoxyuridine
C/EBP A or B	CCAAT/Enhancer-Binding-Protein alpha or beta
CAM	Cell adhesion molecules
caMAP3K4	Constitutive active mitogen-activated protein kinase kinase kinase 4
caMKK6	Constitutive active mitogen-activated protein kinase kinase 6
CAR	CXCL12 abundant reticular
CD	Cluster of differentiation
CDKI	Cyclin-dependent kinase inhibitors
cDNA	Complementary DNA
CHO	Chinese hamster ovary
Chx	Cyclohexemid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CR6	Cytokine response gene 6
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
dNTP	Deoxy-Nucleoside triphosphate
ELP	Early lymphoid progenitors
EPO	Erythropoietin
FACS	Fluorescence- activated cell sorting
G45G	GADD45G; Growth arrest and DNA damage inducible 45 gamma
Gadd45	Growth arrest and DNA damage inducible 45
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte macrophage (GM) progenitor
HDAC	Histone deacetylase
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IFN G	Interferon gamma
IL	Interleukin
IR	Ionizing radiation

IRES	Internal ribosome entry site
IT	Intermediate-term repopulating
JNK	c-Jun N-terminal kinase
L80E	GADD45G L80E mutant
LMPP	Lymphoid primed Multi potent progenitor
LSK	Lineage$^-$, SCA-1$^+$, c-KIT$^+$ (CD117)
LT-HSC	Long-term repopulating hematopoietic stem cell
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
Mek/E	Megakaryocyte-/ erythrocyte-
MEP	Megakaryocyte erythrocyte (ME) progenitor
min	minutes
MKK	Mitogen-activated protein (MAP) kinase kinase
MMS	Methyl-methanesulfonate
MOI	Multiplicity of infection
MPP	Multi-potent progenitor
mRNA	Messenger Ribonucleic acid
MSC	Mesenchymal stem cell
MyD	myeloid differentiation primary response
NER	Nucleotide excision repair
NF-κB	nuclear factor- kappa B
NK	Natural killer (cell)
NUP98	Nucleoporin 98
OB	Osteoblast
PB	Peripheral blood
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
qPCR	Quantitative reverse transcription Polymerase chain reaction
qRT-PCR	Quantitative Reverse transcription Polymerase chain reaction
ROS	Reactive oxygen species
SCA-1	stem cell antigen 1
SCF	Stem cell factor
SNS	sympathetic nervous system
SP	Side population
Sry	Sex determining region of Y
ST	Short-term repopulating
T cell	Thymus derived cell
T79E	GADD45G T79E mutant
TNFA	tumor necrosis factor alpha
TPO	Thrombopoietin
TTT	Timm's Tracking Tool
UV	Ultra violet radiation
w/v	Weight per volume
WT	Wild type

8.2 Vector maps

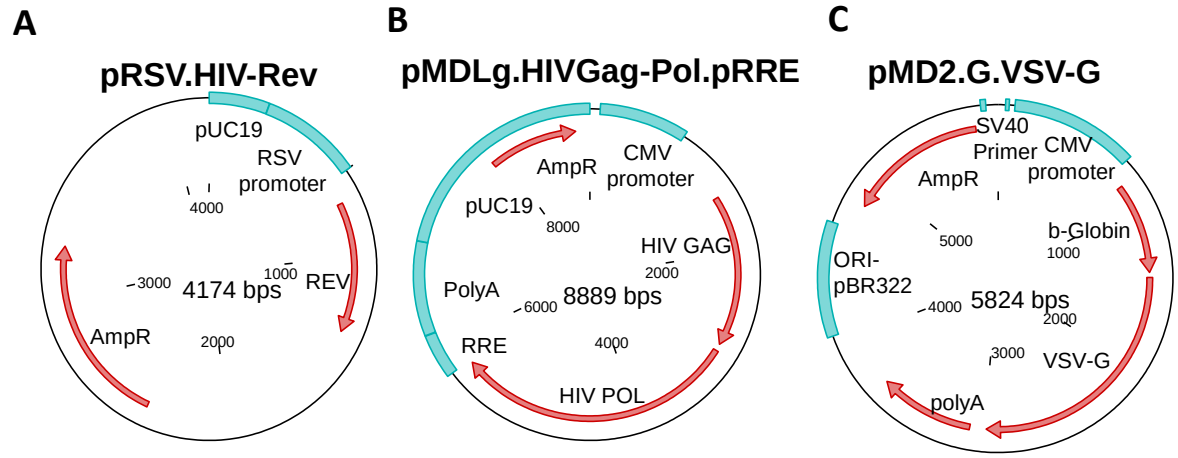

Figure 34 Packaging plasmids for lentiviral production.

A) Expression plasmid pRSV.HIV-Rev for lentiviral reverse transcriptase (*REV*) of HIV-1; driven by RSV promoter. B) Expression plasmid pMDLg.HIVGag-Pol.pRRE for structural genes (*gag/pol*) of HIV-1 for lentiviral particle assembly. C) Expression plasmid pMD2.G.VSV-G for VSV-G (*env*) for pseudo typing of lentiviral particles. RSV: Rous sarcoma virus promoter; CMV: cytomegalo virus promoter; Amp: Ampicillin Resistence gene; HIV: human immunodeficiency virus; RRE: Rev response element; VSV-G: glycoprotein G of VSV. Kindly provided from Axel Schambach, Hannover medical school.

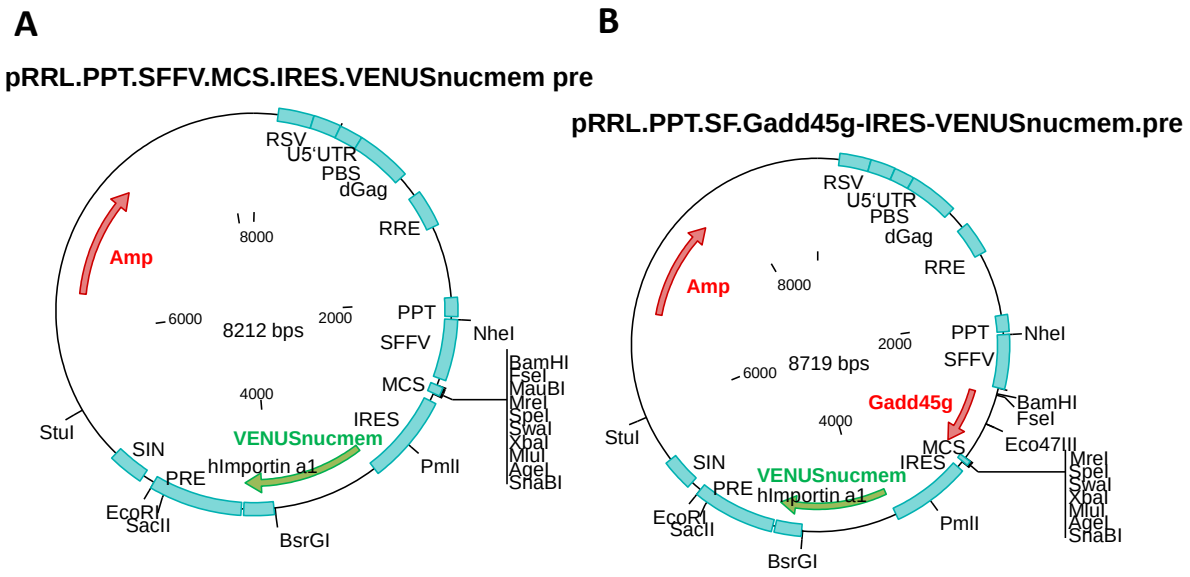

Figure 35 Lentiviral expression plasmids.

A) Lentiviral control expression vector used for control transduction and as basis for other lentiviral expression vectors. B) Lentiviral Gadd45g-expressing vector for GADD45G expression and cloning of L80E and T79E mutants. Restriction enzymes used for cloning and analysis are shown on the outside of the plasmid ring. RSV: Rous sarcoma virus enhancer; PBS:Primer binding site ; Gag: Group specific antigen; UTR: Untranslated region; RRE: Rev response element; PPT: Polypurine tract; SFFV: Spleen focus-forming virus, Promoter; MCS: Multiple cloning site; IRES: Internal ribosome binding site; VENUS: green fluorescent protein; PRE: posttranscriptional regulatory element; SIN: Self inactivating UTR; Amp: Ampicillin Resistence gene.