# Transgenic models to study TGF-β function in hematopoiesis

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## *Summary*

Studies of hematopoietic pathologies involving the growth factor TGF-β have provided important evidence of its keyrole in the regulation of human hematopoietic stem/progenitor cell quiescence, proliferation, and differentiation. The inactivation of one of the various genes involved in the TGF-β signal transduction pathway may represent a possible mechanism by which some early hematopoietic progenitors, which are normally quiescent, escape from cell-cycle inhibition. Abnormalities in the expression of TGF-β receptors have been described in proliferative syndromes including both early myeloid and lymphocytic leukemia [1,2]. In these cases the loss of the growth inhibitory TGF-β signal might provide a selective advantage to the malignant cell. Additional autocrine TGF-β production and thereby inhibition of neighboring cells leads to an overgrowth of the malignant clone. In patients with myeloproliferative disorders, reduced mRNA levels of the TGF-β signaling components Smad4 and type II TGF-β receptor were reported [3-5] further establishing a role of abolished TGF-β signaling in the pathogenesis of hematopoietic malignancies. The role of TGF-β in the regulation of hematopoiesis has also been analyzed *in vivo* using different mouse models. For example, the administration of TGF-β in mice revealed an inhibition of thrombopoiesis and erythropoiesis [6]. A variety of knockout mice have been generated to study the effect of TGF-β *in vivo*. The most of these approaches were hampered by the early lethality of the knockout like in the case of the Smad proteins and the TGF-β receptors I and II [7,8]. Homozygous TGF-β1 knockout mice have a 50% intrauterine death rate because of severe developmental retardation. The other 50% die within several weeks after birth due to a severe inflammatory autoimmune disease [9]. Nevertheless, TGF-β knockout mice display defective hematopoiesis with elevated platelet counts and reduced numbers of erythroid cells [9]. However, as most of the knockout approaches for TGF-β signaling components resulted in early embryonic lethality, the exact functions of the different elements of the TGF-β signaling cascade in hematopoiesis are still controversial.

In this thesis work I describe different transgenic approaches to gain insight into the function of TGF-β signaling components in hematopoiesis, with a focus on megakaryopoiesis. In the first part I describe the generation of a transgenic mouse strain for the tissue-specific deletion of target genes in megakaryocytes and platelets. Many of the genes potentially involved in megakaryopoiesis are difficult to study by conventional knockout approaches, as they are ubiquitiously expressed and therefore their germline deletion is embryonically lethal. One

way to circumvent the obstacles of early embryonic lethality is the use of the Cre/loxP system for tissue-restricted target gene deletion [10]. Hence, we generated a transgenic mouse for the megakaryocyte-specific expression of the Cre recombinase. As short plasmid based transgenes are often hampered by position variegation effects, like mosaic expression or transgene silencing, we decided to modify a large genomic DNA fragment using ET-recombination in *E.coli* [11]. The coding sequence of the Cre recombinase was placed under the control of the *Pf4* gene embedded in a 100kB bacterial artificial chromosome (BAC). The modified BAC-insert was used to generate PF4Cre transgenic lines. Analysis of the resulting transgenic lines revealed differences in tissue-specific expression of the Cre recombinase, dependent on copy numbers. Accordingly, strains with low copy numbers revealed very specific Cre expression in megakaryocytes and platelets, while strains with higher copy numbers displayed ectopic Cre expression. The evaluation of excision efficiency in megakaryocytes of the different PF4Cre strains revealed that the strain with 5 integrations excised with 90%, whereas the strains with 1 or 2 copies excised with 60-70% efficiency. However, I used these strains to delete the TGF-β signaling components type II TGF-β receptor (TBRII) and Smad4 in megakaryocytes by mating the PF4Cre strains with either TBRIIlox/lox or Smad4lox/lox mice. Homozygous offspring was analyzed for peripheral blood counts. Surprisingly, no change in the numbers of circulating platelets was detected in any of these mice in comparison to control mice. I confirmed these results using the transgenic Mx1Cre mouse for inducible deletion of target genes in hematopoietic stem cells. Again, no changes in the numbers of circulating platelets were detected neither in TBRIIlox/lox-Mx1Cre mice, nor in Smad4lox/lox-Mx1Cre mice. Together these results argue against an involvement of TGF-β signaling components in the onset of myeloproliferative disorders and additionally reveal that TGF-β signaling is dispensable for functional megakaryopoiesis.

In a second mouse model we intended to disrupt Smad-mediated TGF-β signaling in hematopoiesis by the induced deletion of the TGF-β signal transducer Smad4. We used the Mx1Cre transgenic strain to induce Smad4 deletion in the bone marrow of Smad4lox/lox-Mx1Cre mice. Smad4 deleted mice developed a severe haemolytic anemia 4-5 weeks after the induction of Cre recombinase expression, accompanied by extramedullary hematopoiesis and splenomegaly. Anemia in Smad4lox/lox-Mx1Cre mice was not autoimmune-mediated as revealed by a negative direct antiglobulin test (DAT). The hyperplasia of the spleens in Smad4lox/lox-Mx1Cre mice was due to a massive increase of immature myeloid cells. FACS analysis revealed the myeloid cells in the spleen are TER119$^{high}$/CD71$^{high}$ erythroblasts, which

argues for a maturation block in erythropoiesis as the cause for anemia in Smad4lox/lox-Mx1Cre mice. Transplantation of Smad4lox/lox-Mx1Cre bone marrow into lethally irradiated C57BL/6 recipients revealed that the anemia is not transplantable and thus can be compensated by host-derived factors. Furthermore, Smad4lox/lox-Mx1Cre bone marrow transplanted recipients did not develop a wasting syndrome. This is in complete contrast to the previously described induced deletion of TBRII and TBRI in TBRIIlox/lox- and TBRIlox/lox-Mx1Cre mice. In both of these mouse models deletion of the TGF-β signaling caused a severe inflammatory phenotype, which is transplantable. Together, these results implicate that the autoimmune phenotype in TGF-β receptor deleted mice is not Smad-mediated, as Smad4 is the quintessential for signaling through activated Smads.

In the last part of my thesis I describe the generation of a new tool to study gene function in human hematopoietic stem/progenitor cells. For this purpose I took advantage of the rapid advances in the RNA-interference field and the demonstrated capability of lentiviruses to infect non-cycling human hematopoietic stem cells. I modified a lentiviral vector by the insertion of a expression cassette for short-interfering RNAs (siRNA), which drives siRNA expression under the control of the H1 promotor. Originally thought to target TBRII in human hematopoietic stem cells, the system was first established to target the human p53 mRNA, as a functional siRNA sequence for this target was available at that time. Human cord blood derived $CD34^+$ cells were infected with the lentiviral construct pWPXLp53si and p53 mRNA from infected cells was analyzed by quantitative real-time PCR. Infection efficiencies were typically around 50% as revealed by the enhanced green fluorescent protein reporter gene (EGFP). Infected $CD34^+$ cells not only revealed p53 mRNA reduction to 3-10% of the control levels, but also functional p53 silencing was demonstrated by the increased resistance to apoptotic stimuli of pWPXLp53si-infected $CD34^+$ cells. We also demonstrated that the lentiviral system was able to silence p53 in early hematopoietic progenitors by growing infected CD34+ cells under long-term culture initiating cell (LTC-IC) conditions. In summary, we revealed that lentiviral delivery of siRNA can be used for efficient and stable gene silencing in human hematopoietic progenitors. This system will be very valuable to study the function of key regulatory genes in human hematopoiesis.

## *General Introduction*

## *Hematopoiesis*

The continuous production of peripheral blood cells throughout lifetime is provided from a rare population of pluripotent bone marrow stem cells, called hematopoietic stem cells (HSCs). Two major features characterize HSCs. First, they can undergo differentiation to progenitor cells that give rise to all the different hematopoietic lineages of the peripheral blood. Second, they maintain the stem cell pool throughout lifetime by their self-renewal capacity, thus giving rise to additional HSCs [12]. The properties of HSCs are best described by their ability to reconstitute the hematopoietic system of a recipient individual, where they can sustain long-term multilineage hematopoiesis.

## *Origins of the hematopoietic system*

The hematopoietic system derives from the embryonic mesoderm. As far as vertebrates are concerned, hematopoiesis takes place at successive anatomic sites. The earliest embryonic site of hematopoiesis occurs in the blood islands of the yolk sac at around embryonic day 7.5-11 in the mouse. From embryonic day 11 to 16, definitive or adult hematopoiesis is transiently found in the fetal liver before it moves into the bone marrow where it stays throughout lifetime [13]. An alternative scenario derives from chick-quail experiments where an independent site of hematopoiesis was found to originate from the para-aortic splachnopleura/aorta, gonad, mesonephros region (AGM) of the embryo proper. Different from yolk-sac derived progenitors, AGM precursors of hematopoietic cells provide multilineage differentiation upon transplantation into irradiated adult recipients. In the mouse, analogous AGM-derived cells were identified [14,15]. This indicates that the AGM is a region where HSCs are “born”, and dismisses a contribution of yolk-sac derived progenitors in the adult hematopoiesis. However, the debate about the contribution of the yolk-sac and AGM regions to the adult hematopoiesis continues.

## *Hematopoietic markers*

An even bigger challenge is provided by the goal of the phenotypic description of the HSC. In the mouse, cells of the c-kit$^{+}$, sca-1$^{+}$, thy-1$^{lo}$, lineage-negative phenotype were shown to

possess the ability to reconstitute hematopoiesis in an irradiated recipient [12]. Dyes such as Hoechst 33324 and Rhodamine 123 have also been used to identify populations within the HSCs, which are greatly enriched. At first, one proceeded on the assumption that in the human system $CD34^+$ is present on all HSCs, but there is evidence for the existence of $CD34^-$ HSCs [16]. There is still a vivid debate amongst researchers in the field about the phenotypic description and the molecular characterization of the HSC.

Although the definitive phenotypic characterization of the HSC is still pending there are several transcription factors which have been shown to be required either for the generation or the maintenance/proliferation of HSCs. Data for the requirement of these transcription factors came from gene targeting experiments of ES cells in mice. These experiments demonstrated that the transcription factors SCL/tal-1, AML-1/Runx1 and Lmo2 are essential for the generation of hematopoietic stem cells either at the yolk sac stage or at later stages [17-19]. It was shown later that the transcription factor SCL/tal-1 is mainly required in adult hematopoiesis, where it is essential for erythropoiesis and megakaryopoiesis, but is dispensable for the production of myeloid cells in definite hematopoiesis [20]. The transcription factor AML-1, which was also believed to play a fundamental role in hematopoiesis, was recently shown to be a crucial factor for megakaryopoiesis and T- and B-cell development. An effect of AML-1 on the maintenance of hematopoietic stem cells in definite hematopoiesis was not proposed by these experiments [21]. The requirement for other transcription factors like GATA-1 and GATA-2 in normal hematopoiesis were also demonstrated by gene-targeting experiments. Due to functional redundancies of GATA-1 and GATA-2, the precise role of the GATA-family of transcription factors has yet not been ultimately resolved. Finally, the are probably several other so far unknown factors involved in functional hemetopoiesis and their exact functions are still an unsolved issue.

## *Megakaryopoiesis*

Mature megakaryocytes are giant (15-30μm in diameter), polyploid cells that contain a unique set of cell organelles, namely α–granules, dense bodies, and an extensive system of internal membranes, the so called demarcation membrane system (DMS), which consists of narrow channels homogenously distributed in the cytoplasm. Likewise all the other cells of the different hematopoietic lineages, the megakaryocytes derive from a pluripotent hematopoietic progenitor cell. The most primitive progenitor of megakaryocytes was originally described in the murine system as a mixed high-proliferative-potential

megakaryocyte cell. These cells have a high proliferative capacity giving rise to large colonies of megakaryocytes when cultured *in vitro* [22-24]. Later stages, as the colony-forming unit megakaryocyte (CFU-Meg) can undergo 1-8 cell divisions forming colonies consisting of 16-32 cells [22,23].

The first morphologically recognizable megakaryocyte in the bone marrow is characterized by high nuclear-to-cytoplasmic ratio and plasma membrane blebbing. With ongoing maturation, the nuclear-to-cytoplasmic ratio decreases as the amount of cytoplasm drastically increases with abundant cytoplasmic granules. At this stage of maturation the DNA content of the cells exceeds 4*N*. Further maturation is characterized by an increase in the DNA content, ranging from 4*N* to 64*N*. This unique feature of megakaryocytes within the hematopoietic compartment occurs through a process termed endomitosis. Endomitosis is defined as DNA replication and a mitotic event with sister chromatid separation in the absence of subsequent cytokinesis. Polyploidization starts in the morphologically unrecognizable immature stages of megakarypoiesis and is completed in the immature basophilic stage [25]. The level of polyploidization is inversely correlated to the amount of circulating platelets as revealed by experimental thrombocytopenia and thrombocytosis [26-31].

At the late stages of megakaryocyte maturation, an extensive system of membrane demarcation is visible in the cells, the demarcation membrane system. The DMS is thought to compartmentalize the platelet cytoplasm into platelet territories, which are then released into the circulation as platelets. The complete maturation from the earliest recognizable megakaryocyte to the release of platelets takes 2-3 days in rodents and 5 days in humans.

## *Platelets*

The products of megakaryocyte maturation are the platelets. The platelet is a disc-shaped piece of membrane cytoplasm endowed with all organelles found in other mammalian cells except for a nucleus, a Golgi zone, and a mitotic apparatus. The average diameter of platelets is 2-3µm and their number in the human circulation ranges from 150-400 $x10^9$/L. The half-life of these circulating platelets is about 4 days in rodents and 7-10 days in humans. Proteoglycans on the plasma membrane of platelets function as receptors for ligand binding or mediate interaction with external surfaces. From the large number of integrins detectable on the platelet surface, the GPIIb/IIIa complex is of special interest. First it is a specific marker for the megakaryocyte/platelet lineage and second it is the prime receptor for fibrinogen, thus most significant for platelet function.

The major function of platelets is to activate blood coagulation upon vessel injury. In the case of injury, platelets migrate and adhere to the site of damage and here they aggregate to form a plug, which seals the defective blood vessel, thereby avoiding major blood-loss. In a second step, activated platelets induce the blood coagulation system that replaces the platelets plug with a fibrin clot. Platelets are the store for several mediators involved in hemostasis, wound repair and inflammation. These molecules are either produced during megakarypoiesis and then stored in the released platelets, or taken up by endocytosis from megakarycytes and platelets. One of the molecules produced early in megakaryopoiesis and then stored in circulating platelets is the CXC-subfamily chemokine platelet factor 4 (PF4). Expression of this 7.8 kd protein of 70-amino acid length starts at the early stages of megakaryocyte maturation, namely the promegakaryoblast stage and thus later as the earliest megakaryocyte marker glycoprotein IIb [32,33]. As far as the expression of PF4 was demonstrated to be almost exclusively restricted to the maturing megakaryocyte and the platelets, this molecule can be considered as a good molecular marker for megakaryopoiesis.

## *Molecular regulation of megakaryocyte maturation*

Among the transcription factors known to have functional influence on megakaryopoiesis, the zinc-finger proteins GATA-1 and GATA-2 were shown to be the major transcriptional regulators of erythro-megakaryocytic differentiation. Virtually every examined gene known to be expressed specifically in megakaryocytes, was shown to have a GATA-binding site within its promotor region. Further insight into the function of GATA-1 and GATA-2 came from gene targeting experiments in mice. Mice lacking GATA-1 selectively in megakaryocytes revealed a severe reduction in circulating platelet numbers to about 15% of the normal. Platelets of such mice were increased in size and bleeding time was prolonged [34], while the megakaryocytes revealed an enhanced proliferation rate *in vitro*. The increased numbers of megakaryocytes in these animals showed an abnormally small and immature cytoplasm, which harbours only a small amount of platelets. This indicates the importance of GATA-1 for functional megakaryopoiesis, although the critical GATA-1 regulated targets in megakaryopoiesis are still unknown. Another factor shown to have fundamental impact on megakaryocyte differentiation is the cofactor Friend of GATA (FOG-1). Mice with a germline deletion of FOG-1 completely lack megakaryocytic progenitors [35]. Other transcription factors like Fli-1 and NF-E2 were demonstrated by gene targeting experiments to play a crucial role

in the maturation of megakaryocytes [36,37], and it is likely that further transcriptional regulators of megakaryopoiesis exist.

## *Cytokines involved in megakaryopoiesis*

Several factors were shown to exert a critical effect on megakaryopoiesis. The most prominent among them is the lineage-restricted growth factor thrombopoietin (TPO). The role of TPO in megakaryopoiesis was mainly demonstrated by gene-targeting experiments in the mouse [38,39]. The generation and analysis of Mpl, the cognate receptor for TPO, and TPO deficient mice revealed a decrease of platelet and megakarycyte numbers of >80%, demonstrating the pivotal role of TPO and Mpl in the regulation of megakaryopoiesis. Other non-lineage specific growth factors were also shown to have an influence on megakaryocytopoiesis. Of those, IL-3 was shown to have a strong stimulatory effect on Colony Forming Unit-Megakaryocyte (CFU-Meg) and also burst forming cells (BFC-MK) [40,41]. The same was demonstrated for Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), although to a lesser degree [42-44]. It was also shown that Stem Cell Factor (SCF), IL-11 and erythropoietin (EPO) can synergize with TPO to stimulate megakaryocyte colony formation [45]. Megakaryocyte maturation *in vitro* is also promoted by LIF, SCF, OSM and EPO as determined by their effects on megakaryocyte number, ploidy and size [46-50].

Amongst the inhibitory factors, type-β transforming growth factor 1 (TGF-β1) and CXC chemokines like platelet factor (PF)-4 or the close related IL-8, but also the interferon family, were shown to exert the most prominent effect on *in vitro* megakaryopoiesis [42,51-53]. Several studies indicated that TGF-β1 has a strong inhibitory effect on megakaryocyte development *in vitro*. As far as α-granules of platelets are the main source for the storage of inactive TGF-β1, it remains unclear how the destruction of platelets and thus the release of TGF-β1 can have a stimulatory effect on platelet production. One possible explanation is provided by the observation that TGF-β1 induces TPO mRNA production in bone marrow stromal cells, which then stimulates bone marrow stem cells to commit to the megakaryocytic compartment [54]. PF-4 was shown to exert its function already on hematopoietic stem cells, where it supports the survival of stem and progenitor cells and to suppresses the development and maturation of cells from the megakaryocytic lineage [55]. Other CXC chemokines like neutrophil-activating product-2 (NAP-2) and IL-8, but even more the distantly related CC chemokines MIP-α and MIP-1β were also shown to have direct inhibitory effects on in vitro megakaryocyte colony formation.

## *TGF-β signaling*

TGF-β belongs to a large superfamily of structurally related polypeptides that includes activins, bone morphogenetic proteins (BMPs) and the growth differentiation factors (GDFs) [56], which have fundamental functions in cellular behaviour, like migration, adhesion, survival, proliferation and differentiation [1,57,58]. TGF-βs were first discovered by De Larco and Todaro in 1978 and originally termed "sarcoma growth factors". To date 28 genes in the human genome that encode members of this family are known [59]. There are three isoforms of TGF-β in mammals (TGF-β 1, 2 and 3). TGF-βs are synthesized as biologically inactive precursor proteins. The earliest forms, the pre-pro-peptides, require sequential processing to give rise to the active TGF-β peptide [60]. A first proteolytic cleavage cuts off the hydrophobic signal peptide, yielding the pro-TGF-β form. The second cleavage eliminates the pro-region from the now mature TGF-β peptide. Bioactive forms of TGF-βs consist of two mature TGF-β peptides linked by disulfide bonds. The linked peptides mostly exist as homodimers, but heterodimeric forms have also been reported. The processed form of TGF-β is released from the cells as a latent complex, which has no biological activity. A small and large form of the latent complexes has been described. The small complex consists of one mature TGF-β peptide, which is noncovalently associated with one disulfide-bonded pro-peptide dimer called latency associated protein (LAP). In the large form of the latent complex LAP is linked by disulfide bonds to a member of high molecular weight proteins called latent TGF-β-binding proteins (LTBP) [61,62]. The LTBPs mediate the ability of the LAPs to associate with the extracellular matrix, thus facilitating the storage of TGF-β. Proteolytic enzymes like chymase, plasmin and elastase are then able to cleave LTBPs and releasing the LAP from the extracellular matrix [63-65]. The active form of TGF-β can be released by proteolytic nicking of the N-terminal region of the LAP by plasmin [66]. Thrombospondin, which is a platelet α-granule and extracellular matrix protein, has also been shown to activate the latent forms of TGF-β by inducing conformational changes in the LAP, resulting in the release of active TGF-β [Schultz-Cherry, 1994 #56].

## *The TGF-β signal transduction pathway*

The TGF-β family members bind to their cognate heteromeric receptor complex, which consists of two types of transmembrane serine/threonine kinases known as type I (TβRI or ALK) and type II receptors (TβRII) (Fig.1). These transmembrane receptors represent two families of serine/threonine kinase receptors of 53 to 65 kd [67]and 80 to 95 kd, respectively. In mammals five type I receptors and seven type II receptors were identified (Fig. 1).

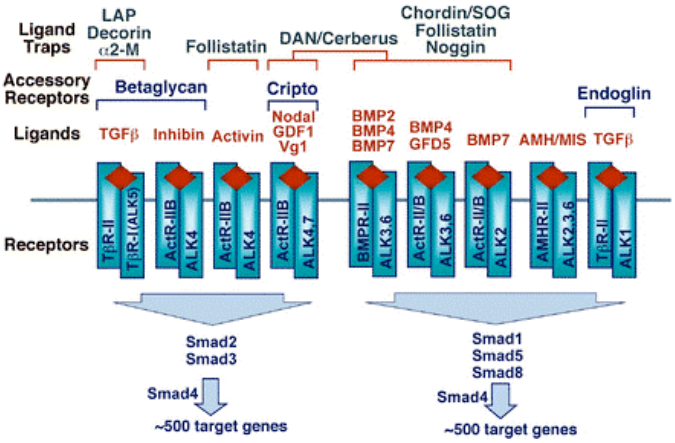


**Figure 1.** Graphic of the TGF-β family members and their cognate receptors.

Ligand binding to the type II receptor, a constitutively active kinase, leads to dimerization with the type I receptor and phosphorylation of the Glycin-Serine domain (GS) [68]. Phosphorylation of the GS domain activates the C-terminal kinase domain, which phosphorylates and thereby activates the so-called receptor Smads (R-Smads). In the absence of ligand binding the receptors where demonstrated to exist as homodimers on the cell surface. Although ligand binding to type II receptors could induce autophosphorylation, signaling in the absence of the type I receptor component has not been reported. Thus the formation of the heterotrimeric receptor/ligand complex is a prerequisite for functional signaling. Formation of different heteromeric receptor complexes with different ligands has been reported [69,70]. This way divergent signaling responses upon the binding of the same ligand are possible. For example, TGF-β1 can not only bind the type II receptor TβRII, which can dimerize with ALK5 or ALK1, but also to the ActRI/ALK2 on endothelial cells. This way complexity of the signaling complex can be further increased. Substrate specificity for the R-Smad phosphorylation is determined by the L45 loop within the type I receptors and primarily within the L3 loop of the R-Smad MH2 domain. Accordingly, the TGF-β and

activin receptors phosphorylate Smad2 and Smad3, while BMP receptors phosphorylate Smad1, Smad5 and Smad8 [71].
The phosphorylated R-Smads dimerize with the co-Smad (Smad4) and translocate to the nucleus where they exert their function as transcription factors [72]. Here, the decision whether Smads activate or repress target gene transcription is determined by cofactors that confer specific properties to the SMAD complex [73].

TGF-β is the quintessential growth-inhibitory cytokine, but growth inhibition is only one of the various functions TGF-β can exert on different tissues. It helps to restrain growth of mammalian tissues through its cytostatic and apoptotic effects [1]. Escape of epithelial cells from TGF-β growth control is a hallmark of many cancers. The role of TGF-β signaling as a tumor suppressor pathway in early carcinogenesis is illustrated by the presence of inactivating mutations in genes encoding TGF-β receptors and Smads in human carcinomas, and by studies of tumor development in mouse models [2]. On the other side, tumor cells, which are relieved from the inhibitory effect of TGF-β were shown to overproduce this cytokine and thus create a local immunosuppressive microenvironment that supports tumor growth and metastatic invasion [74]. The function of TGF-β signaling components in development and tumorigenesis was elucidated in several deletion and overexpression mouse models (Table 2). These experiments did not indicate a role for TGF-β as a growth inhibitor early in embryogenesis. Later in maturation many cell types gain the ability to respond to TGF-β with growth arrest or cell death.

## *TGF-β signaling is mediated via a conserved familiy of signal transducers*

The intracellular effectors of TGF-β signaling are the Smads. These substrates for type I receptor kinases were first identified as products of the *Drosophila Mad* and *C.elegans Sma* genes, which were shown to be downstream of the BMP-analogous ligand-receptor systems in these animals [75]. So far eight vertebrate Smads are known: Smad1 to Smad8. Smads are ubiquitously expressed throughout development as well as in the most adult tissues [76].
The Smad family of signal transducers can be functionally subdivided into three different groups. The first group comprising Smad1, Smad2, Smad3, Smad5 and Smad8 are termed receptor-Smads (R-Smads), which are phosphorylated by the type I receptors. Within this group of R-Smads it could be shown that Smad1, Smad5 and Smad8 are restricted to signaling through the BMP-pathway, whereas Smad2 and Smad3 are believed to be restricted

to the Activin/TGF-β signaling pathway [71]. The second group, the common mediator Smads (Co-Smads), consist so far only of one known member, namely Smad4. The Co-Smads bind to the receptor-activated R-Smads and form heterodimers and these complexes then translocate to the nucleus.

| *Type II receptors* | *Type I receptors* | *Receptor Smads* |
|---|---|---|
| BMPRII | ALK-2 (ActRI)<br>ALK-3 (BMP-RIA)<br>ALK-6 (BMP-RIB) | Smad1, Smad5, Smad8 |
| ActRII, ActRIIB | ALK-4 (ActRIB) | Smad2 |
| ActRIIB | Alk-7 | Smad2 |
| TBRII | Alk-5 (TBRI)<br>Alk-1<br>Alk-2 | Smad2, Smad3<br>Smad1, Smad5 |
| AMHR | Alk-3<br>Alk-2<br>Alk-6 | Smad1, Smad5 |

**Table 1.** Summary of well characterized interactions of type II and type I receptors and their R-Smads.

The last group comprising Smad6 and Smad7 are the inhibitory Smads (I-Smads), which upon TGF-β induction compete with the R-Smads for receptor binding and target bound receptors for degradation.

The Smads resemble two highly conserved Mad homology (MH) domains, the N-terminal MH1-domain and the C-terminal MH2-domain. The MH1 domain was shown to be responsible for DNA-binding, nuclear import and interaction with other nuclear proteins. It is conserved within the R-Smads and Co-Smads, whereas the I-Smads show only a weak sequence homology to the MH1 domain. The MH2 domain is highly conserved throughout all the known Smads. The MH2 domain was shown to contain a forkhead-associated domain (FHA), which is a common phosphopeptide-binding domain among transcription and signaling factors [77]. The MH2 domain is crucial for type I receptor recognition, oligomerization with other Smads and interaction with cytoplasmic adaptors such as Axin and Smad Anchor for receptor activation (SARA) and with transcription factors like Lef1/Tcf, Runx/AML and the Evi-1 oncoprotein. Type I receptor recognition by R-Smads leads to the phosphorylation of the most C-terminal serine residues within the MH2-domain, which forms an evoluntionary conserved SSXS motif, together with a third non-phosphorylated serine residue. The two Mad homology domains of the Smad family members are bound through a variable proline-rich linker region, which is mainly bound by proteins mediating ubiquitination of the Smads.

## *Smad function is regulated by phosphorylation*

As already mentioned above, Smads become phosphorylated by the kinase domain of type I receptors. Once the receptor Smads are phosphorylated on their C-terminal MH2-domain, they form homo-oligomers, which rapidly convert to hetero-oligomers containing the Co-Smad, Smad4. Non-phosphorylated Smads exist primarily as monomers, which are intrinsically auto-inhibited through an intramolecular interaction between the MH1 and the MH2 domains. The receptor-mediated phosphorylation of R-Smads induces conformational changes that relieve the auto-inhibition [78,79]. The I-Smads are phosphorylated by as-yet uncharacterized kinases[80].

The composition of the Smad-complexes was originally demonstrated to consist of a Smad trimer. Later studies revealed that the Smad2-Smad4 complex exists as a hetero-dimer [78]. Thus, different complexes of R-Smads and Co-Smads are possible with different stoichiometries.

Inactive cytoplasmic Smads are retained in the cytoplasm by interaction with scaffolding proteins. In the case of Smad2/3 it was shown that the protein SARA regulates the subcellular distribution of Smad2/3[81]. SARA is bound to the inner leaflet of the plasma membrane via its FYVE domain, which mediates the interaction to the membrane phospholipids. The SARA/Smad interaction assists in the phosphorylation of Smad2/3 by forming a bridge between Smad2/3 and the receptor complex, and at the same time prevents nuclear import of non-phosphorylated Smad2/3 to the nucleus. Phosphorylation of Smad2/3 by the activated type I receptor kinase leads to dissociation of Smad2/3 from the receptor and SARA. Another FYVE domain containing scaffoling protein, Hrs/Hgs, was also shown to participate in the Smad presentation to the receptor[82].

TGF-β receptor internalization is required for the presentation of Smads to the TGF-β receptor by SARA. The finding that Caveolin-1, a principal component of caveolae membranes, cofractionates with TGF-β receptors and Smad2, implies an internalization of the TGF-β receptor complex via caveolin-rich vesicles[83,84].

Microtubules were identified as another subcellular regulator of Smads. Interaction of Smad2/3 and Smad4 with tubulin was demonstrated and disruption of the Smad interaction with microtubules by nocodazole, which destabilizes the microtubule network, was shown to induce TGF-β mediated Smad2 phosphorylation and Smad mediated transcription [85].

## *Negative regulation of R-Smads*

Functional signaling through the activated receptor Smads and the Co-Smad is tightly regulated. One mechanism of regulation is exerted by of the last group of the Smad family, the inhibitory (I-) Smads [86-88]. So far two I-Smads are known in the vertebrate system, Smad6 and Smad7. Whereas Smad7 acts as a general inhibitor of TGF-β signaling pathway, Smad6 preferentially blocks BMP signaling. One way how I-Smads block signaling is through the competition of I-Smads with R-Smads for the interaction with activated type I receptors [86]. In contrast, Smad7 was shown to constitutively interact with the HECT-domain ubiquitin ligases Smurf1 and Smurf2 [89]. Recruitment of this I-Smad/Smurf complex to the TGF-β receptor leads to proteasomal or lysosomal degradation of the receptor complex and thus to a block in TGF-β signaling. Further, I-Smads can inhibit by competing with Smad4 for complex formation with phosphorylated Smad(Imamura T 1998).

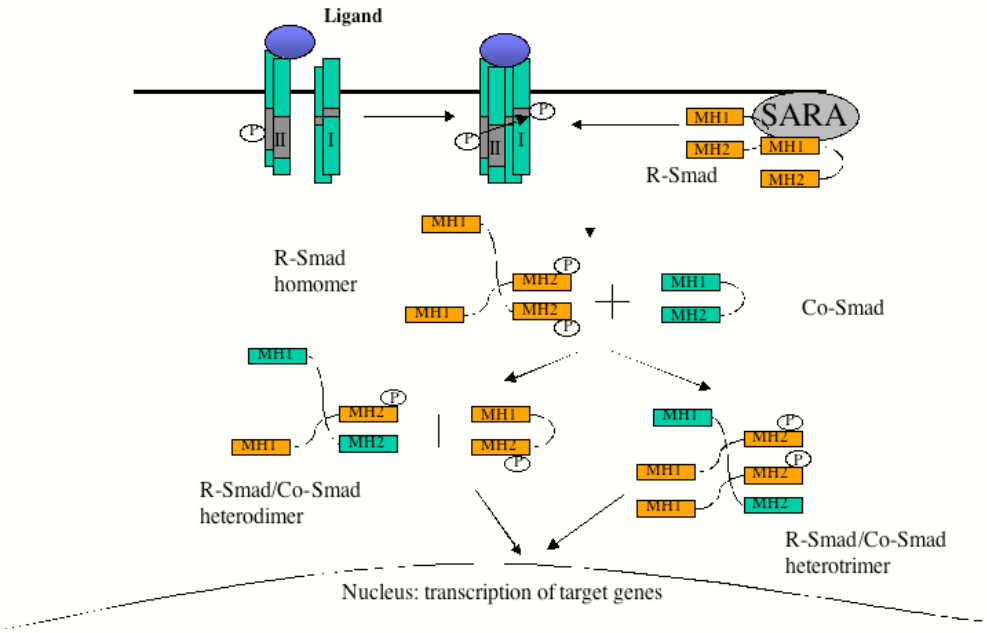


**Figure 2.** General mechanism of TGF-β receptor and Smad activation.

## *Smad-independent signaling pathways*

TGF-β signaling is mainly mediated through the intracellular activation and subsequent nuclear translocation of Smads. Nevertheless, TGF-β also activates other signaling cascades like the MAP-kinase (MAPK) pathways. It was shown that TGF-β can activate Erk, JNK and p38 MAPK pathways and the, in some cases rapid activation of these pathways suggests independence from Smad-mediated transcription [90,91]. The Smad-independent activation of the MAPK pathway was additionally proven in Smad4-deficient cells, where the activation of the MAPK pathway upon TGF-β stimulation was still detectable. Furthermore, mutated TGF-β type I receptors, unable to activate R-Smads, still activate p38 MAPK signaling in response to TGF-β[92].

It was shown that both, TGF-β and BMP4 activate TGF-β-activated kinase 1 (TAK1), which is a MAPK kinase kinase (MEKK). Because TAK1 can activate IκB, thus stimulate NF-κB signaling, TGF-β/BMP signaling may induce NF-κB signaling.

## *Nuclear Smad-complexes control the transcription of a plethora of target genes*

The mechanisms for the nuclearcytoplasmic shuttling of the R-Smads Smad1, Smad2 and Smad3 are well characterized [93,94]. The MH1 domain of all known Smads contain a lysine-rich motif, which was demonstrated to function as nuclear localisation signals for Smad1 and Smad3. In contrast, Smad2 was shown to require a region within the MH2 domain for functional nuclear translocation [95]. A completely different scenario was demonstrated for the Co-Smad, Smad4. Analysis of the dimeric *Xenopus* Smad4 revealed that Smad4 is constitutively entering the nucleus and that cytoplasmic localisation of Smad4 in unstimulated cells is due to active nuclear export [96].

All Smads have transcriptional activity [97]. R-Smads as well as Co-Smads can bind to their cognate DNA-sequences, termed Smad binding elements (SBE), with relatively low affinity. Whereas Smad4 can directly bind to the SBE via its MH1 domain, binding of Smad3 to the SBE needs the relief of the auto-inhibitory interaction between the N-terminal and C-terminal domains through phosphorylation of the C-terminal SSXS motif. Interestingly, it was shown for BMP receptor Smads that they can bind with low affinity to GC-rich sequences, suggesting that the DNA-binding specificity of Smads is not so strict [98].

Microarray analysis revealed that the TGF-β induced and the BMP induced Smads control the expression of about 500 target genes each. Specificity of Smad-mediated transcription in a tissue- and context dependent manner is believed to depend on several transcriptional co-activators or co-repressors, which interact with Smads. One example of Smad-interaction with co-activators is the ligand-dependent recruitment of the structurally related transcription factors p300 and the core-binding factor CBF to the MH2 domain of R-Smads [99,100]. Both of these transcription factors have intrinsic acetyltransferase activity (HAT), which facilitates transcription by decreasing chromosome condensation through histone acetylation and by increasing the accessibility of Smads with the basal transcription machinery. In contrast, Smad nuclear interacting protein 1 (SNIP1) binds Smad4 upon TGF-β receptor activation and supresses the TGF-β/Smad pathway by competing for the binding of Smad4 to the co-activators p300 and CBF [98]. Two proto-oncogenes, Ski and SnoN were found to exhibit transcriptional co-repressor activity for activated Smads. Whereas Ski was shown to be a competitor for p300 binding to Smad, SnoN interacts with non-activated Smads in a ligand-independent fashion [101,102].

All these different interactions of Smads with transcriptional co-activators and co-repressors further increase the complexity of the readout from the incoming TGF-β or BMP signal to confer context dependent specificity of TGF-β/BMP signaling. The exact way, how TGF-β/BMP signaling induces these other signaling pathways needs further investigations.

## *Mutations of TGF-β signaling pathway components are frequently detected in cancer*

Several mouse models for the functional loss of TGF-β signaling components are hampered by their early lethality. Nevertheless, there is a growing set of data available from overexpression experiments and conditional deletion of TGF-β signaling components. Much of this data underline the tumor-suppressor effect of TGF-β and demonstrate that the functional loss of TGF-β signaling in many analysed tissues leads to uncontrolled cell expansion either due to insensitivity to growth inhibition or due to the resistance to apoptotic stimuli.

Several studies were initiated to find out, if TGF-β signaling components are mutated in human malignancies. In hereditary non-polyposis colorectal cancer (HNPCC) the TGF-β

receptor II locus (TBRII) is frequently mutated by microsatellite instability [103,104]. Other mutations within the TBRII were also detected in gastric tumors, gliomas and liver cancers [105,106].

Mutations targeting the TGF-β receptor I (TBRI) have been found in ovarian, breast, pancreatic and T-cell lymphomas [71,107,108]. Studies to detect possible alterations of TGF-β signaling components in the onset of blood cancers, revealed that at least in some patients with myeloid malignancies a decrease in mRNA levels for TBRII could be found [4,5]. These observations indicate that the loss of TGF-β signaling might be implicated in the formation of certain tumors.

The downstream mediators of TGF-β family signaling, the Smads, were also found to be involved in the onset of cancer. For example *SMAD4* was originally described as a classical tumor suppressor that was homozygously deleted in over 50% of pancreatic carcinomas and thus named deleted in pancreatic carcinoma locus 4 (DPC4) [109]. Germline mutations in *SMAD4* were demonstrated to be associated with Familial Juvenile Polyposis, an inherited disease that is characterized by the development of benign polyps in the colon [110].

Some rare *SMAD2* mutations are also found in human colorectal and lung cancers [111,112], but screening of over 50 primary lymphoid and myeloid leukemia cells did not reveal any genetic defects within this gene [113]. However, it was shown in the case of Smad3 that overexpression of the oncogene Evi-1, a repressor of Smad3 transcriptional activity, leads to blocking of the TGF-β signaling in some cases of chronic myeloid leukaemia [114]. As Evi-1 expression in hematopoietic cells is normally restricted to a transient stage of myeloid differentiation, its constitutive expression might contribute to leukemic transition. Studies of murine gene deletion models to further elucidate the function of Smads in the development of cancer were mainly hampered by the early lethality of Smad gene deletions. An overview of published phenotypes from Smad deletion models in mouse is shown in table 3. However, the exact function of different Smad family members in varying tissues remains to be discovered.

Therefore, experimental models for the conditional gene-targeting of Smads will most likely reveal more functions of Smads and TGF-β signaling components in specific tissues.

| | *Mutant phenotype* | *Adult* |
|---|---|---|
| **Smad2** | Lethal before E8.5<br>Defect in egg cylinder elongation,<br>Mesoderm formation and gastrulation | |
| **Smad3** | | Metastatic colorectal cancer (4-6 months of age). Impaired immunity and chronic infection. Accelerated wound healing. |
| **Smad4** | Lethal before E7.5<br>Growth retardation, failure in egg cylinder formation and elongation,<br>gastrulation and mesoderm formation | Conditional deletion in bone marrow leads to severe anemia, extramedullary hematopoiesis and splenomegaly (unpublished results). |
| **Smad5** | Lethal around E10.5 to E11.5 due to multiple embryonic and extraembryonic defects | Cardiovascular abnormalities. Defect in endocardial cushion transformation |
| **Smad6** | Embryonic lethal (E9.5-10.5). Defect in angiogenesis, left/right asymmetry, Increased mesenchymal apoptosis | |

**Table 2.** Summary of phenotypes from gene targeting experiments for germline or conditional deletion of Smad family members.

## *TGF-β in murine and human hematopoiesis*

Several *in vitro* studies established the role of TGF-β as a potent inhibitor of proliferation in human and murine hematopoiesis [115,116]. It was shown that the addition of TGF-β1 to colony forming assays of murine and human hematopoietic progenitors inhibits colony formation of early progenitors but not of late progenitors. On a single-cell based assay it was demonstrated that addition of TGF-β directly inhibits early human hematopoietic progenitor cell proliferation in the presence of various cytokines. Using antisense oligonucleotides to block autocrine TGF-β1 or exogenous TGF-β1 showed that primitive $CD34^+CD38^-$ cells are highly sensitive to TGF-β1 mediated cell-cycle inhibition [117,118]. More mature $CD34^+CD38^+$ cells were only affected to a minor degree or even stimulated by TGF-β1 addition.

Long-term culture initiating cells (LTC-IC) are a subpopulation of primitive human hematopoietic stem/progenitor cells capable of the continuous production of progenitor cells for a period of at least 8 weeks when cultured on bone marrow stromal cells. The addition of anti-TGF-β antibody to these cultures reactivates the proliferation of LTC-IC, showing that TGF-β1 is a potent endogenous inhibitor of hematopoietic progenitor cells [119]. The reverse experiment, namely the addition of TGF-β1 to the LTC-ICs demonstrated the direct inhibitory effect of TGF-β1 on these cells [120].

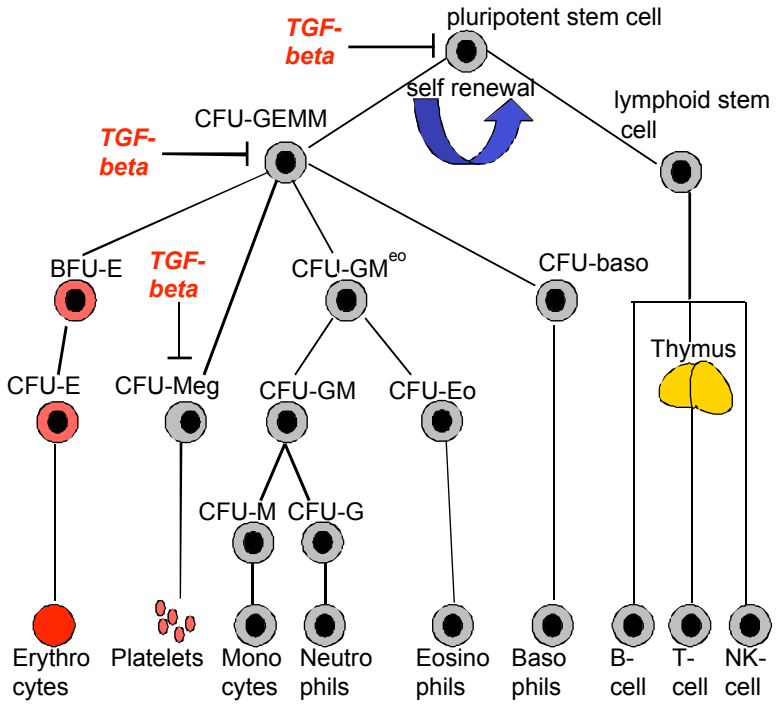


**Figure 3.** Hematopoietic differentiation into the lineages of the peripheral blood. Well characterized inhibitory functions of TGF-β signaling on the different stages and lineages of hematopoiesis are indicated. baso, basphil; BFU, burst-forming unit; CFU, colony-forming unit; E, erythroid; Eo, eosinophil; GEMM, granulocyte, erythroid, monocyte and megakaryocyte; GM, granulocyte, monocyte; Meg, megakaryocyte; NK, natural killer.

## *TGF-β signaling in megakaryopoiesis*

The exact functions of TGF-β in murine or human megakaryopoiesis is still not completely understood. Most data available to date are based on *in vitro* assays, where the inhibitory function of TGF-β on the formation of CFU-Megs was demonstrated [121,122]. The inhibitory effect of TGF-β was also demonstrated using animal *in vivo* models, where for example the constant administration of TGF-β by subcutaneous injection for 2 weeks led to a drastic decline in circulating platelet counts and an increase of megakaryocyte numbers [123]. Similar conclusions on the potential impact of TGF-β on megakaryopoiesis were derived from gene targeting experiments of TGF-β in mice: TGF-β -/- mice displayed elevated platelet numbers and increased megakaryocyte counts[9,124]. Because of TGF-β -/- mice develop a severe autoimmune phenotype, the observed elevation in platelet numbers may be secondary to the first phenotype. A second mouse model from Letterio et al. [125], in which TGF-β was deleted in a MHC-II deficient background, thus avoiding the immunological primary phenotype, showed an excess of megakaryocyte counts and expansion of the myeloid lineage within the bone marrow.

| *Gene* | *Mutant phenotype* | *Adult* |
|---|---|---|
| TGF-β1 | Die postnatally (approx. 4 weeks of age) from multifocal inflammatory disease. 50% die at E10.5 due to defective yolk sac vasculogenesis and hematopoiesis | Inflammation and autoimmune disorder, mainly CD4+ T cell mediated.<br>Platelet aggregation defect.<br>In MHCII ko background: Expansion of the myeloid compartment, myeloid metaplasia associated with splenomegaly and anemia. |
| TBRI | Lethal around E10.5 due to defects in vascular development of the yolk sac<br>No defects in hematopoiesis detectable.<br>Normal number of CFU-GM and mixed myeloid colonies. Increase in erythroid colonies | Bone marrow stem cells from induced ko mice showed higher proliferation rate in vitro.<br>Normal hematopoietic capacity in vivo (transplantation) with normal numbers and differentiation ability of progenitors. Fatal inflammatory autoimmune phenotype 8-10 weeks after transplantation. |
| TBRII | Lethal around E10.5 due to defects in yolk sac hematopoiesis and vasculogenesis. | Conditional deletion in bone marrow leads to a transplantable inflammatory autoimmune phenotype as observed in the TGF-β1 ko.<br>Conditional deleted mice die on wasting syndrome 10-12 weeks post induction |

**Table 3.** Summary of phenotypes from gene targeting experiments for germline or conditional deletion of TGF-β signaling components.

Recent studies demonstrate a strong induction of TPO mRNA expression in bone marrow stromal cells activated by TGF-β1 [54]. It was shown that TGF-β, which is mainly stored in megakaryocytes and platelets, has a pronounced impact on the thrombopoitin (TPO) production of bone marrow stromal cells. The TPO production leads then to the expansion of the megakaryocytic progenitor cells within the bone marrow. Furthermore, TPO induces the expression of type I and type II TGF-β receptors on the cell surface of megakaryoblasts. This result suggests that TGF-β1 might be involved in the pathophysiological feedback regulation of megakaryopoiesis.

## *Potential implication of TGF-β signaling components in the progression of myeloproliferative disorders*

The myeloproliferative disorders (MPDs), comprising polycythemia vera (PV), essential thrombocytosis (ET) and idiopathic myelofibrosis (IMF) are characterized by a clonal expansion of the myeloid lineages, involving erythrocytes, granulocytes and platelets. The clonal expansion of the myeloid lineages is most likely due to a single transformed hematopoietic stem cell (HSC), which aquired a proliferative advantage and thus overgrows

the normal HSC pool. The proliferative advantage of the MPD clone is believed to originate either from a cytokine-hypersensitivity or insensitivity to inhibitory signals. One cytokine exerting a strong inhibitory function in hematopoiesis is TGF-β. Therefore, several investigators speculated that the observed decreased sensitivity of hematopoietic cells from MPD patients to TGF-β signaling causes the expansion of the MPD clone. Reports, on reduced expression of the type II TGF-β receptor in patients with MPD [4,5] further underlines the possibility that a diminished TGF-β sensitivity of the MPD clone is the reason for the clonal expansion. The mouse models for targeted disruption of TGF-β signaling give controversial answers to the question, whether the above proposed model for clonal expansion in MPD due to TGF-β insensitivity are correct. Therefore, conditional deletion of TGF-β signaling components in hematopoiesis and megakaryopoiesis might be a valuable tool to create mouse models for MPD. A definite answer, whether TGF-β plays a role in the clonal expansion in MPD might come from experiments, where human HSCs are silenced for TGF-β gene expression. The loss of functional TGF-β signaling in these cells can be subsequently analyzed *in vivo* by transplantation into immunocompromised mice. Recent advances in the use of RNA-interference (RNAi) in HSCs might be valuable to gain insight into the function of TGF-β signaling in human hematopoiesis.

# *Results*

## *The platelet factor 4-Cre mouse: a new tool to study megakaryopoiesis* in vivo

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Running title: The new transgenic mouse strain PF4Cre directs Cre recombinase expression into the megakaryocytic lineage

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## *Abstract*

Many genes potentially involved in megakaryopoiesis are difficult to study by conventional gene targeting models, because their germ line deletion is embryonically lethal. Therefore, we generated a new mouse model for tissue-specific deletion of target genes in megakaryocytes and platelets using the Cre/loxP system [126]. Taking advantage of the ET-cloning system for the modification of large genomic DNAs in bacterial artificial chromosomes (BACs), we inserted the improved Cre recombinase (iCre) sequence into the first exon of the CXC-chemokine platelet factor 4 (PF4). The injection of the modified 100kb genomic BAC-insert harbouring the PF4Cre transgene and the regulatory sequences for tissue-specific expression in megakaryocytes and platelets, resulted in 3 transgenic founder strains with megakaryocyte-restricted Cre expression. We have used the PF4Cre mouse for megakaryocyte-restricted deletion of the TGF-β receptor II (TBRII) and the Smad4 gene. This way, we aimed to prove the hypothesis that the loss of functional TGF-β signaling in megakaryopoiesis promotes elevated platelet counts. This was suggested by studies establishing the *in vitro* and *in vivo* inhibitory effect of TGF-β on megakaryopoiesis [121,122]. Megakaryocyte-specific deletion of TBRII was verified by southern analysis in homozygous TBRIIlox/lox-PF4Cre mice. Peripheral blood counts of these mice were analyzed and no alterations of platelet counts were detectable in TBRIIlox/lox-PF4Cre or Smad4lox/lox-PF4Cre mice arguing against a function of TGF-β signaling in normal megakaryopoiesis. Furthermore, the analysis of TBRIIlox/lox-Mx1Cre mice for conditional deletion of target genes in bone marrow progenitor cells indicates that TGF-β signaling does not affect very early stages of megakaryopoieis.

## *Introduction*

Many of the genes that are thought to control megakaryopoiesis are expressed ubiquitously, which makes it difficult to determine their specific contribution to megakaryopoiesis by a classical gene knockout approach. In order to investigate functions of genes, which are potentially involved in megakaryopoiesis, we intended to target Cre recombinase expression to megakaryocytes and platelets by exploiting the *Pf4* gene. The α-chemokine platelet factor 4 (*PF4*) is expressed during megakaryocytic differentiation, where it is activated during the late stages of megakaryopoiesis [32,33]. Due to the lineage-restricted expression pattern of the *Pf4* gene, we used the PF4 promotor for tissue-specific expression of the Cre recombinase in megakaryocytes and platelets. To circumvent the known obstacles of conventional plasmid-based transgenes, namely position variegation effects as silencing of the transgene or mosaic expression, we modified a Bacterial Artificial Chromosome (BAC) harboring the entire PF4 gene and regulatory regions, which are responsible for tissue-specific expression. Using the ET-recombination system in E.coli [11] the BAC was modified to contain the improved Cre (iCre) recombinase sequence, replacing exon I of the PF4 gene [127]. Here, we describe the generation of the PF4Cre mouse, which serves as a valuable tool to study gene function in megakarypoiesis by tissue-restricted gene targeting.

We then asked the question whether the loss of the TGF-β signaling pathway during early stages of megakaryocyte differentiation promotes elevated platelet counts. Several *in vitro* and *in vivo* studies, where the inhibitory function of TGF-β on megakaryopoiesis and platelet production was demonstrated, implied that the functional loss of TGF-β signaling in megakarypoiesis might lead to megakaryocytic hyperproliferation and thus increased platelet counts in the peripheral blood [121,122]. Furthermore, reports where mRNA levels of TGF-β signaling components were shown to be decreased in patients with myeloproliferative disorders (MPD) set up the idea that the loss of TGF-β signaling leads to the clonal expansion of myeloid cells in these patients [4,5]. We sought to examine the above-mentioned hypothesis by the megakaryocyte restricted deletion of the type II TGF-β (TBRII) using the PF4Cre mouse. As the full knockout of TBRII is embryonically lethal at day E10.5 [128], tissue-specific deletion of TBRII in megakaryocytes facilitates the study of abolished TGF-β signaling during adult megakarypoiesis. Binding of the ligand to the type II receptor is the crucial initial step for the formation of a functional heteromeric TGF-β signaling complex, deletion of

TBRII leads to a complete block of TGF-β signaling. We crossed PF4Cre mice with TBRIIlox/lox mice and received viable homozygous TBRIIlox/lox-PF4Cre offspring. Analysis of peripheral blood counts from TBRIIlox/lox-PF4Cre mice revealed no alteration in the numbers of circulating platelets, demonstrating that functional TGF-β signaling is dispensable in megakarypoiesis. We obtained the same results, when we crossed the PF4Cre mice with Smad4lox/lox mice where loxP sites flank exon 8 of the Smad4 gene [129]. This result additionally underlines that TGF-β signaling is dispensable for functional megakaryopoiesis, as Smad4 is the crucial down stream transducer of Smad-mediated TGF-β signaling.

## *Material and Methods*

### Screening of a mouse genomic BAC filter library

For the identification of a BAC (Bacterial Artificial Chromosome) clone containing the platelet factor 4 (PF4) gene as well as its 5' and 3' regulatory regions a genomic BAC filter library from Incyte Genomics was screened using a PCR generated PF4 promotor specific radioactive labelled probe, which was hybridzed to the BAC filters. Positive clones were identified after autoradiography as double-spots on the filter grid and the corresponding clone identities were ordered as agar stabs from Incyte Genomics. Three clones were ordered and verified to contain the PF4 gene by PCR with the primers PF4 forw. 5'-TACAGCATACCTTTTGCTAA-3' and PF4 rev. 5'-GTCAAGAGGGTGCCACTGGA-3'. The insert size is 120kb on average, ranging from 40-240kb. One positive clone, 117b02, was subsequently used to insert the improved Cre recombinase (iCre) sequence by ET-recombination in *E.coli.*

### BAC DNA preparation

BAC DNA from the maternal clone as well as from the modified versions was purified using the Nucleobond AX DNA purification kit from Machery-Nagel.

### Generation of the PF4Cre mouse

The PF4 gene was modified by homologous recombination using the ET-cloning system from F.A.Stewart [11]. A DNA fragment containing the complete improved Cre-recombinase (iCre) sequence and 54 nucleotides of the proximal *Pf4* promotor as well as 54 nucleotides of the *Pf4* first intron, the bovine growth hormone polyadenylation signal, an ampicillin resistance cassette (bla) flanked by frt sites, was PCR generated, the resulting 2.8kb PCR fragment was DpnI digested to remove any contaminating vector DNA and subsequently gel purified for homologous recombination in ET-cloning. The PCR primers for the insert generation were: 5'-CATTTCCTCAAGGTAGAACTTTATCTTTGGGTCCAGTGGCACCCTCCTGACATGGTGCCCAAGAAGAAGAGGAAAGTC-3' and 5'-CACCTGAGGCTCCTGAACTGTCTTCCTGTCCCTAGCATCCCTTCACCCAATCTCACTTGATGAGTTTGGACAAACCACAACTAGAATCCA-3'. The ampicillin cassette was removed by the transiently expressed Flp recombinase. The modified 100 kB BAC insert was

removed by NotI digest, separated by pulsed-field gel electrophoresis and purified by agarase digestion and microdialysis [130]. DNA was injected into the male pronucleus of fertilized C57Bl/6 mice. Transgenic offspring was analysed by PCR using a PF4 promotor specific 5' oligonucleotide and a Cre recombinase specific 3' oligonucleotide. Copy numbers were determined by real time PCR using Cre recombinase specific primers and HPRT primers which served as a one copy gene control.

**Mice**

The TBRIIlox/lox mouse was kindly provided by Dr.J.Roes from the University College London. The Smad4lox/lox mouse was a gift from Dr.CX.Deng from National Institutes of Health, Bethesda, Maryland. ROSA26lacZ mice were kindly provided from Dr.S.Zuklys from the Pediatric Immunology Department, Basel University.

**Analysis of recombination efficiency**

DNA from homozygous TBRIIlox/lox-PF4Cre mice was prepared from MACS-purified megakaryocytes, the flow through fraction from the megakaryocyte isolation or full bone marrow of heterozygous mice by overnight incubation at $50^0$C in Lysis buffer ( 10mM Tris-HCl, pH 8.0, 0.1M EDTA, pH 8.0, 0.5% (w/v) SDS and 20$\mu$g/ml Dnase-free pancreatic Rnase) and subsequent Phenol extraction. For southern blotting typically 5-10$\mu$g genomic DNA were digested by Nco I for 5 hours and size-fractionated on a 1% agarose gel. After overnight blotting the membrane was hybridized with a PCR generated radiolabeled probe for 12 hours in hybridisation buffer SLURP (48% formamide, 5x SSC, 0.2M TrisHCl pH 7.6, 1x Denhardt's, 10% dextran sulfate and 0.1% SDS). Primers for the probe were: 5'-CATGAAGTCTGCGTGGCCGTGTG and 5'-TGTAATCGTTGCACTCTTCCATGT-3'. Bands for the wild type (2.7kb), floxed (2.9kb) or the recombined allele (1.8kb) were detected by autoradiography and quantified on a BioRad phosphoimager.

**Cre recombinase expression analysis**

Mice of all 5 independent PF4Cre strains were crossed into the ROSA26lacZ reporter strain to detect expression of Cre recombinase by lacZ staining. Heterozygous PF4Cre/ROSA mice were killed by cervical dislocation and organs were removed and subsequently embedded in OCT compound for fixation. The samples were frozen on dry ice and used to prepare cryosections at the desired thickness (10$\mu$). Organ sections were fixed in lacZ fixation buffer

(0.2% glutaraldehyde, 5mM EGTA, pH 7.3, 100mM $MgCl_2$ in phosphate buffered saline) for 10 min at room temperature and washed three times for 5 min in lacZ wash buffer (2mM $MgCl_2$, 0.01% NaDOC, 0.02% Nonidet P40 in phosphate buffered saline), followed by lacZ staining over night in lacZ staining buffer (100mg X-gal dissolved in DMSO, 0.21g K-ferrocyanide and 0.16g K-ferricyanide were dissolved in 96ml lacZ wash buffer). The next day sections were washed in phosphate buffered saline (PBS), post-fixed in lacZ fixation buffer for 10min at room temperature and PBS washed again. After rinsing the sections with deionized water for 5min they were stained with nuclear fast red to visualize the nuclei. Mounted slides were analyzed microscopically for lacZ positive cells.

**Peripheral blood counts**

For the quantification of peripheral blood mice were either bled by tail vein sections or heart punctures. For tail bleeding the most proximal tip of the tail was cut, blood was collected in EDTA-coated capillaries (BRAND) and diluted in 0.9% NaCl. Blood from cardiac heart punctures was collected without anticoagulants and approximately 500$\mu$l were immediately mixed with EDTA and blood counts were performed with an automated blood counter (Technicon H-3, Bayer Diagnostics, Tarrytown, NY).

**CFU-Meg assay**

Bone marrow was flushed from femurs and tibiae with IMDM using a syringe with a 25 gauge needle. Cells were seeded at $2x10^6$ cells/ml on 3.3% agar supplemented with 7.8$\mu$g/ml cholesterol, 25$\mu$g/ml soybean lipids, 10mg/ml BSA, 5.6$\mu$g/ml linoleic acid, 1mM sodium pyruvate, 2mM L-glutamine, 100$\mu$M $\alpha$–thioglycerol and 300$\mu$g/ml human transferrin. Plates were incubated for 7 days at 37C in 5% $CO_2$ and colonies were scored microscopically after acetylcholinesterase stain.

**Bone marrow cytospins**

A total of $3\text{-}5x10^5$ cells from freshly isolated bone marrow cells was resuspended in phosphate buffered saline (PBS) and centrifuged at 500g in a Cytospin 3 fuge from Shandon onto a glass

slide. The slide was dried at $50^0$C for 1min and stained with Whright stain or used for over night lacZ stains. Stained cells were analyzed microscopically.

**Acetylcholinesterase stain**

Bone marrow and purified megakaryocyte cytospins were stained for enzymatically active acetylcholinesterase by overlaying the cytospins for 3.5 hours with Ach-stain (0.3mg/ml acetylthiocholine iodide, 3mM sodium citrate, 2mM copper sulfate, 300$\mu$M potassium ferricyanide disolved in phosphate buffer). After staining for 3.5 hours the slides were briefly rinsed in deionized water and megakaryocytes, appearing brown, were detected microscopically.

**Purification of bone marrow derived megakaryocytes**

Bone marrow from the femurs and tibiae of TBRIIlox/lox-PF4Cre mice was flushed with a 25 gauge needle into RPMI/10% fetal calf serum (FCS). Cells were sedimented by centrifugation at 1200rpm for 5 min. and subsequently washed once in MACS-buffer (phosphate buffered saline, pH 7.2, 0.5% bovine serum albumin and 2mM EDTA). Cells were resuspended in 300$\mu$l MACS buffer and stained with anti-CD41-FITC conjugated antibody for 15 minutes at 4$^o$ C. The cells were washed again in MACS buffer and resuspended in 300$\mu$l MACS buffer for the incubation with the anti-FITC magnetic immunobeads. After incubation at 4$^o$ C for 20 min, the cells were washed twice in MACS buffer, resuspended in 500$\mu$l MACS buffer and separated over a MACS MS column. The separated megakaryocytes were either used for acetylcholinesterase staining or for the preparation of genomic DNA.

# *Results*

## *Generation of the PF4Cre mouse*

It has been shown before that a promotor construct of the chemokine platelet factor 4 ( PF4) is able to drive tissue-specific gene expression of a reporter gene into the megakaryocytic lineage [131,132]. These short, plasmid-based transgenes were hampered in their expression by position variegation effects. While expression of these transgenes was efficiently detectable in bone marrow derived megakaryocytes, splenic megakaryocytes showed no transgene expression. We isolated a Bacterial Artificial Chromosome (BAC) containing the entire platelet factor 4 gene from a mouse genomic BAC filter library with the intention to generate a transgenic PF4Cre mouse with tissue-specific expression of the Cre recombinase in megakaryocytes and platelets. The use of large genomic DNA inserts from a BAC for transgene delivery into mouse oocytes circumvents position effect variegations, namely silencing or mosaic expression demonstrated for plasmid based transgenes. To gain maximal tissue-specificity of Cre expression, we modified the BAC by inserting the coding sequence of the improved Cre recombinase (iCRE) into the first exon of the *Pf4* gene in frame with the ATG of the endogenous *Pf4* gene by ET recombination in *E.coli* [11,127]. Homologous recombination between the 5' and 3' homology arms of the PCR-generated Cre-cassette and the circular BAC-DNA led to the insertion of the iCre sequence into the first exon of the *Pf4* gene. The iCre sequence was followed by the bovine growth hormone sequence and an ampicillin resistance cassette flanked by Frt-sites ( Fig.1).

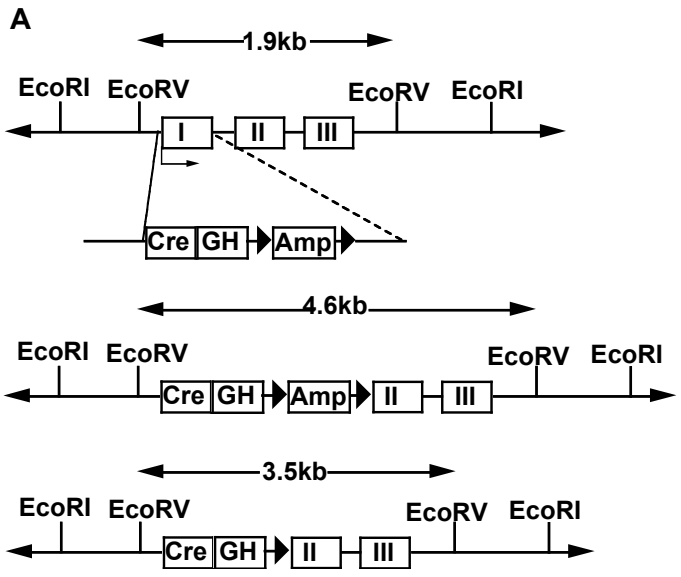


**Figure 1.** Modification of a Bacterial Artificial Chromosome (BAC). The transgene construct was amplified by PCR using the vector pCre.myc.nuc.FRT.NotI.ampFRT as a template.
Open boxes I-III indicate platelet factor 4 exons 1-3. *Cre* sequence of the improved Cre recombinase, *GH* bovine growth hormone polyadenylation signal, *EcoRI* and *EcoRV* indicate the position of analytical restriction-sites. The arrows indicate the sizes of restriction fragments for southern analysis. Arrowheads show the position of the FRT-sites.

The resulting modified BAC clone was subsequently analyzed by Southern blotting (Fig.2). Analytical EvoRV-digests of the modified BAC DNA revealed effective recombination of the Cre cassette into exon I of the *Pf4* gene (Fig.2). Ten positive clones were further modified by the excision of the ampicillin resistance cassette via Flpe recombinase mediated recombination. Recombined clones were detected by PCR and Southern blotting and subsequently analyzed by sequence analysis. Out of 5 clones only one passed all controls and was used for the generation of transgenic PF4Cre mice. The 100 kb genomic BAC-insert harboring the transgene construct was microinjected into the male pronuclei of fertilized C57BL/6 oocytes. Out of 12 founder lines 6 lines were tested PCR-positive for transgene integration and were subsequently used for further analysis.

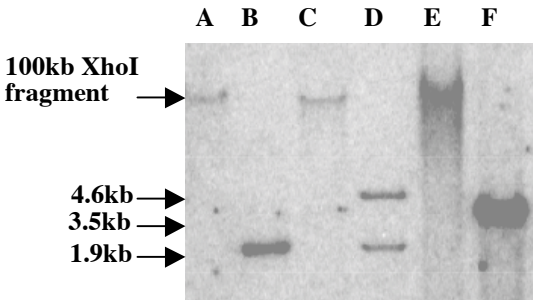


**Figure 2.** Southern blot from the parental and modified BAC. Arrows indicate the sizes of analytical EcoRV restriction fragments; in lane **B**, for the maternal clone; lane **D** for the recombined with Amp-cassette and **F** for the recombined clone without the Amp-cassette. The size of a XhoI restriction fragment to monitor BAC-integrity is shown in lane **A** for the maternal clone and **C,E** for modified versions.

### *Tissue-specific Cre expression in PF4Cre mice*

Copy number detection in transgenic PFCre strains by quantitative real time PCR using Cre specific primers revealed integration numbers between 1 and 22 copies (table 1). The F1 progeny of all six lines was tested for Cre mRNA expression in peripheral blood by RT-PCR (data not shown). Only one strain (Q10) had no detectable Cre mRNA expression in peripheral blood although it was tested positive for the integration of the construct. The remaining 5 lines were than analyzed for tissue-specific Cre expression in the megakaryocytic lineage by crossing them into the ROSA26lacZ reporter mouse strain [133]. In the heterozygous offspring, expression of functional Cre recombinase leads to the deletion of a stop-cassette within the ROSA locus and thereby to the expression of enzymatically active β-galactosidase. Bone marrow cytospins and tissue-sections were analyzed for the expression of β-galactosidase by lacZ staining. We found that β-galactosidase expression was restricted to megakaryocytes and platelets of the bone marrow and spleen in the two lines with

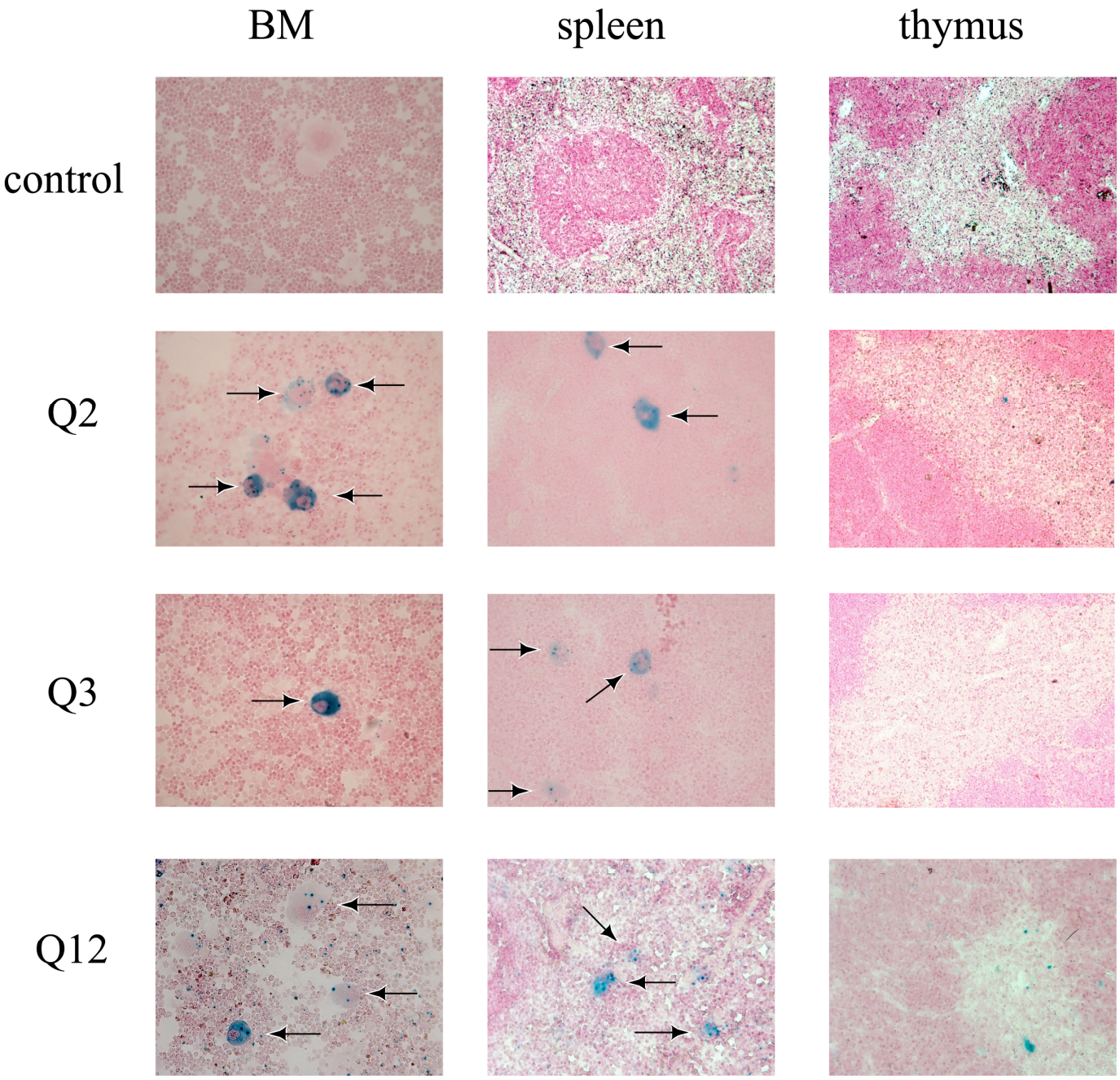


**Figure 3.** Analysis of Cre recombinase expression in tissues from ROSA26lacZ/PF4Cre mice by X-gal staining. Sections were counterstained with nuclear fast red after lacZ-staining. Black arrows indicate megakaryocytes stained for β-galactosidase expression. ***BM*** bone marrow, ***Q2***, ***Q3*** and ***Q12*** transgenic PF4Cre strains crossed with ROSA26lacZ reporter mice.

one or two integrations (Q3 and Q2), whereas ectopic expression was detectable in the lines with 5 or 22 copies (Q12 and Q1), respectively (Fig.3).

The Q12 strain showed ectopic blue staining in spleen, thymus, bone marrow (Fig.3) and brought blue staining in the alveolae of the ovary (not shown). The analysis of Cre expression in the 22 copy Q1 strain revealed that almost all organs showed positive lacZ staining to different degrees (data not shown), therefore this strain was excluded from further experiments. Monitoring β-galactosidase activity in the one copy Q11 line revealed no β-galactosidase positive cells in any of the analyzed tissues and was excluded from further analysis. The various degree of Cre expression in different strains is in agreement with previous reports of copy number dependent expression of BAC-derived transgenes [134].

To evaluate the excision efficiency of a floxed target gene in megakaryocytes, we analyzed the percentage of excision in purified megakaryocytes from PF4Cre mice crossed with TGF-beta receptor II lox (TBRIIlox) mice where exon 3 is flanked by loxP sites. Cre mediated recombination of the floxed exon 3 leads to direct splicing of exon 2 to exon 4 generating a frameshift mutation and a stop codon at position 187 of the open reading frame, resulting in termination of translation [135]. Genomic DNA from purified, bone marrow derived megakaryocytes of TBRIIlox/lox-PF4Cre or TBRIIlox/+-PF4Cre mice was used to detect the recombined allele by southern blotting. The purity of the megakaryocyte preparation was typically between 70-80%, as revealed by acetylcholinesterase stain of the purified megakaryocytes. Determination of the band intensities indicated relatively low excision efficiencies for the 2 copy Q2 strain, e.g. 65% in the purified megakaryocytes, and no detectable excision for the one copy Q11 strain, which is in agreement with the previously obtained negative β-galactosidase detection in this strain. The 5 copy Q12 strain showed an excision efficiency of 90% in the purified megakaryocytes, but ectopic excision in the megakaryocyte-depleted bone marrow flow through of the Q12 strain was also detectable to 50% (Fig.4). This result correlates the previous Cre expression data obtained by lacZ stains (Fig.3). Unfortunately, analysis of the one copy Q3 strain was only possible in heterozygous mice, as this strain had no homozygous TBRIIlox/lox-PF4Cre offspring.

| Transgenic strains | Copy numbers | Tissue-specific Cre expression | % excision efficiency in megakaryocytes |
|---|---|---|---|
| **Q3** | 1 | **+++** | 70% |
| **Q2** | 2 | **+++** | 65% |
| **Q12** | 5 | **+** | 90% |

**Table 1.** Summary table of transgenic PF4Cre strains. +++ Cre expression only in megakaryocytic lineage, + Cre expression in megakaryocytic lineage; ectopic expression in other hematopoietic and non-hematopoietic tissues.

The excision efficiency of this strain was 60-70% in purified megakaryocytes and excision was undetectable in megakaryocyte-depleted bone marrow or any other organ. A possible reason for the lack of viable homozygous TBRIIlox/lox-PF4Cre offspring in this strain, namely co-segregation of the transgene with the endogenous TBRII gene was excluded by the fact that we found Cre-negative heterozygous mice, which should be impossible in the above described scenario.

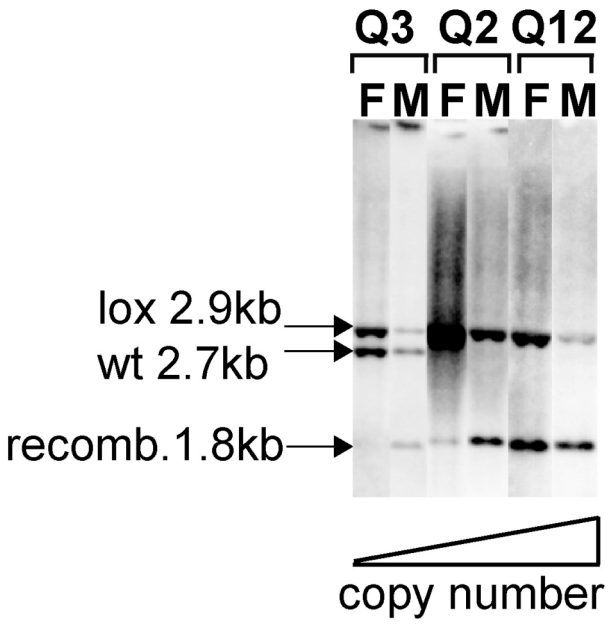


**Figure 4.** Southern blot for the quantification of excision in megakaryocytes. TBRIIlox/lox-PF4Cre genomic DNA from purified megakaryocytes and depleted bone marrow flow through was analyzed by Southern blotting. Fragment sizes are indicated. ***lox*** conditional allele, ***wt*** wild type allele, ***recomb.*** recombined allele. ***Q3***, ***Q2*** and ***Q12*** indicate the analyzed transgenic strains, which are plotted according to the copy numbers. ***F*** flow through from megakaryocyte depleted bone marrow, ***M*** purified megakaryocytes

### *Deletion of the TGF-β receptor II in megakaryocytes has no effect on circulating platelet counts*

To determine the functional role of transforming factor beta (TGF-β) in megakaryopoiesis, we crossed our PF4Cre transgenic strains with a TGF-β receptor II lox (TBRIIlox/lox) mouse where exon 3 is flanked by loxP-sites [135]. Upon Cre-mediated recombinaton between the loxP-sites, exon 2 is fused to exon 4 generating a stop codon and thus a null-allele. We received

viable TBRIIlox/lox-PF4Cre offspring from strains Q2 and Q12, but no homozygous Cre-positive mice from matings with Q3. However, analysis of homozygous TBRIIlox/lox-PF4Cre offspring from the Q2 strain revealed no phenotypic abnormalities compared to littermate controls. Analysis of peripheral blood from TBRIIlox/lox-PF4Cre mice showed no alterations in circulating platelet counts or any other lineage of peripheral blood (Table 2). The fact that mice from the Q2 strain were shown to have incomplete excision of the floxed TBRII in megakaryocytes (Fig.4) might explain the complete lack of phenotypic alterations in this strain.

Next, we analyzed TBRIIlox/lox-PF4Cre mice derived from the Q12 strain. Homozygous mice were viable at birth and had no obvious phenotypic differences in comparison to their littermates. Peripheral blood counts from 5 weeks old homozygous mice revealed no alterations in circulating platelet counts, but slight changes in the numbers of white blood cells (WBC). With increasing age, these mice developed obvious phenotypic alterations. At the age of 10 weeks these mice were clearly phenotypically distinguishable by size from heterozygous littermates. At the age of 15-17 weeks TBRIIlox/lox-PF4Cre (Q12) mice displayed a severe weight loss of up to 30% as compared to their heterozygous littermates.

| **Strain** | **n** | **Age** | **RBC** ($x10^6$/ml) | **Hb** **(g/l)** | **Hct** **(%)** | **WBC** ($x10^6$/ml) | **platelets** ($x10^6$/ml) |
|---|---|---|---|---|---|---|---|
| **control** | 6 | 6-8 weeks | 9.6 (±0.2) | 156 (±4.3) | 49 (±1) | 7.7 (± 1.5) | 1295 (± 187) |
| **Q2** | 4 | 8-10 weeks | 9.6 (±0.5) | 155 (±9.7) | 46 (±3) | 4.7 (± 1) | 1230 (± 134) |
| **Q12** | 2 | 13-17 weeks | 8.4 (±0.04) | 125 (±2.12) | 42 (±3) | 19.9 (± 0.13) | 1435 (± 37) |

**Table 2.** Peripheral blood counts in wild type and transgenic TBRIIlox/lox-PF4Cre mice. Given are the mean values of the indicated mouse strains. Numbers in parenthesis represent ± SD. ***RBC*** Red Blood Count, ***Hb*** hemoglobin value, ***Hct*** hematocrit in %, ***WBC*** White Blood Count, ***platelets*** number of platelets

Homozygous TBRIIlox/lox-PF4Cre (Q12) mice appeared lethargic and showed inflammation of the eyes with corneal opacity. Peripheral blood counts obtained from these mice showed an increase of the previously detected leukozytosis. This was revealed by the double amount of circulating WBCs compared to littermate controls (Table 2). The increase of WBC counts was due to elevated numbers of neutrophils and lymphocytes in the circulating blood.

Nevertheless, these mice did not show any alterations in circulating platelet counts in agreement with the previously analyzed Q2 strain.

### *Deletion of the TGF-β signaling mediator Smad4 in the megakaryocytic lineage*

To further underline the results obtained with the deletion of the TGF-β receptor II in megakaryopoiesis, we asked the question whether the targeting of a downstream signaling component of the TGF-β pathway would have the same outcome as the deletion of the TGF-β receptor II. Therefore, we decided to analyze the consequences of the conditional deletion of the mediator of TGF-β family signaling, Smad4, in megakaryopoiesis. Smad4 lox mice, where exon 8 is flanked by loxP-sites, were kindly provided by Dr.CX Deng [129]. Smad4lox/lox mice were crossed with PF4Cre mice to receive homozygous Smad4lox/lox-PF4Cre mice. We received viable homozygous offspring for two lines, Q3 and Q12 and analyzed peripheral blood counts from these mice. Similar to TBRIIlox/lox-PF4Cre mice, the number of circulating platelets were not altered in the analyzed strains (Table 3).

| Strain | n | Age | RBC ($x10^6$/ml) | Hb (g/l) | Hct (%) | WBC ($x10^6$/ml) | platelets ($x10^6$/ml) |
|---|---|---|---|---|---|---|---|
| **control** | 3 | 8-10 weeks | 9.7 (±0.6) | 154 (±9.2) | 48 (±2) | 9.7 (±1.8) | 1182 (±217) |
| **Q12** | 3 | 8-10 weeks | 9.3 (±0.64) | 152 (±8.7) | 48 (±2) | 11.2 (±0.48) | 1127 (±98) |

**Table 3.** Peripheral blood counts in wild type and transgenic Smad4lox/lox-PF4Cre mice. Given are the mean values of indicated mice. Numbers in parenthesis represent ± SD. ***RBC*** Red Blood Count, ***Hb*** hemoglobin value, ***Hct*** hematocrit in %, ***WBC*** White Blood Cell, ***platelets*** number of platelets $x10^6$/ml

### *Induced deletion of TBRII in bone marrow stem/progenitor cells has no influence on megakaryopoiesis*

Using the PF4Cre transgenic mouse strain for conditional gene deletion in the megakaryocytic lineage, we clearly demonstrated that the loss of TGF-β signaling in early megakaryopoiesis does not lead to the expected increase of circulating platelets. To study the consequences of non-functional TGF-β signaling in the earliest stages of megakaryopoiesis, the transgenic Mx1Cre mouse strain was used to induce deletion of TBRII in bone marrow stem/progenitor cells. In Mx1Cre mice the expression of the Cre recombinase is controlled by the interferon-

α/β inducible Mx1-promotor [10], which mediates close to 100% target gene excision in bone marrow. Induction of Cre recombinase expression in TBRIIlox/lox-Mx1Cre mice by polyinosinic/polycytidylic acid (pIpC) resulted in the expected 100% deletion of TBRII in bone marrow, as revealed by RT-PCR (data not shown). In a previous report from Leveen *et al.*[7], the induced deletion of TBRII in homozygous TBRIIlox/lox-Mx1Cre mice led to an inflammatory phenotype, characterized by weight loss, immobility and inflammation of the eyes. Pathologies of phenotypic mice 8-10 weeks after pIpC-injection, revealed organ infiltrations by granulocytes and T/B-cells in many of the analysed organs, mainly in stomach, pancreas, and liver, accompanied by tissue destruction. The phenotype was described as a severe inflammatory disorder affecting multiple organs. Interestingly, 6-8 weeks post injection we could not detect alterations in peripheral blood counts of these mice for all analyzed blood lineages (Table 4), which is in line with the reported phenotype from Leveen *et al*. Especially the lack of altered platelet counts further underlined our data obtained with the TBRIIlox/lox-PF4Cre mice. Taken together, both mouse models for the conditional deletion of the TGF-β receptor II demonstrate that TGF-β signaling is dispensable for functional megakaryopoiesis and that the loss of the inhibitory TGF-β signal does not lead to the expected thrombocytosis.

| **Strain** | **n** | **Age** | **RBC** (x$10^6$/ml) | **Hb** **(g/l)** | **Hct** **(%)** | **WBC** (x$10^6$/ml) | **platelets** (x$10^6$/ml) |
|---|---|---|---|---|---|---|---|
| **TBRII$^{-/+}$/Cre** | 6 | 10-15 weeks | 9.9 (±0.5) | 151 (±9.5) | 50 (±10) | 7.5 (±3.8) | 1371 (±140) |
| **TBRII$^{-/-}$/Cre** | 6 | 10-15 weeks | 9.5 (±0.85) | 152 (±21) | 48 (±7) | 5.76 (±2.9) | 1467 (±390) |

**Table 4.** Peripheral blood counts from pIpC-injected TBRIIlox/lox-Mx1Cre and TBRIIlox/+-Mx1Cre mice. Given are the mean values of indicated mice 6-7 weeks after the last pIpC-injection. Numbers in parenthesis represent ± SD. ***RBC*** Red Blood Count, ***Hb*** hemoglobin value, ***Hct*** hematocrit in %, ***WBC*** White Blood

However, mating the PF4Cre/Q12 mouse with the TBRIIlox/lox strain, we found that homozygous TBRIIlox/lox-PF4Cre mice developed a leukozytosis as demonstrated by increased numbers of peripheral white blood cells (Table 2). Particularly, the numbers of neutrophils and lymphocytes in peripheral blood were elevated in comparison to littermate controls. The detected leukocytosis increased with age ranging from 50% increase in 5-10

week old mice, to an 100% increase detected at the age of 17 weeks. Other phenotypic features like weight loss and inflammation of the eyes were comparable to the TBRIIlox/lox-Mx1Cre mice. As induced TBRIIlox/lox-Mx1Cre mice die around 8-10 weeks after the first pIpC-injection, there is an obvious difference in the severity of the phenotype developed by these mice in comparison to TBRIIlox/lox-PF4Cre mice, which survived at least to 17 weeks after birth. The exact cause of the leukozytosis in TBRIIlox/lox-PF4Cre mice from the Q12 line has to be investigated in detail. Nevertheless, it is most likely that the observed ectopic Cre expression in the Q12 line accounts for the similarities in the phenotype compared to the previously described TBRIIlox/lox-Mx1Cre and TGF-β-/- mice.

## *Discussion*

We were interested to study the function of genes, which are potentially involved in megakaryopoiesis. As gene-targeting experiments for many of these genes resulted in embryonic lethality of the knockout mice, these approaches are not informative for gene-function studies in megakaryopoiesis. Therefore, we intended to generate a transgenic mouse model for the conditional deletion of target genes specifically in the megakaryocytic lineage using the Cre/loxP-system [126]. We chose the promotor of the α-chemokine platelet factor 4 (PF4) to drive tissue-specific expression of the improved Cre recombinase (iCre) into megakaryocytes and platelets [127]. Several reports demonstrated that expression of PF4 is almost exclusively restricted to the megakaryocytic lineage, beginning at early stages of megakaryopoiesis [32,33]. As far as short plasmid based transgenes are often restricted by positional effects, like silencing or mosaic expression, we decided to modify a BAC containing the entire PF4 gene and locus control region by using the ET-recombination technology [11]. The iCre sequence was inserted into the first exon of the PF4 gene and the modified BAC insert was microinjected into fertilized oocytes. The resulting 12 founder lines were first analyzed for transgene integration and 6 PCR positive founders, with transgene integration numbers ranging from 1-22 copies, were bred into F1 for further analysis. One line could be excluded by being negatively tested for Cre mRNA expression in peripheral blood. The remaining 5 lines were bred into the ROSA26 reporter strain to examine Cre recombinase expression on a cellular level [133]. We found a clear correlation between the copy number of integrated transgenic constructs and tissue-specific expression. Mice having only one or two transgene integrations (Q3 and Q2) expressed the transgene only in megakaryocytes and

platelets, whereas mice with higher copy numbers had more scattered expression profiles. Especially the Q1 strain with the highest number of integrations showed ectopic expression in most of the analyzed organs. The analysis of the Q12 strain with 5 transgene integrations revealed ectopic Cre expression in spleen, thymus, bone marrow and, surprisingly, in the alveolae of the ovary. Nevertheless, we show for the first time that a transgenic mouse strain expressing Cre recombinase under the control of the PF4 promotor is able drive tissue-specific transgene expression into megakaryocytes of the bone marrow and spleen. Earlier reports of PF4 driven transgenes, where either the human or rat PF4 promotor was used to control transgene expression, failed to show copy number dependent transgene expression in bone marrow megakaryocytes and especially in splenic megakaryocytes [131,132].

Next, we asked the question whether the deletion of the growth factor TGF-β1 in megakaryocytes has an effect on *in vivo* megakaryopoiesis. Several reports established a strong inhibitory effect of TGF-β1 on CFU-Megs *in vitro* [121,122]. Similarly, *in vivo* experiments in the mouse showed that intravenous TGF-β1 administration leads to a decrease in platelet counts [119,136]. The observation that in some patients with myeloproliferative disorders (MPD), TGF-β signaling components were decreased at the mRNA levels further strengthened the hypothesis that the loss of TGF-β signaling in megakaryopoiesis would lead to elevated platelet counts [4,5]. To examine this hypothesis, we functionally deleted the TGF-β receptor II (TBRII) via Cre-mediated recombination in megakaryocytes and platelets. As TGF-β1 directly binds to the type II receptor, the deletion of the TBRII completely abrogates TGF-β1 induced signaling [68]. None of the different TBRIIlox/lox-PF4Cre strains showed an alteration in platelet counts. As the excision efficiency in megakaryocytes of the Q2 TBRIIlox/lox-PF4Cre strain was only 60%, the lack of phenotype might be due to incomplete deletion of the TBRII in megakaryocytes. Nevertheless, looking at the Q12 strain, which showed good excision efficiency in megakaryocytes, demonstrates that TGF-β signaling in normal megakarypoiesis is dispensable, because the numbers of platelets is unchanged in TBRII/Q12 compared to littermate controls. This result implicates that although the *in vitro* proliferation capacity of megakaryocytes can be inhibited by the addition of exogenous TGF-β1, the destruction of TGF-β signaling *in vivo* does not lead to hyperproliferation of megakaryocytes and thus elevated platelet counts. Crossing the PF4Cre mouse with Smad4lox/lox mice for the conditional deletion of the TGF-β signal mediator Smad4, further underlined the previous results from the TBRII deletion. Again, no changes in circulating platelet counts in Smad4lox/lox-PF4Cre mice were observed. This demonstrates that the disruption of Smad-

mediated TGF-β family signaling in early stages of megakaryopoiesis does not lead to elevated platelet counts.

To answer the question, whether the deletion of TBRII in earlier stages of megakarypoiesis results in thrombocytosis, we analyzed TBRIIlox/lox-Mx1Cre mice. In this mouse model TBRII can be deleted in hematopoietic stem cells by inducted expression of the Cre recombinase in bone marrow [7]. It is therefore possible to study the consequences of TBRII deletion in the earliest stages of megakaryopoiesis. However, analysis of induced TBRIIlox/lox-Mx1Cre mice revealed no changes in platelet numbers compared to control mice. This demonstrates that deletion of the TGF-β signaling in early hematopoietic progenitors does not lead to a hyperproliferation of megakaryocytes and high platelet counts. Similar results were also obtained by the targeted disruption of the TGF-β receptor I in murine bone marrow stem cells [8]. As already shown for the TBRIIlox/lox-Mx1Cre, no alterations in peripheral blood counts were detectable in TGF-β receptor I deficient mice. In summary, the deletion of TGF-β signaling components in the above-discussed mouse models, clearly argues against a relevance of TGF-β signaling in megakaryopoiesis. Furthermore, a direct involvement of abolished TGF-β signaling in the molecular pathogenesis of myeloproliferative diseases is questionable. Even though several reports suggested that non-functional TGF-β signaling is responsible for the onset of MPD, the above-discussed mouse models demonstrate the opposite. There is still the possibility that the discussed mouse models for conditional deletion of the TGF-β signaling pathway do not resemble the phenotype observed in patients with myeloproliferative disorders where reduced TBRII or Smad4 mRNA levels were detected. The definitive prove that the loss of functional TGF-β signaling in human hematopoietic stem/progenitor cells *in vivo* leads to an escape from cell cycle inhibition and hyperproliferation of these cells is still lacking. One possible way to answer this question is the block TGF-β signaling in human hematopoietic stem cells using siRNA. This could be achieved by lentivirus-mediated delivery of siRNA targeting TBRII and the subsequent transplantation of infected cells into the NOD/SCID mouse model. This way, the function of TGF-β signaling in human hematopoiesis could be studied *in vivo* using a gene-silencing approach.

## *Induced deletion of Smad4 in mouse leads to a non-transplantable haemolytic anemia*

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Running title: Anemia develops in Smad4lox/lox-Mx1Cre mice after conditional Smad4 disruption in bone marrow

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## *Abstract*

The inactivation of one of the various genes involved in the TGF-β signal transduction pathway may represent a possible mechanism how normally quiescent early hematopoietic progenitors, escape from cell-cycle inhibition. In patients with myeloproliferative disorders (MPD) a reduction of Smad4 and TGF-β receptor II mRNA levels was recently demonstrated [3-5], arguing for an involvement of these TGF-β signaling components in the pathology of these diseases. As Smad4 is the crucial mediator of signaling through TGF-β family members, its conditional deletion in bone marrow might result in a phenotype reminiscent of MPD. To analyze the function of Smad4 in adult hematopoiesis by conditional deletion using the Cre/lox technology and an interferon-inducible Cre transgenic mouse [10,126] we crossed the previously described [129] Smad4lox/lox mouse with the Mx1Cre mouse for inducible deletion of Smad4 in bone marrow. Surprisingly, we found that the induced deletion of Smad4 in Smad4lox/lox-Mx1Cre mice leads to a rapidly developing hemolytic anemia, which is not autoimmune-mediated. Furthermore, the observed anemia in Smad4lox/lox-Mx1Cre mice was not transferable by bone marrow transplantation into lethally irradiated recipient mice. This result strongly implicates that the hematopoietic deficiency in Smad4lox/lox-Mx1Cre mice is not cell autonomous and thereby can be compensated by a host-derived factor. Additionally, mice transplanted with Smad4lox/lox-Mx1Cre bone marrow did not develop an autoimmune mediated inflammatory phenotype like we would have expected from previous reports from conditional TGF-β receptor I and II knockouts. Therefore, we speculate that the observed inflammatory phenotype in these mouse models might not be Smad-mediated, but rather mediated by an alternative TGF-β signaling pathway. In summary, we created a mouse model for secondary anemia by the induced deletion of Smad4 and this model also implicates that the function of TGF-β in immunity and inflammation might be regulated via Smad-independent pathways.

## *Introduction*

Smad4 is the central mediator of signals coming from the TGF-β family of growth factors. The human *SMAD4* was originally described as a classical tumor suppressor that was homozygously deleted in over 50% of pancreatic carcinomas and thus named deleted in pancreatic carcinoma locus 4 (DPC4) [109]. Extensive studies on the function of Smads in the TGF-β signaling pathway revealed that Smad4 functions as a Coactivator-Smad (Co-Smad) binding to receptor-activated R-Smads. The heteromeric Smad-complex then translocates into the nucleus, where it exerts its function as a transcriptional activator/repressor of target gene transcription together with other accessory transcription factors [79,104]. It was shown that activated R-Smads can still translocate to the nucleus without prior binding to Smad4, but the ability to activate transcription of target genes is dependent on Smad4 binding to the activated R-Smad complex [2,137]. Thus, Smad4 is the key-mediator of Smad-dependent TGF-β signaling and deletion of Smad4 abrogates the transcriptional activation of many Smad family induced target genes. Gene-targeting experiments in the mouse revealed a major role for Smad4 in embryonic development, as Smad4 deleted mice die *in utero* before E7.5 due to defects in gastrulation and abnormal visceral endoderm development. Because of the early embryonic lethality of Smad4 null mice, the detailed analysis of Smad4 function in different tissues *in vivo* remains difficult. With the introduction of the Cre/loxP-system for the conditional deletion of loxP flanked target genes via Cre-mediated recombination [2,126], it is possible to study the function of genes by tissue-specific deletion. Recently, the Smad4lox/lox mouse strain was published [129], where exon 8 of the Smad4 gene is flanked by loxP-sites for the conditional inactivation of Smad4 via Cre-mediated recombination. Using this Smad4lox/lox mouse and the Mx1Cre mouse strain, where the expression of Cre-recombinase is under the control of the interferon-αβ-inducible Mx1 promotor, we were interested to gain insight into the function of Smad4 in early hematopoiesis by its conditional inactivation. Similar experiments using TBRIIlox/lox-Mx1Cre or TBRIlox/lox-Mx1Cre mice, where either TGF-β receptor I (TBRI) or TGF-β receptor II (TBRII) were deleted in bone marrow(Karlsson), demonstrated that the loss of TGF-β signaling in hematopoiesis leads to a autoimmune-like phenotype. Between weeks 8-10, injected TBRIIlox/lox-Mx1Cre and TBRIlox/lox-Mx1Cre mice developed a severe autoimmune inflammatory condition with organ infiltrations of lymphoid cells [7,138]. The phenotype observed in induced TBRIIlox/lox-Mx1Cre mice was similar to phenotypic features of the TGF-β1 null mouse, even though differences were detected. We now asked

the question, whether the induced deletion of the downstream mediator of TGF-β family signaling, Smad4, would result in a similar phenotype as previously observed in the conditional deletion of the type II TGF-β receptor, or an even more severe and rapidly developing phenotype. Furthermore, this mouse model potentially answers the question, whether abolished TGF-β signaling is involved in the molecular pathology of myeloproliferative disorders (MPD) [3].

As Smad4 does not only mediate signaling through the TGF-β receptors, but also integrates signaling from the bone morphogenetic proteins (BMPs) and activins, we expected that the deletion of Smad4 in bone marrow would manifest in a more severe and also different phenotype as compared to the TBRII or TBRI deletion. However, the previously described inflammatory phenotype in TBRII and TGF-β 1 deleted mice was expected to be reproducible by the conditional deletion of Smad4 in Smad4lox/lox-Mx1Cre mice.

We show here that homozygous inactivation of the Smad4 gene in the bone marrow of Smad4lox/lox-Mx1Cre mice leads to a severe haemolytic anemia 3-4 weeks after the last pIpC injection, accompanied by splenomegaly and extramedullary hematopoiesis. Anemia in Smad4lox/lox-Mx1Cre mice was tested negative for IgG and IgM binding (Coombs-Test) on the surface of erythrocytes, which implies that anemia in Smad4 deleted mice is not autoimmune-mediated. Furthermore, the phenotype in deleted Smad4lox/lox-Mx1Cre is clearly different from the one detected in mice with conditional deletion of the TBRII and TBRI, as they do not show signs of an inflammatory autoimmune disease. Finally, the induced deletion of Smad4 does not lead to increased platelet counts. This result strongly argues against a relevance of abolished TGF-β signaling in the pathology of MPD.

## *Material and Methods*

### Mice

Smad4lox/lox mice were kindly provided by Dr.C.X.Deng from the National Institutes of Health, Bethesda, Maryland. In brief, a targeting vector was introduced into the embryonic stem cell line TC-1 to create Smad4lox mice. The vector was designed to target exon 8 of the endogenous Smad4 gene and to insert flanking loxP-sites. Homozygous Smad4lox/lox mice were mated to interferon-inducible Mx1-Cre transgenic mice, which express the Cre recombinase under the control of the interferon α/β-inducible Mx1 promotor. Mx1-Cre expression in 5-6 weeks old mice was induced by the intraperitoneal injection of 300µg polyinosinic/polycytidylic acid for 3 times in 2 day intervals. The C57Bl/6 Ly5.1 strain was used for bone marrow transplantation experiments into irradiated recipients.

### Genotyping

For genotyping, tail sections of Smad4lox/lox-Mx1Cre mice were lysed in proteinase K-buffer (10mM Tris-HCl, pH8, 100mM NaCl, 50mM EDTA, 0.5M SDS, 200µg/ml proteinase K) at $55^0$C for 12 hours followed by DNA extraction by phenol/chloroform. DNA was amplified using the SMAD4f primer 5'-GGGCAGCGTAGCATATAAGA-3' and Smad4r primer 5'-AAGAGCCACAGGTCAAGCAG-3'. For the detection of the Mx1-Cre transgene primers Cre-f 5'-CACCATTGCCCCTGTTTCACTATC-3' and Cre-r 5'-GCCAGGCGTTTTCTGAGCATAC-3' were used.

### Bone marrow transfer

Donor mice were killed by cervical dislocation and bone marrow was flushed from femurs and tibiae using a 27 gauge needle and RPMI/2% fetal calf serum (FCS). The cell suspension was filtered through a 70µM mesh to remove clumps and T-cells were removed by complement lysis. For T-cell removal, bone marrow cells were incubated for 30min on ice with monoclonal antibodies against mouse CD3, CD8 and Thy1.2. Cells were washed once with RPMI/2%FCS and T-cells were lysed by the addition of low toxic rabbit complement diluted 10-fold in RPMI/2% FCS. Cells were incubated for 45 min at $37^0$C. After T-cell depletion, cells were washed twice in HBSS and resuspended in 500µl HBSS and containing 4-10x$10^6$ cells, which were injected into the tail vein of lethally irradiated (1000 cGy) 7-10 weeks old recipient mice. Chimerism of the transplanted mice was

analysed on peripheral blood by flow cytometric analysis of CD45.1 and CD45.2 positive cells.

**Histologic analysis**

Organs were incubated in PBS buffered 4% paraformaldehyde for 12 hours, washed once in PBS and stored in 70% ethanol. Fixed organs were paraffin-embedded, sectioned and typically stained with Erlich eosin for microscopic examination.

**Flow cytometry**

Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)- conjugated monoclonal antibodies against TER119, CD71, CD3, B220, Mac-1, GR1, c-kit and Thy-1 (Pharmingen, BD, San Diego, CA)were diluted in PBS/1% calf serum and used for staining of single cell suspensions derived from bone marrow and spleen. In order to get single cell suspensions cells were filtered and organs were grinded through a 70µM nylon mesh. Dead cells and debris were eliminated by gating on forward scatter (FSC) and side scatter (SSC).

**Analysis of peripheral blood cells**

For the quantification of peripheral blood mice were either bled by tail vein sections or heart punctures. For tail bleeding the most proximal tip of the tail was cut, blood was collected in EDTA-coated capillaries (BRAND) and diluted in 0.9% NaCl. Blood from cardiac heart punctures was collected without anticoagulants and approximately 500µl were immediately mixed with EDTA and blood counts were performed with an automated blood counter (Technicon H-3, Bayer Diagnostics, Tarrytown, NY).

**Bone marrow cytospins**

A total of 3-5x$10^5$ cells from freshly isolated bone marrow was resuspended in phosphate buffered saline (PBS) and centrifuged at 500g in a Cytospin 3 fuge from Shandon on a glass slide. The slide was dried at 50$^o$C for 1min and stained with Whright stain for microscopic analysis.

**Immunomagnetic cell sorting (MACS)**

Bone marrow was flushed from femurs and tibiae using 27 gauge needles and RPMI/10% fetal calf serum (FCS). Sedimented cells were washed once in MACS-buffer (phosphate buffered saline, pH 7.2, 0.5% bovine serum albumin and 2mM EDTA) and resuspended in 300µl for the labelling with the anti-TER119 immunomagnetic beads from Miltenyi Biotec (Miltenyi, Auburn, CA). After incubation for 15 min. at 4-8$^{o}$C cells were washed in MACS buffer and resuspended in 500µl. The cell suspension was separated over a MACS MS magnetic column and the eluted TER119-positive cells were used for RNA preparation.

**RNA preparation and quantitative PCR**

Total RNA from TER119-positive bone marrow cells was isolated with Trizol reagent (Invitrogen, Carlsbad, CA), and reverse transcribed after random hexamer priming using the Omniscript RT kit (Qiagen, Germany) following the manufacture's protocol. In brief, 1µg RNA was reverse-transcribed in a 20µl reaction volume containing 100U of Omniscript RT for 60min at 37$^{0}$C followed by a 2 min denaturation step at 95$^{0}$C. Quantitative PCR (q-PCR) was carried out on an ABI Prism 7700 sequence detector using the SYBR Green PCR Master Mix chemistry (Applied Biosystems, Warrington, UK). Primers for GATA-1 were 5'-GTCAGAACCGGCCTCTCATC-3' and 5'-TGCCTGCCCGTTTGCT-3', for GATA-2 5'-GGCTCTACCACAAGATGAATGGA-3' and 5'-GTCGTCTGACAATTTGCACAACA-3', for GATA-3 5'-GAACCGGCCCCTTATCAAG-3' and 5'-CAGGATGTCCCTGCTCTCCTT-3', for FOG-1 5'-CCTTGCTACCGCAGTCATCA-3' and 5'-GTACCAGATCCCGCAGTCTTTG-3'. The primers for the internal control, mouse ribosomal protein L19 (RPL) were 5'-ATCCGCAAGCCTGTGACTGT-3' and 5'-TCGGGCCAGGGTGTTTTT-3'. The $\Delta C_T$ values were derived by the subtraction of the threshold cycles ($C_T$) values for the target cDNAs from the $C_T$ value from the mouse RPL19.

## *Results*

### *Cre recombinase mediated Smad4 deletion in Smad4lox/lox-Mx1Cre mice leads to severe anemia*

To generate mice, where Smad4 is conditionally deleted in the bone marrow, we crossed the previously described Smad4lox/lox mice [129], where exon 8 is flanked by loxP sites, with Mx1Cre transgenic mice. Deletion of Smad4 in 5-6 week old Smad4lox/lox-Mx1-Cre was induced by intraperitoneal injection of polyinosinic-polycytidylic acid (pIpC). For control, heterozygous Smad4lox/+-Mx1-Cre mice were injected at the same time. Induction of the Cre recombinase in bone marrow from Smad4lox/lox-Mx1Cre mice resulted in the deletion of the loxP-flanked exon 8 of the Smad4 gene. Deletion efficiency was determined by PCR analysis of bone marrow derived colonies grown on semisolid medium. From 40 analysed hematopoietic colonies 39 (97%) were shown to be positive for the Smad4-null allele and negative for the conditional allele (Fig1).

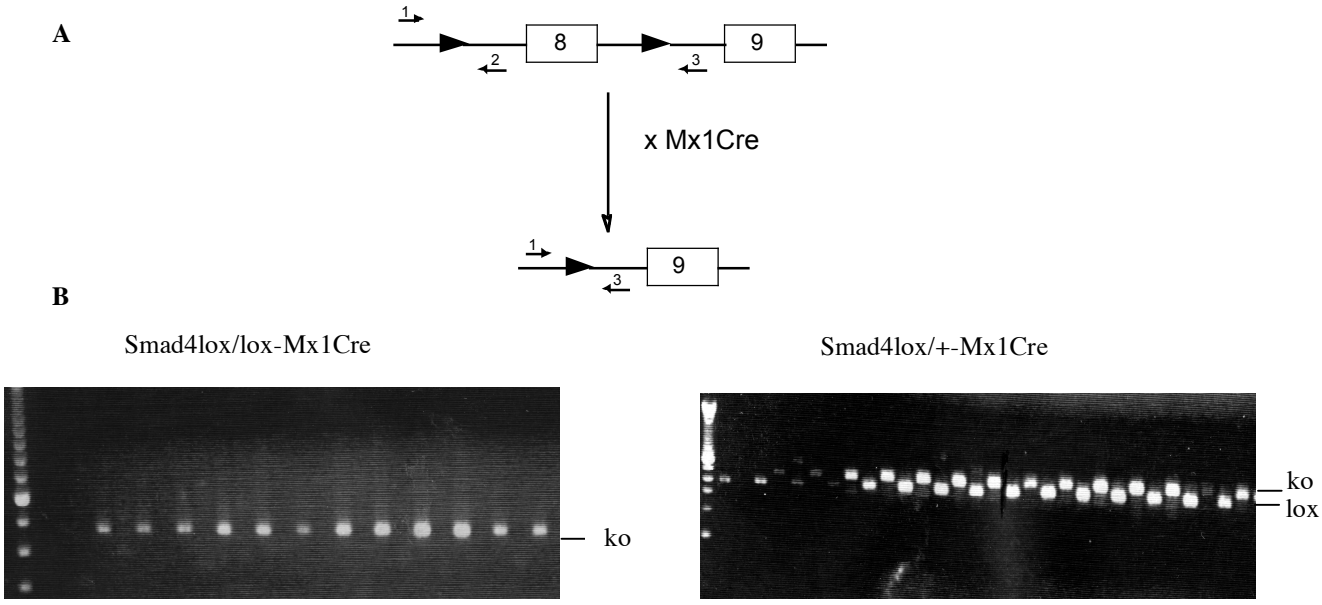


**Figure 1.** Inducible targeting of the Smad4 gene in bone marrow. **A** Schematic representation of Smad4 gene targeting**.** Exon 8 and 9 of the Smad4 gene are shown in boxes. Big arrow heads represent loxP sites, small arrows 1-3 show location of the primers used for ko-detection and genotyping. **B** Bone marrow of Smad4lox/lox-Mx1Cre and Smad4lox/+-Mx1Cre mice was plated on semi-solid medium and single colonies were picked for the PCR detection of the recombined allele. ***ko*** recombined allele, ***lox*** conditional allele. Representative picture for over 30 colonies analyzed.

Smad4 deleted mice showed no obvious symptoms of disease within the first 4 weeks after the last pIpC injection. However, analysis of peripheral blood counts from Smad4lox/lox-Mx1Cre and control mice starting one week after the last injection, revealed a rapidly

developing anemia in Smad4 deleted mice beginning at 2-3 weeks post-injection. 4-5 weeks after the last injection, peripheral blood counts from Smad4lox/lox-Mx1Cre showed that these mice were suffering from a severe anemia and therefore were killed for further analysis. At that time, hemoglobin values of the most affected mice were reduced to 20% of that from healthy controls, while the hematocrit values were decreased by half (Fig 2). At this stage, mice were sacrificed for further analyses.

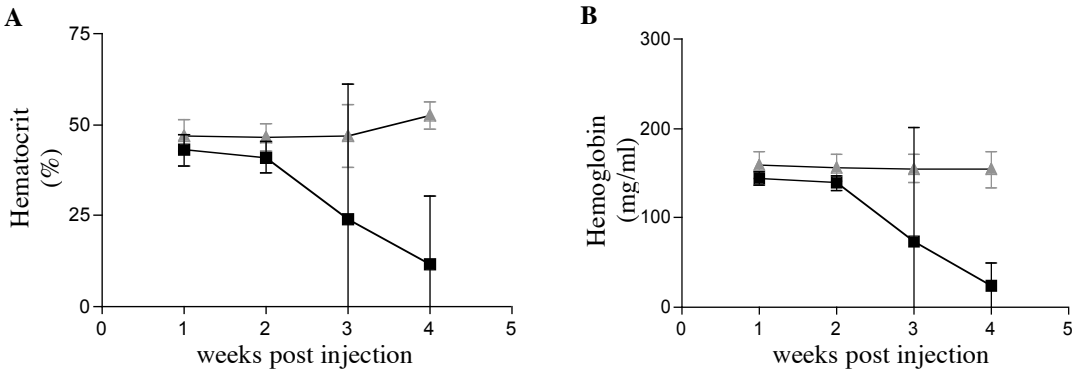


**Figure 2.** Developing anemia in Smad4lox/lox-Mx1Cre mice. **A** Hematocrit values are reduced to 50% of control mice 4 weeks after the last pIpC-injection. **B** Hemoglobin values 4 weeks post injection are at 20% of control mice. Black squares show values for Smad4 deleted mice, grey triangles indicate controls.

Furthermore, the amount of reticulocytes was increased 2-5 fold and the numbers of white blood cells were elevated in some, but not all analyzed Smad4lox/lox-Mx1Cre mice compared to injected heterozygous controls. The increase of white blood cells was due to a high number of granulocytes and lymphocytes in peripheral blood. Analysis of pIpC treated homozygous Smad4lox/lox-Mx1Cre mice 4 weeks after the last injection revealed that not all mice displayed the same severity of anemia, which might be due to background variations in these mice. Nevertheless, more than 80% of the injected Smad4lox/lox-Mx1Cre mice developed a severe anemia, beginning at 3-4 weeks post injection (Table 1).

| **Strain** | **n** | **Age** | **RBC** ($x10^6$/ml) | **Reticulocytes** ($x10^6$/ml) | **Hb** (g/l) | **Hct** (%) | **WBC** ($x10^6$/ml) | **platelets** ($x10^6$/ml) |
|---|---|---|---|---|---|---|---|---|
| **control** | 5 | 10-12 weeks | 9.4 (±0.3) | 326 (±93) | 149 (±6) | 48 (±3) | 8.5 (±1.7) | 1353 (±125) |
| **Smad4lox/lox-Mx1Cre** | 8 | 10-12 weeks | 3.6 (±2.5) | 1026 (±534) | 59 (±42) | 22 (±14) | 11.6 (±7) | 1216 (±164) |

**Table 1.** Peripheral blood counts from induced Smad4lox/lox-Mx1Cre mice and controls. ***RBC*** red blood counts, ***Hb*** Hemoglobin, ***Hct*** Hematocrit, ***WBC*** white blood counts.

**Haemolytic anemia in Smad4lox/lox-Mx1Cre mice is not autoimmune-mediated**

Anemia in Smad4lox/lox-Mx1Cre mice was further characterized by the analysis of the serum levels of the lactate dehydrogenase (LDH), which is increased in patients with haemolytic anemia due to the massive destruction of erythrocytes. Therefore, serum LDH levels serve as a diagnostic marker for haemolytic anemia. Serum LDH levels of homozygous Smad4lox/lox-Mx1Cre mice were elevated 2-3fold compared to the heterozygous control mice (data not shown), further establishing the diagnosis of a haemolytic anemia in Smad4lox/lox-Mx1Cre mice. Nevertheless, as elevated LDH levels can also be detected due to general tissue destruction, the possibility that inflammation-mediated tissue destruction is the reason for increased serum LDH levels cannot be excluded.

Using a direct antiglobulin test (DAT) for the detection of autoantibodies of the IgG/IgM-type on the membrane of erythrocytes, we sought to find out whether the detected anemia in Smad4lox/lox-Mx1Cre is autoimmune-mediated. Blood from the anemia mouse model New Zealand Black (*NZB)* served as positive control and Smad4lox/lox as negative control for DAT on Smad4lox/lox-Mx1Cre blood. We could not detect IgM or IgG surface antibodies on erythrocytes from Smad4lox/lox-Mx1Cre and the Smad4lox/lox controls, while the *NZB* mice displayed strong surface immunoglobulin staining as revealed by flow cytometry (not shown). This result argues against an autoimmune-mediated haemolytic anemia in Smad4 deleted mice. However, the possibility that autoantibodies of the IgA class bind to the surface of the erythrocytes and thereby mediating haemolysis remains open.

**Smad4lox/lox-Mx1Cre mice display splenomegaly due to myeloid hyperplasia**

Further analysis of Smad4lox/lox-Mx1Cre mice revealed that spleen sizes of these mice were increased up to 10-fold compared to heterozygous controls (Fig. 3).

Histopathologic examination of Smad4lox/lox-Mx1Cre organs demonstrated a massive expansion of immature myeloid elements with varying degrees of differentiation in the spleen. In some of the analysed Smad4lox/lox-Mx1Cre spleens the white pulp was completely replaced by immature myeloid cells (Fig. 3). The immature myeloid cells were also detectable in the liver of Smad4lox/lox-Mx1Cre mice, demonstrating extramedullary hematopoiesis in Smad4 deleted mice.

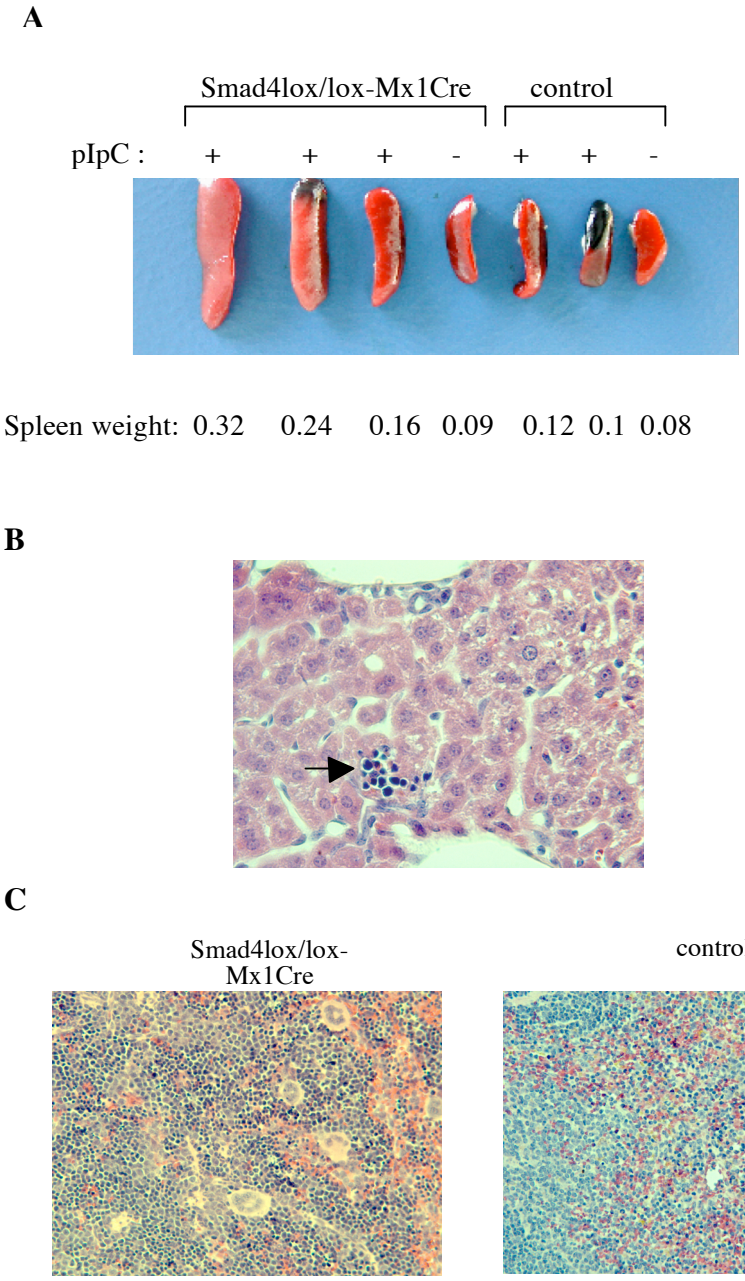


**Figure 3.** Smad4lox/lox-Mx1Cre mice display splenomegaly by 4 weeks after the last pI/pC injection due to the expansion of immature myeloid cells. **A** Spleens of Smad4lox/lox-Mx1Cre mice and controls. Genotypes are indicated. ***polyI/C ±*** indicates induction with polyinosinic/polycytidylic acid. Spleen sizes are shown in g. **B** Liver section from a Smad4lox/lox-Mx1Cre mouse showing infiltrations of immature erythroblasts indicated by the arrow. **C** Representative spleen sections from Smad4lox/lox-Mx1cre mice show expansion of immature myeloid cells compared to a heterozygous control. Sections were made 4 weeks after the last polyI/C injection. Magnification is 200x.

Flow cytometric analysis from enlarged spleens from Smad4lox/lox-Mx1Cre mice revealed that the content of TER119-positive cells was drastically increased in Smad4lox/lox-Mx1Cre spleens. While spleens of control mice displayed between 1-2% TER119-positive cells, the

TER119-positive cells in spleens of Smad4lox/lox-Mx1Cre mice were increased up to 70% (Fig. 4). This result is in line with the previous histological observation of massive myeloid expansion in Smad4lox/lox-Mx1Cre spleens shown in Figure 3.

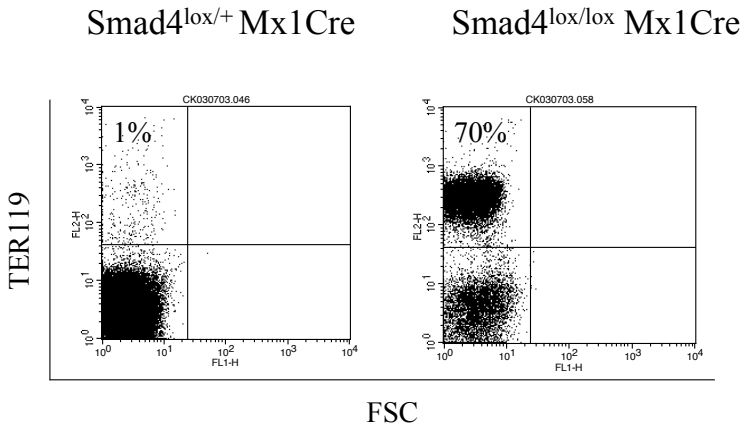


**Figure 4.** Flow cytometric analyis of spleens from Smad4lox/lox-MxCre and Smad4lox/+-MxCre mice 4 weeks after induction demonstrates drastically increased numbers of erythroid cells in homozygous mice. Erythroid cells with TER119 positive surface staining are gated in the upper left corner. ***FSC*** forward site scatter.

Analysis of the maturation status of the TER119-positive cells by additional staining for CD71 demonstrated that most of the cells within the spleen (50-70%) were off the TER119$^{high}$/CD71$^{high}$ phenotype, which is characteristic for immature erythroblasts (Fig.5). In contrast, the most mature erythroid population of TER119$^{high}$/CD71$^{low}$ was strongly reduced as compared to control mice. However, we also found Smad4lox/lox-Mx1Cre mice with normal spleen sizes. These spleens appeared pale in comparison to controls and FACS-analysis revealed that TER119-positive cells were almost absent. Especially cells of the TER119$^{high}$/CD71$^{high}$ phenotype were undetectable within the spleens of these mice. These results imply a potential maturation arrest of erythrocytes at the erythroblast stage as the cause for haemolytic anemia in Smad4lox/lox-Mx1Cre mice.

The histological observation that the white pulp in Smad4lox/lox-Mx1Cre spleens is almost entirely missing was verified by FACS analysis of the splenic B- and T-cell content. While control mice displayed 45-50% B220-positive cells within the spleen, Smad4lox/lox-Mx1Cre spleens showed only 10-17% B220-positive cells. The same observation was made

for the CD3-positive T-cells, which were reduced to 20% of the numbers of normal controls (Fig.5).

The analysis of bone marrow from injected Smad4lox/lox-Mx1Cre mice revealed that in contrast to the spleens of affected animals, the amount of TER119-positive cells was decreased (Fig.5). Again, the main population of TER119-positive cells were of the TER119$^{high}$/CD71$^{high}$ phenotype representing immature erythroblasts, while the TER119$^{high}$/CD71$^{low}$ cells were reduced to 10% in comparison to control mice. Especially the cell population with a TER119$^{high}$/CD71$^{dim}$ phenotype were almost undetectable by FACS analysis. Similar to the situation observed in the spleens of Smad4lox/lox-Mx1Cre mice, the amount of B220-positive cells in bone marrow was decreased when compared to normal controls. Nevertheless, we also detected a slight increase of GR1-positive granulocytes in the bone marrow of Smad4lox/lox-Mx1Cre mice, which is in agreement with the previously observed increase of neutrophils in the peripheral blood of these mice.

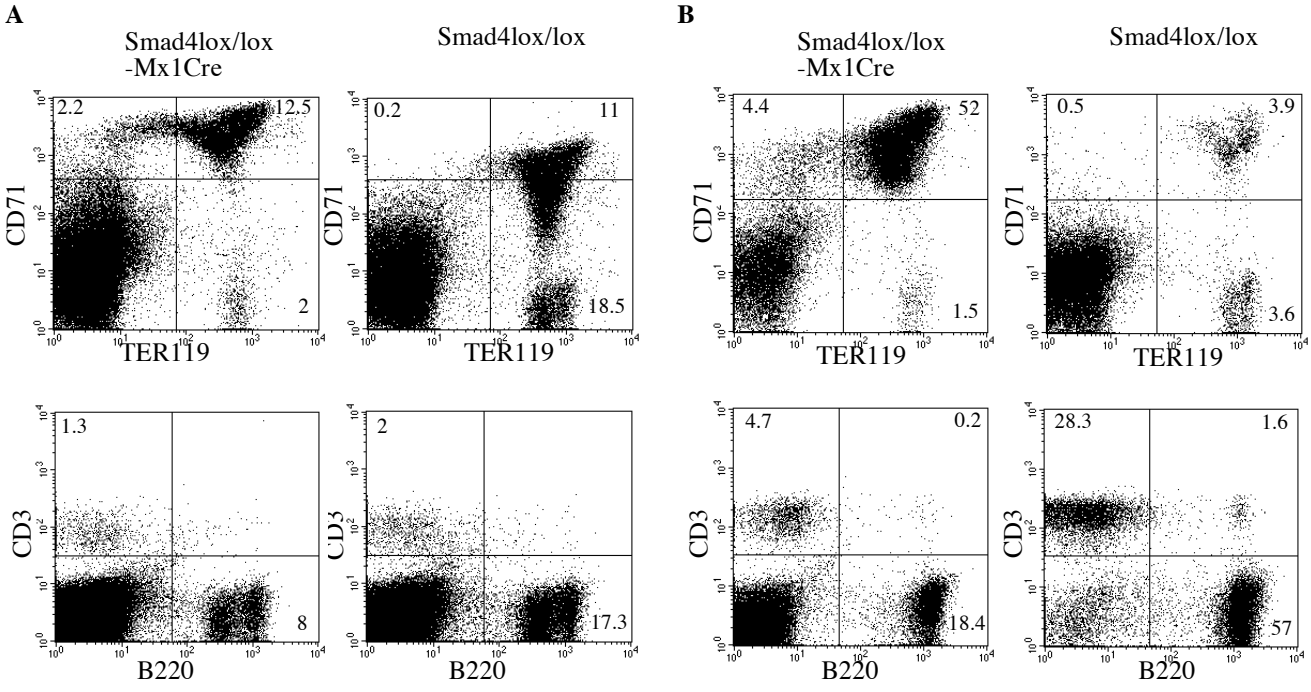


**Figure 5.** Erythroid and lymphoid cell populations in BM and spleen are altered in Smad4lox/lox-Mx1Cre mice. **A** Bone marrow cells were stained for CD71/TER119 or CD3/B220 to analyze the maturation status of bone marrow erythroid cells and the T-and B-cell ratios, respectively. **B** Same as in **A** with spleen derived cells. Percentages of the different cell populations are given.

In summary, most of the analyzed Smad4lox/lox-Mx1Cre mice displayed splenomegaly due to an increase of myeloid cells, which were shown to originate from the erythroid lineage. The expanded TER119-positive cell population mainly consisted of immature erythroblast as revealed by their TER119$^{high}$/CD71$^{high}$ phenotype. The same population of cells was

detectable in the bone marrow of Smad4lox/lox-Mx1Cre mice, but here the overall content of TER119-positive cells was decreased. These results implicate that deficient erythropoiesis in the bone marrow Smad4lox/lox-Mx1Cre mice is compensated by the enormous increase of immature erythroblasts in the spleens, which might account for the decreased numbers of splenic B- and T-cells. The phenotype above- of Smad4lox/lox-Mx1Cre mice described above is reminiscent of phenotypic features found in patients with a myelodysplastic syndrome (MDS). MDS is believed to originate either in the hematopoietic stem cells or the bone marrow stromal cells and is characterized by cytopenia of varying lineages due to bone marrow failure. These patients often display anemia or thrombocytopenia and the disease can transform into leukaemia, whereas splenomegaly in patients with MDS was shown to be a rare event.

***The mRNA level of transcription factor GATA-2 is decreased in TER119-positive cells from Smad4lox/lox-Mx1Cre mice***

To gain further insight into the molecular pathogenesis of the Smad4 deletion phenotype, we analyzed the mRNA levels of the GATA transcription factors 1, 2 and 3 as well as from Friend Of Gata-1 (FOG-1). GATA-1 and GATA-2 were shown to be required for normal hematopoieis in mouse and human [139]. The loss of either GATA-1 or GATA-2 causes embryonic lethality because of the failure of erythroid maturation and the expansion of progenitors, respectively. The functional overlap of GATA-1 and GATA-2 in primitive hematopoiesis was recently demonstrated [140], but the exact functions in adult hematopoieis are still poorly understood. Recent findings demonstrated that signaling through BMP4/Smad5 induces GATA-2 transcription [141] and therefore the deletion of Smad4 in Smad4lox/lox-Mx1Cre mice might lead to deregulated GATA-1/-2 transcription, as Smad4 is crucial for transcriptional activation of Smad5 target genes. The mRNA levels of GATA-1/-2/-3 and FOG-1 were determined in purified TER119-positive cells from spleen and bone marrow of Smadlox/lox-Mx1Cre and control mice by quantitative real-time PCR (Fig.6). As expected from the previous works mentioned above, we found decreased levels of GATA-2 in the spleen and bone marrow TER119-positive cell population.

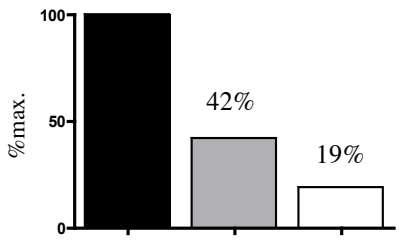


**Figure 6.** Relative mRNA expression of GATA-2 in purified TER119-positive cells. Black bar represents 100% GATA-2 expression in Smadlox/lox control mice. The grey bar represents GATA-2 mRNA expression in pooled bone marrow from 3 Smadlox/lox-Mx1Cre mice; white bar represents GATA-2 mRNA expression from spleens of the same three mice.

erythroid maturation is blocked in the bone marrow and probably even more in the spleen.

***Anemia is not transferred by bone marrow transplantation***

As TGF-β is known to exert different functions in a wide variety of organs and tissues, we were interested to limit the Smad4 deletion to bone marrow cells. We therefore transplanted bone marrow from Smad4lox/lox-Mx1Cre mice into lethally irradiated C57BL/6Ly5.1 recipients. In a first round of transplantations, bone marrow from 3 Smad4lox/lox-Mx1Cre, Smad4lox/+-Mx1Cre and Smad4lox/lox mice was transplanted into 4 recipient mice for each group. Engraftment of the transplanted bone marrow was analyzed 5 weeks after transplantation. Chimerism of the recipient mice was shown to be approx. 80% as detected by flow cytometric analysis of the CD45.1/CD45.2 ratio in peripheral blood (data not shown). All three groups of recipient mice were induced by pIpC-injection and peripheral blood counts were analyzed beginning at 1 week after the last injection. Unexpectedly, we could not detect anemia in any of the analyzed mice. Four weeks post injection peripheral blood counts from all 3 groups of transplanted mice were normal, while at that time injected Smad4lox/lox-Mx1Cre mice already displayed severe anemia. Further analysis of peripheral blood counts over time revealed no change in hemoglobin and hematocrit levels. Even 9 weeks post injection, mice transplanted with bone marrow from Smad4lox/lox-Mx1Cre mice showed no alterations in peripheral blood counts, arguing against bone marrow as the origin of the haemolytic anemia in Smad4lox/lox-Mx1Cre mice. The possibility that pIpC-induced Smad4 deletion failed in transplanted mice was excluded by PCR on peripheral blood cells. In mice transplanted with Smad4lox/lox-Mx1Cre bone marrow no wild type band for the endogenous Smad4 gene was detectable by PCR, demonstrating that all cells in the periphery originated from the donor bone marrow. In a second PCR reaction the recombined allele was detected in peripheral blood from all 4 Smad4lox/lox-Mx1Cre recipient mice. Therefore, a failure in inducing recombination can be ruled out as the reason for the lack of phenotype in transplanted mice. Thus the hematopoietic deficiency in Smad4lox/lox-Mx1Cre mice is not BM cell-autonomous and can be compensated by the host. This strongly argues for the deregulation of a soluble factor, expressed from bone marrow stromal cells or a different organ such as liver and kidney, as the target of Smad4 deletion.

## *Discussion*

The major mediators of signals coming from TGF-β family members are the Smads. While the bone morphogenetic proteins (BMPs) mainly signal through Smad1, Smad5 and Smad8, TGF-β signaling is mediated through Smad2/3. These receptor-activated Smads (R-Smads) are phosphorylated from the type I receptors and mediate the incoming signal to the nucleus. Before activated R-Smads translocate to the nucleus, they heterodimerize with Smad4. Dimerization of activated R-Smads with Smad4 is not a prerequisite for nuclear entry, but only heteromeric R-Smad/Smad4 complexes display full transcriptional activity. While phosphorylated R-Smads can still translocate to the nucleus, their function as transcriptional activators is dependent on Smad4 binding [2,137]. Thus, Smad4 is the key-regulator of Smad-dependent signaling through TGF-β family members. Due to the multifunctional nature of TGF-β family members in a wide variety of organs and tissues, we were interested to delete Smad-dependent signaling through TGF-β family members in hematopoietic tissues. Previous reports, were TGF-β signaling was abrogated by targeted disruption either of the TGF-β1 ligand or the receptors I and II strongly underlined the importance of TGF-β signaling for immune functions and inflammation [7,9,138]. Nevertheless, differences within the phenotypes of these knockout models exist. For example, the targeted disruption of TGF-β1 leads to increased white blood cell (WBC) counts due to an elevated number of lymphocytes and neutrophils. The conditional deletion of the type II TGF-β receptor (TBRII) in bone marrow also leads to an autoimmune-mediated inflammatory phenotype as it was detected in TGF-β1 knockout mice. In contrast to TGF-β1 null mice, TBRII-/- mice have no increase of peripheral WBCs or other blood lineages. Whether these differences are due to different genetic backgrounds or originate from a possible different receptor-usage of TGF-β1 is difficult to answer. We therefore asked the question, whether the induced disruption of Smad-mediated signaling through TGF-β family members in hematopoietic tissues results in an inflammatory autoimmune phenotype. As the deletion of TGF-β signaling components leads to a transplantable inflammatory phenotype, we expected that the conditional deletion of Smad4 would result in a more severe and rapidly developing wasting syndrome. Furthermore, several reports demonstrated reduced levels of either Smad4 or TBRII in hematopoietic cells from patients with MPD [3-5]. These reports underlined the hypothesis that abolished TGF-β signaling might be involved in the molecular pathogenesis of MPD. Therefore, a mouse model for conditional deletion of

Smad4 in bone marrow might reveal the consequences of abolished TGF-β signaling in MPD.

Unexpectedly, the induction of Smad4 deficiency in Smad4lox/lox-Mx1Cre mice leads to a severe anemia, detectable as early as 3 weeks after the last pIpC injection. At 4-5 weeks post-injection mice were killed due to the severity of the phenotype and analyzed in detail. Anemia in these mice was progressed to a stage, where hemoglobin levels in some mice was reduced to 10% of normal control mice. As the LDH levels of these mice were increased 2-3fold, possibly dependent on increased erythrocyte destruction due to haemolytic anemia, we speculated that anemia in Smad4lox/lox-Mx1Cre mice is autoimmune-mediated. However, erythrocytes from Smad4lox/lox-Mx1Cre mice were negatively tested for the direct antiglobulin test (DAT), which detects surface-binding of IgG and IgM on erythrocytes. As autoimmune-mediated haemolytic anemia is characterized by hemolysis associated with the presence of the immunoglobulins IgG, IgM and components of the complement system on the red cell membrane, the observed anemia in Smad4lox/lox-Mx1Cre mice is not autoimmune-mediated. Further analysis of the phenotype from Smad4 deleted mice revealed extramedullary hematopoiesis with foci of erythropoiesis in the liver of Smad4lox/lox-Mx1Cre mice. Additionally, Smad4lox/lox-Mx1Cre mice showed splenomegaly due to a massive expansion of immature erythroblasts in the spleen. Expression of the transcription factor GATA-2 was found decreased in TER119-positive cells from Smad4 deleted mice. As GATA-2 was shown to have fundamental functions in erythroid maturation from hematopoietic progenitor cells, the observed diminished mRNA levels of GATA-2 might explain the maturation block of the erythroid lineage at the erythroblast stage [139].

To inquire whether this phenotype is due to an intrinsic cell defect in erythroid progenitors and thus cell autonomous, we transplanted bone marrow from Smad4lox/lox-Mx1Cre mice into lethally irradiated C57BL/6 recipients. After bone marrow engraftment reached 80%, Smad4 mutagenesis was induced by pIpC injection. Surprisingly, none of the Smad4lox/lox-Mx1Cre bone marrow recipients developed an anemia within the 10 weeks after the last injection. This result implies that hematopoietic deficiency in Smad4lox/lox-Mx1Cre mice is not BM cell autonomous and is rescued by a recipient derived factor. As conditional target gene deletion via Mx1Cre was shown to efficiently work in other organs than the bone marrow, such as spleen, kidney and liver [10], the anemic phenotype in Smad4lox/lox-Mx1Cre mice might result from the deregulation of a Smad activated factor produced in one of the mentioned organs. To test this hypothesis, transplantation models where bone marrow

from healthy donors is transferred into Smad4lox/lox-Mx1Cre mice have to be set up (the results were not available at the time of writing this manuscript).

Another important observation was the fact that transplantation of bone marrow from Smad4lox/lox-Mx1Cre mice did not cause the inflammatory phenotype observed in the transplantation model of TBRII deleted bone marrow. At 10 weeks after the induction of Smad4 deletion in the recipient mice, no phenotypic alteration in these mice was detectable in comparison to control transplanted mice. This result demonstrates that the phenotype observed in TBRII-deleted mice is different from mice with a conditional deletion of Smad4. Hence, the effect of TGF-β signaling on immune function and inflammation is not Smad mediated. Even though Smads are the major mediators of TGF-β family signaling, signaling of TGF-β family members also activates other pathways. Among these pathways, signaling through the MAPK pathway might be a potential candidate for the observed inflammatory phenotype in TBRII and TBRI deleted mice. TGF-β can activate extracellular signal-regulated kinase (ERK), c-jun $NH_2$-terminal activated kinase (JNK) and p38 MAPK pathway in a Smad-independent manner [90,91]. As these kinases are also involved in the regulation of tumor necrosis factor alpha (TNF-α) transcription, which is a crucial cytokine in the establishment of inflammation and in multiple autoimmune diseases, TGF-β-deletion might deregulates the activation of these kinases and thereby TNF-α transcription. The observation that TNF-α levels were elevated in TGF-β1 deleted mice is in line with the above-discussed Smad-independent pathway of TGF-β1 signaling.

Another possible Smad-independent pathway potentially involved in the inflammatory phenotype seen in TGF-β1 knockout mice and conditionally TBRII and TBRI deleted mice is the nuclear factor kappa B (NF-κB) signaling. TGF-β1 and BMP4 were shown to activate the TGF-β-activated kinase 1 (TAK1), which can phosphorylate and activate IκB kinase, thus stimulating NF-κB signaling [142]. As NF-κB is a major mediator of immune responses of innate immunity, the potential deregulation of NF-κB in TGF-β signaling deficient mice is possibly involved in the observed phenotype.

Finally, induced disruption of Smad4 in bone marrow does not lead to a MPD-like phenotype in Smad4lox/lox-Mx1Cre mice. Platelet numbers in Smad4 deleted mice remain unchanged. Therefore, abolished TGF-β signaling might not be the primary reason for clonal expansion of a malignant stem cell clone, characteristic for MPD.

## *Gene silencing by lentivirus-mediated delivery of siRNA in human CD34$^+$ cells*

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Running title: lentiviral gene silencing in CD34$^+$ cells

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## *Abstract*

To derive an efficient system for gene silencing in human hematopoietic stem cells we modified a lentiviral vector for small interfering RNA (siRNA) delivery. For this purpose, an H1-promotor driven siRNA expression cassette was introduced into a lentiviral vector and the p53 mRNA was chosen as a target for siRNA mediated gene silencing. Using the recombinant lentivirus we infected human cord blood derived $CD34^+$ cells and obtained a transfection efficiency of up to 50%, as determined by expression of enhanced green fluorescent protein (EGFP). In EGFP positive LTC-IC and CFU-C derived cells we observed a reduction of p53 mRNA of up to 95%. Importantly, this reduction remained stable during several weeks of cell culture. Furthermore, p53 gene silencing resulted in decreased p21 mRNA levels and reduced the sensitivity of $CD34^+$ cells towards the cytotoxic drug etoposide. Thus, lentiviral delivery of siRNA can allow for efficient and stable gene silencing in human HSC and will be very valuable for further gene function studies.

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## *Introduction*

Gene silencing by siRNA has become a powerful and rapidly evolving experimental method for studying gene function in mammalian cells. [143, 144] The use of siRNA in hematopoietic stem cells (HSC) is limited by the difficulty of delivering RNA or DNA into HSC by conventional transfection methods. Lentiviral vectors have been shown to efficiently transduce human HSC.[145,146,147,148] Lentiviruses are able to infect non-dividing primary cells and transcription from integrated viruses remains stable over time.[149,150] Lentiviral vectors have been used to deliver siRNA in some primary mouse and human tissues. [151,152] Here we show that efficient gene silencing can be achieved in human HSC by a lentiviral system designed for delivering siRNA.

## *Materials and Methods*

### Constructs

To allow efficient transfer of the H1 promoter/siRNA cassette, we introduced a second ClaI site into pSUPER [153] by ligating the adaptor 5'-AATTATCGATGTTGTAAAAC-3' and 5'-AATTGTTTTACAACATCGAT-3' into the unique EcoRI site. The template for human p53 siRNA was generated by ligating the annealed primers 5'-GATCCCCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTGGAAC-3' and 5'-TCGAGTTCCAAAAAGACTCCAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGTCGGG-3' into the BglII and HindIII sites of pSUPER. As a non-relevant control we used the primers 5'-GATCCCCCTGGCATCGGTGTGGATGATTCAAGAGATCATCCACACCGATGCCAGTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAACTGGCATCGGTGTGGATGATCTCTTGAATCATCCACACCGATGCCAGGGG-3', which were derived from the mouse SMAD4, but were non-functional, i.e. did not change SMAD4 mRNA levels, in mouse and human cells (data not shown). The expression cassette for p53 siRNA was excised from the modified pSUPER as a ClaI fragment and subcloned into the ClaI site of the lentiviral vector pWPXL. All constructs were verified by sequence analysis. Lentiviral production with pWPXL, the envelope vector pMD.G and the

packaging vector pCMVR8.91 (all kindly provided by Dr. Didier Trono, University of Geneva) were carried out as described before.[154,155] [148]

**Cell culture and virus infection**

293T cells were seeded in 6-well plates ($3x10^5$ cells per well) and after 24 hours incubated with concentrated virus for 6 hours. The medium was changed and the cells were grown for the indicated times. Human cord blood $CD34^+$ cells were purified as previously described. [148] $CD34^+$ cells were seeded in a 96-well plate at $1x10^5$ cells per well and virus was added twice for 6 hours with a multiplicity of infection (MOI) of 10-30. EGFP positive cells were isolated using a FACSVantage cell sorter (Becton Dickinson Biosciences, San Diego, CA) and cultured in methylcellulose as described before [Wodnar, 1992 #2510] or maintained in liquid culture in IMDM and 10% fetal calf serum supplemented with human recombinant Flt-3 ligand (Amgen Inc., Seattle, WA) and PEGylated megakaryocyte growth and development factor (MGDF; Amgen, Thousand Oaks, CA). Growth factors were used at concentrations of 50 ng/ml for MGDF and 100 ng/ml for Flt-3 ligand. Etoposide (Sigma-Aldrich, St Louis, MI) was used at 25 nM in methylcellulose cultures and at 20 $\mu$M for induction of apoptosis. LTC-IC cultures were performed as described. [Wodnar, 1992 #2510]

**Western blot analysis**

p53 protein was detected by immunoblot using the anti-p53 rabbit polyclonal antibody FL 393 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were reprobed using the monoclonal anti-β-actin antibody AC-15 (Sigma-Aldrich, St Louis, MI). Densitometry was performed on a ChemiImager 5500 (Alpha Inotech, San Leandro, CA) using the Alpha Ease Software.

**Real-time PCR**

Total RNA (2$\mu$g) from EGFP-positive $CD34^+$ cells at day 7 and 20 of liquid culture was isolated with Trizol (Invitrogen, Carlsbad, CA), and reverse transcribed after random hexamer priming. RT-PCR was carried out using the SYBR Green PCR Master Mix chemistry (Applied Biosystems, Warrington, UK). Primers for p53 were 5'-TTCACCCTTCAGATCCGTGG-3' and 5'-CAGCTCTCGGAACATCTCGAA-3', for p21 5'-GGCAGACCAGCATGACAGATT-3' and 5'-AGAAGATCAGCCGGCGTTT-3'. The

conditions for RT-PCR of the ribosomal protein L19 (RPL19) were described elsewhere.[156] All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

**Quantification of apoptosis by flow cytometry**

Apoptosis was assessed using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Diego, CA).

## *Results and discussion*

Since lentiviruses can efficiently transduce human hematopoietic cells,[155] we tested whether the lentiviral vector pWPXL can be used for siRNA mediated gene silencing in human HSC. To allow for a convenient transfer of a cassette comprising the H1 promoter and the siRNA template into pWPXL, we modified the polylinker of the vector pSUPER by introducing a second ClaI site (Fig. 1A). As a target gene we chose the p53 mRNA, because the conditions for efficient p53 gene silencing have already been established in other cell types.[153] The resulting lentiviral vector pWPXL-p53si was packaged and first tested on 293T cells, a human embryonal kidney derived cell line. p53 protein expression was clearly reduced in cells infected with pWPXL-p53si, as compared to parental 293T cells (Fig. 1B). Densitometric analysis at 72 hours post infection revealed that p53 protein was reduced by pWPXL-p53si to 17% of the control. Since 293T cells express high levels of p53 protein due to the presence of SV40 large T antigen, which stabilizes and inactivates p53, the observed reduction of protein levels by pWPXL-p53si should be considered as very effective.

We next examined whether pWPXL-p53si can inhibit p53 expression in human cord blood derived $CD34^+$ cells. An advantage of pWPXL is that infected cells can be detected by the presence of EGFP. Using concentrated virus, up to 54% of $CD34^+$ cells were EGFP positive, as determined by flow cytometry (Fig. 1C). Silencing of p53 gene expression was assessed in infected cells sorted for EGFP expression. Since p53 protein in $CD34^+$ cells was undetectable by Western analysis (data not shown), we used quantitative real-time PCR to assess the expression of p53 mRNA. In cells sorted for EGFP and CD34, we found that p53 mRNA levels were reduced to 3% (Fig. 2A), whereas p53 mRNA levels in EGFP-negative cell

fraction remained unchanged (not shown). After 5 weeks in liquid culture, over 90% of cells remained EGFP positive and 2-4% were CD34+/EGFP+ (not shown). In sorted CD34+/EGFP+ cells p53 expression was 11% of the controls (Fig. 2A). These results demonstrate persistent gene silencing over time.

To determine whether CFU-C progenitors can be targeted, we plated sorted CD34+/EGFP+ cells in methyl cellulose. By real time PCR, we observed an 8 to 10-fold decrease in p53 mRNA in single colonies derived from p53si transduced progenitors (Fig. 2B). Cells that have lost p53 function are less sensitive to agents that normally cause apoptosis.[157,158] A reduction, but not complete resistance to apoptosis can be expected in cells transduced with p53 siRNA, as cells from p53 knockout mice and human p53-deficient cell lines remained partially sensitive to cytotoxic drugs, such as etoposide, indicating that apoptosis can still be induced through p53-independent pathways.[159-161] To provide evidence that gene silencing by pWPXL-p53si interfered with p53 function, we measured apoptosis in response to etoposide. $CD34^+$ cells infected with p53si, control-si and empty vector were EGFP-sorted, plated in semi-solid media in the presence or absence of 25 nM etoposide and colonies were counted after 14 days. In the presence of etoposide, $CD34^+$ cells infected with the empty vector yielded 6% and with control-si vector yielded only 3% of colonies that were observed in the absence of etoposide. In contrast, pWPXL-p53si infected $CD34^+$ cells were more resistant to etoposide, as 36% of colonies survived in the presence of etoposide (Fig. 2C). To demonstrate that this difference in survival is due to apoptosis, we exposed $CD34^+$ cells in liquid culture to etoposide and assessed apoptosis by cell surface expression of annexin V. $CD34^+$ cells infected with the pWPXL-p53si virus displayed reduced sensitivity to etoposide-inducted apoptosis than the controls: the percentage of annexin-positive cells in pWPXL-p53si-infected cells increased by only 17%, as compared to an increase of 31% in the controls (Fig. 2D). To further verify that the observed effects result from p53 gene silencing, we analyzed the expression levels of p21 mRNA, a p53 transcriptional target, in the EGFP-positive fraction of the virus infected cells by real-time PCR. We found that in p53 silenced cells the levels of p21 mRNA were reduced to 16% of the controls (Fig. 2E). Thus, p53 gene silencing by siRNA in human $CD34^+$ cells resulted in the expected reduction of p53 function.

To demonstrate that not only CFU-C, but also earlier hematopoietic progenitors can be transduced by our vectors, we analyzed p53 expression in sorted CD34+/EGFP+ cells grown for 5 weeks on feeder cells under LTC-IC conditions and in single methyl cellulose colonies

derived from LTC-ICs. After 5 weeks of culture, over 90% of cells remained EGFP positive and 0.3% were CD34+/EGFP+ (not shown). We sorted these CD34+/EGFP+ cells and found p53 mRNA expression to be reduced to 9% in pWPXL-p53si transduced cells (Fig. 2F). Furthermore, single methylcellulose colonies derived from LTC-ICs displayed an up to 16-fold reduction in p53 mRNA (Fig. 2G). These results show that early hematopoietic progenitors of the LTC-IC type were transduced and that siRNA expression was persistent in LTC-IC-derived cells.

In summary, we demonstrate that lentiviral delivery of siRNA can be used for efficient and stable gene silencing in human hematopoietic progenitors. This system will be very valuable to study the function of key regulatory genes in human hematopoiesis.

**Acknowledgments**

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**Figure legends**

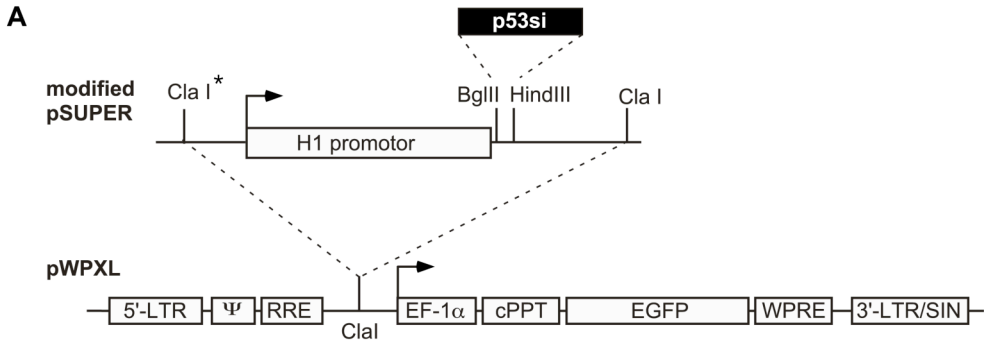
Figure 1. Construction of a lentiviral vector for the expression of siRNA

(A) The template DNA for p53 siRNA (p53si) was directionally inserted into the BglII and HindIII sites of pSUPER. A second ClaI restriction site (asterisk) was created by adaptor ligation. The complete p53si expression cassette was excised from the modified pSUPER as a ClaI fragment and inserted into the lentiviral vector pWPXL (dotted line). LTR, long terminal repeat; ψ psi packaging signal; RRE, Ref-responsive element; cPPT, central polypurine tract; EF1-alpha, human elongation factor alpha; EGFP, enhanced green fluorescent protein; WPRE, post-transcriptional cis-acting regulatory element of the woodchuck hepatitis virus; LTR/SIN, self-inactivating 3' long terminal repeat. (B) Western analysis of p53 protein

expression in 293T cells. Control, non-transduced 293T cells; p53si, 293T cells transduced with pWPXL-p53si. (C) Assessment of lentiviral infection efficiency. $CD34^+$ cells infected with pWPXL (empty vector), pWPXL-control-si (control si) or pWPXL-p53si (p53si) were analyzed for EGFP expression by flow cytometry. Cutoff used for cell sorting of EGFP-positive cells is shown. FSC, forward side scatter

Figure 2. Gene silencing by lentiviral delivery of p53 siRNA

(A) Reduction of p53 mRNA expression by siRNA. $CD34^+$ cells were infected, sorted for EGFP positive cells. Expression of p53 mRNA was assessed by quantitative RT-PCR at the day of sorting (left panel) and after 5 weeks of liquid culture (right panel). The value for the control cells infected with pWPXL virus (black bars) was set as 100% and compared to cells infected with the pWPXL-control-si virus (gray bars) and the pWPXL-p53si virus (open bars). Error bars indicate the SEM when triplicate measurements were done. (B) Sorted CD34+/GFP+ cells were cultured in methylcellulose and single colonies were picked after 14 days for detection of p53 mRNA. Note that ΔCT represents a binary logarithmic scale and higher numbers correspond to lower levels of p53 expression. Each dot represents the result obtained from one single colony. (C) Effects of etoposide on CFU-C formation. Sorted CD34+/GFP+ cells were grown in methylcellulose in the presence (+) or absence (-) of etoposide (25 nM). Error bars indicate SEM of triplicate cultures. (D) Measurement of etoposide-induced apoptosis by flow cytometry. $CD34^+$ cells infected with pWPXL-p53si or the empty vector were sorted for EGFP expression by FACS and cultured for 4 days in liquid culture. After exposure to 20 $\mu$M etoposide (Eto) for 6 hours apoptosis was measured by staining with PE-coupled annexin V. (E) p53 target gene expression in silenced versus control cells. p21 mRNA levels were determined by real-time PCR in sorted EGFP positive cells after 10 days in liquid culture. Annotation as in 2A. (F) Silencing of p53 expression in sorted CD34+/GFP+ cells after 5 weeks of LTC-IC stroma-cell culture. Annotation as in 2A. (G) Silencing of p53 expression in LTC-IC derived colonies. Annotation as in 2B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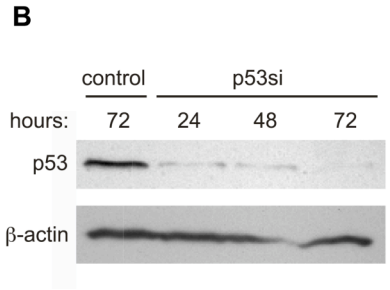
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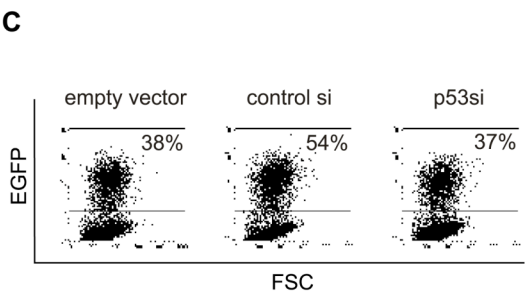
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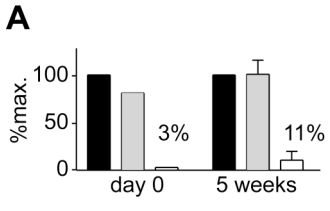
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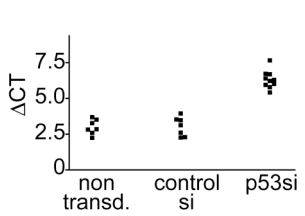
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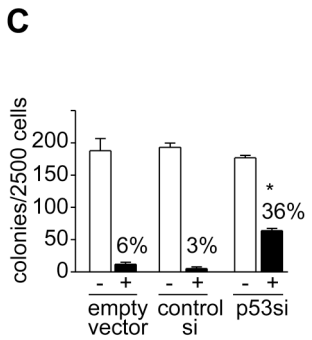
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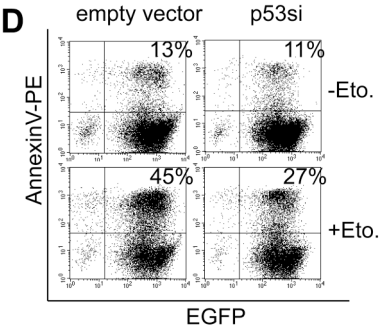
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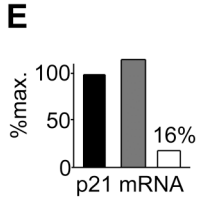
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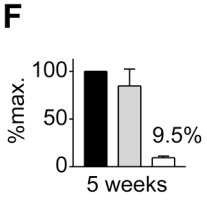
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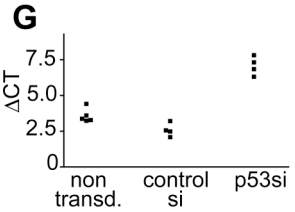
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## *General Discussion*

Functional studies of genes involved in the development and maintenance of specific organs and tissues are very often hampered by the fact that their germ-line deletion in mouse models results in embryonic lethality. To gain further insight into the function of those "lethal" genes in specific organs and tissues, conditional gene targeting approaches have been established using the Cre/lox system [10]. This approach requires two mouse strains, one with the target gene of interest modified to contain the recognition sites for the Cre recombinase, so called loxP-sites and a second mouse strain, which expresses the Cre recombinase in a tissue-specific manner. Crossing such two strains will result in a transgenic mouse strain, where the loxP-flanked gene of interest is deleted by the tissue-specific action of the Cre recombinase. Many mouse strains with loxP-flanked target genes exist today, which would make it easy to study the function of these genes in detail in the desired tissue or organ. The bottleneck for such *in vivo* gene function studies is the availability of transgenic mouse strains for tissue-specific Cre recombinase expression. In the first part of my thesis I described the generation of a transgenic mouse strain, the platelet factor 4 Cre (PF4Cre) mouse. The PF4Cre mouse was generated to express the Cre recombinase specifically in megakaryocytes and platelets. The transgenic PF4Cre mouse strain can be used for tissue-restricted deletion of target genes, which are potentially involved in murine megakaryopoieis, *in vivo*.

### The PF4Cre mouse is a new tool to study megakaryopoieis *in vivo*

The CXC-chemokine platelet factor 4 (PF4) was shown to have a very restriction expression pattern starting from early megakaryopoieis up to the endproducts of megakaryopoieis, the platelets [121,122]. Several attempts to drive the expression of a transgene specifically into megakaryocytes and platelets have been established before [131,132]. In these reports, rat, mouse or human PF4-promotor constructs were used to drive transgene expression into the megakaryocytic lineage. While most of these constructs were sufficient to direct transgene expression into bone marrow megakaryocytes and platelets, expression in megakaryocytes from spleen was not detectable. Furthermore, due to the classical transgenesis protocol using short plasmid-based constructs, these constructs were hampered in their expression by position effects, including gene-silencing or mosaic expression. In our approach we tried to circumvent these problems by placing the improved Cre recombinase (iCre) sequence under the control of the *Pf4* gene embedded in a 110kb bacterial artificial chromosome (BAC). This

BAC is believed to carry all elements (i.e., enhancers, locus control regions and insulators) crucial to drive tissue-specific expression of the *Pf4* gene. Therefore, insertion of the Cre recombinase into exon 1 of the *Pf4* gene should result in a transgenic expression pattern, which is identical to the endogenous PF4 expression in megakaryocytes and platelets. The transgenic mouse strains obtained from pronucleus injections of the 100kb BAC insert into fertilized oocytes showed different Cre expression patterns, according to the number of transgene integrations detected in these mice. Crossing the $F_0$ founders from 5 different PF4Cre strains into the ROSA26lacZ reporter strain [133], I could demonstrate tissue-specific Cre expression in bone marrow and spleen megakaryocytes. The tissue-restricted expression pattern of the Cre recombinase clearly correlated with the previously evaluated copy number. The strain with 22 copies showed ectopic Cre expression in most of the analyzed organs, whereas mice with only 1 copy demonstrated lineage-restricted expression. In both PF4Cre strains with low numbers of transgene integrations Cre expression was only detected in megakaryocytes in the bone marrow and spleen. A third strain with 5 integrated BAC-transgenes revealed some ectopic Cre expression in spleen, thymus, bone marrow and, surprisingly, in the alveolae of the ovary. Nevertheless, this work showed for the first time a functional transgenic mouse model for megakaryocyte restricted Cre recombinase expression. In contrast to previous reports where the PF4 promotor was used to drive expression of a transgene into megakaryocytes and platelets [131,132], transgenic PF4Cre mice expressed Cre recombinase in bone marrow megakaryocytes and splenic megakaryocytes.

Analysis of the excision efficiency in megakaryocytes revealed that the excision was not complete in PF4Cre strains with 1 or two copies, respectively, while the strain with 5 transgene integrations showed almost complete excision of the target gene. Two aspects have to be considered in the evaluation of excision efficiency. First, it is a known phenomenon that recombination of the loxed target gene by Cre recombinase varies dependent on the targeted gene [162]. Therefore, the excision efficiency of particular PF4Cre strains has to be evaluated for every gene targeted. Second, I used the PF4 regulatory regions to drive tissue specfic transgene expression into the megakaryocytic lineage. Expression of the *PF4* gene was shown to start at early stages of megakaryopoiesis, where the first endomitotical duplications already occur [121,122]. Thus, with the increasing ploidy of the maturing megakaryocyte, more copies of the loxed target gene have to be excised. Therefore, excision at the earliest stage of megakaryopoiesis (2*N*) will most likely result in good efficiencies, while excision at later stages has to work against increasing ploidy. However, this work describes for the first time a functional transgenic mouse strain for the targeted disruption of loxP flanked genes

specifically in megakaryocytes from bone marrow and spleen. Therefore, the PF4Cre transgenic mouse strain will be a valuable tool to study gene function *in vivo* by the conditional gene deletion in megakaryocytes and platelets.

**Conditional deletion of the TGF-β receptor II in megakaryocytes does not lead to increased platelet counts**

Several reports established the inhibitory function of TGF-β on hematopoietic stem/progenitor cells [115-118,163]. These data were mostly derived from *in vitro* assays, where the influence of TGF-β addition to cultured cells was analyzed. In methylcellulose cultures of hematopoietic progenitors from either human or murine origin, the addition of TGF-β1 was shown to inhibit colony formation from early progenitors, while late progenitors seemed to be unaffected from TGF-β1 addition [115-117].

Studies of the *in vivo* function of TGF-β on hematopoiesis further proofed these above-mentioned *in vitro* data. For example, in a study designed to evaluate the function of TGF-β on hematopoietic stem cell proliferation *in vivo*, mice were either injected with 5-fluorouracil (5-FU) or TGF-β1 and 5-FU. The chemotherapeutic drug 5-FU selectively kills the cycling cells, while non-cycling stem cells survive the treatment. The simultaneous injection of 5-FU and TGF-β1 into mice led to a delay of hematologic recovery as compared to mice only treated with 5-FU [164]. These data clearly implied the inhibitory effect of TGF-β1 on stem cell proliferation in murine hematopoiesis. Furthermore, the targeted disruption of TGF-β1 in mouse showed defective hematopoiesis resulting in a reduced number of erythroid cells and increased numbers of circulating platelets in TGF-β1-null mice [9].

Evidence for the inhibitory action of TGF-β on human hematopoiesis came from the observation that in some patients with either early myeloid or lymphocytic leukemias decreased expression of TGF-β receptors was detected [5]. Reduced levels for the TGF-β receptor II mRNA or protein have been reported in several patients with myeloproliferative disorders (MPDs), as well as decreased mRNA levels for the mediator of TGF-β signaling, Smad4, in patients with essential thrombocythemia (ET). Together, these data suggested that unblocking of the inhibitory effect of TGF-β leads to a hyperproliferation of the affected cell compartment in hematopoiesis. In my thesis I set up different mouse models to gain insight into the function of TGF-β on hematopoiesis and megakaryopoiesis. In a first approach, I used the above-described PF4Cre mouse for the megakaryocyte-restricted deletion of the type II TGF-β receptor and Smad4. As TGF-β1 directly binds to TBRII, conditional disruption of

TBRII completely abolishes functional signaling through TGF-β1. The deletion of Smad4 in megakaryopoiesis leads to the disruption of any Smad-mediated signal transduction coming from TGF-β family members. Surprisingly, both mouse models for conditional deletion of TGF-β signaling components in megakaryocytes revealed no differences on peripheral platelet counts compared to control mice. These results strongly suggest that the loss of functional TGF-β signaling in murine megakaryopoiesis does not lead to the expected hyperproliferation of the megakaryocytic lineage and thereby to elevated platelet numbers. As the PF4Cre mouse strains with low numbers of transgene integrations were shown to mediate incomplete excision of the TBRII in megakaryocytes, one could argue that the lack of phenotype is due to incomplete TBRII or Smad4 disruption. However, the PF4Cre strain with 5 transgene integrations showed the same results regarding the platelet numbers. Surprisingly, homozygous TBRIIlox/lox-PF4Cre mice from this strain showed an increasing leukocytosis over time with a doubling of white blood cells at the age of 17 weeks. The increase of white blood cells was mainly due to an elevation of granulocytes and lymphocytes. Clinically, these mice showed progressive weight loss, immobility and corneal opacity when analyzed with 17 weeks after birth. Symptomatically, this phenotype was reminiscent on the induced deletion of the TBRII in bone marrow using the Mx1Cre mouse model [7]. In this model, inducible deletion of TBRII in bone marrow leads to an autoimmune-mediated inflammatory phenotype characterized by organ infiltrations of T-/B-cells and granulocytes. In contrast to the above-described elevation of white blood cell numbers in TBRIIlox/lox-PF4Cre mice from the 5 copy strain, TBRIIlox/lox-Mx1Cre mice revealed normal blood counts from all peripheral blood lineages. Using the TBRIIlox/lox-Mx1Cre mice to prove the results obtained from the TBRIIlox/lox-PF4Cre mice additionally demonstrated that TGF-β signaling is dispensable for functional megakaryopoiesis. All of the 3 described mouse models (TBRIIlox/lox-PF4Cre/-Mx1Cre and Smad4lox/lox-PF4Cre) argue against a functional implication of the TGF-β signaling pathway in normal megakaryopoiesis. Furthermore, the possibility that TGF-β signaling in TBRIIlox/lox-PF4Cre mice still occurred with a different receptor-usage and thereby compensating the loss of TBRII, was ruled out by the megakaryocyte specific disruption of Smad4 in Smad4lox/lox-PF4Cre mice.

In summary, the above-discussed results strongly argue against a fundamental function of TGF-β in murine megakaryopoiesis and implicate that the observed reductions of TBRII and Smad4 in patients with MPD are not the cause for the onset of the disease. These results are further strengthened by a screening for alterations of mRNA levels from TGF-β signaling components in patients with MPD (Fig.1).

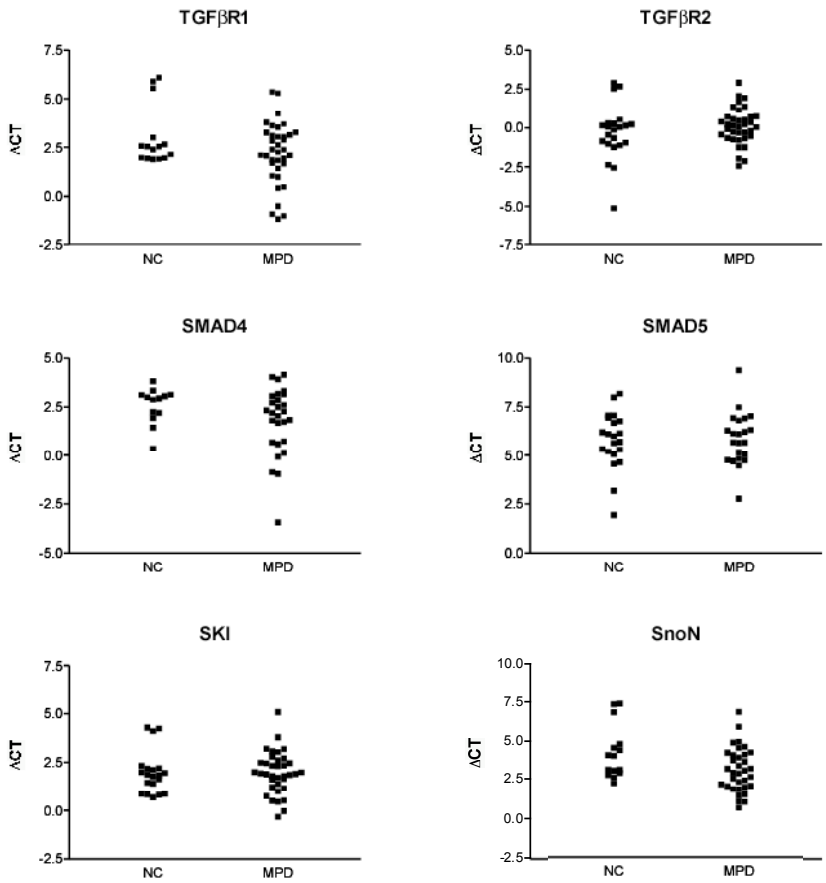


**Figure 1.** Analysis of mRNA levels from TGF-β signaling components in MPD patients. Purified granulocytes from either patients or healthy controls served as source for mRNA. **NC** normal controls, **MPD** patients with myeloproliferative disorders. **ΔCT** subtracted threshold cycles (CT) for the individual analyzed mRNAs from the threshold cycle values for the internal control, the ribosomal protein L19 (RPL19).

In contrast to previous studies showing decreased levels of either TBRII or Smad4 in patients with MPD, we could not confirm these data in the patient cohort used for this screening (Fig.1). Even more surprising was the fact that some patients had elevated mRNA levels of the type I TGF-β receptor or Smad4. Whether these increased levels are involved in the pathogenesis of these patients has to be further studied. Nevertheless, the co-repressor proteins SnoN and Ski, which were shown to block TGF-β signaling by competing with R-Smads for receptor binding or direct binding to R-Smads, showed elevated expression levels in some of the analyzed patients. These increased levels of inhibitory components of TGF-β signaling might render the affected cells unresponsive to TGF-β. This way, cells with elevated SnoN or Ski expression could escape negative regulation of growth by TGF-β and overgrow normal hematopoiesis. However, the above-discussed transgenic mouse models for the disruption of TGF-β signaling components either in hematopoietic stem/progenitor cells or in megakaryocytes strongly argue against such a scenario. In this context, it would be interesting

to evaluate whether the exogenous application of active TGF-β1 into TBRIIlox/lox-PF4Cre mice leads to the expansion of the TBRII defective megakaryocytic lineage. A recent report showed that in a patient cohort with essential thrombocythemia not only Smad4 mRNA levels were decreased, but also serum levels of active and latent TGF-β were significantly increased [3]. This observation is consistent with the notion that tumor cells are not only insensitive to TGF-β mediated growth inhibition, but also produce themselves TGF-β probably to inhibit growth of neighboring cells.

Somewhat unexpectedly, even the targeted deletion of Smad4 in megakaryopoiesis via the PF4Cre mouse or using the Mx1Cre mouse for stem cell targeting did not induce thrombocytosis as it is seen in patients with MPD. As Smad4 funnels signaling from all TGF-β family members, the possibility that TGF-β signaling can be compensated through signaling from other TGF-β family members is highly unlikely.

It seems to be more likely that a malignant stem cell clone in MPD additionally acquires unresponsiveness to growth inhibition by TGF-β signaling through a yet unknown mechanism. Such a mechanism would most likely involve transcriptional downregulation of TGF-β signaling components, which then provides the MPD clone with further growth advantage. The fact that no genetic or epigenetic alterations of TGF-β family genes were reported so far in patients with MPD, makes it also improbable that loss of TGF-β signaling is the primary reason for myeloproliferative disorders.

**Induced deletion of Smad4 in Smad4lox/lox-Mx1Cre mice leads to a rapidly developing haemolytic anemia**

Signaling of TGF-β family members is mainly mediated through the Smad-family of signal transducers. Ligand binding to the type II receptor leads to the formation of a receptor-duplex of type II and type I TGF-β receptors. The constitutively active type II receptor kinase phosphorylates the type I receptor, which then activates members of the R-Smads via phosphorylation. The activated R-Smads form hetero- or homoduplexes with other activated R-Smads and bind the Co-Smad, Smad4, before they translocate to the nucleus. Here, Smad complexes activate or repress transcription of several hundreds of target genes in cooperation with other transcription factors. Most of the previously discussed inhibitory functions of TGF-β signaling on hematopoietic cells are probably mediated through activated Smad-complexes. Blocking of TGF-β signaling by repression of Smad3 activity has been reported in chronic

myeloid leukemia. In this case, dysregulation was not due to mutations in the *Smad3* gene, but was correlated with an abnormal expression of Evi-1, a zinc-finger oncoprotein that interacts with Smad3 and suppresses its transcriptional activity [114]. The potential involvement of other Smad-family members in hematopoietic hyperproliferative diseases needs further investigations.

The germline deletion of Smad4 in mouse results in embryonic lethallity at day E 8.5. Therefore, the study of Smad4 function in hematopoiesis is difficult. In order to investigate the functions of Smad4 in hematopoiesis, we intended to delete Smad4 in hematopoietic tissues. The transgenic Mx1Cre mouse strain was mated with Smad4lox/lox mice and Smad4 deletion was induced in Smad4lox/lox-Mx1Cre mice. Inducible Cre/loxP-mediated disruption of the *Smad4* gene in bone marrow of homozygous Smad4lox/lox-Mx1Cre mice was analyzed for phenotypic alterations of peripheral blood lineages. The expectation was that the induced deletion of Smad4 in bone marrow shows a comparable phenotype as it was observed in TBRIIlox/lox-Mx1Cre mice, which died around 10 weeks after the induced deletion of TGF-β type II receptor due to multifocal autoimmune-mediated inflammations [7]. Unexpectedly, peripheral blood counts from Smad4lox/lox-Mx1Cre mice 3 weeks after the last polyinosinic/polycytidylic acid injection revealed decreased values for hemoglobin, hematocrit and red blood cell numbers. With 4-5 weeks post injection, Smad4lox/lox-Mx1Cre mice displayed severe hemolytic anemia based on the above-mentioned blood parameters. A simultaneous increase in the numbers of reticulocytes as well as in the serum levels of the lactate dehydrogenase further underlined the clinical phenotype of hemolytic anemia. Additional characterization of Smad4lox/lox-Mx1Cre mice revealed splenomegaly due to extramedullary hematopoiesis. Histological examination of organ sections from Smad4 deleted mice showed spots of extramedullary hematopoiesis in the liver and a massive expansion of immature myeloid cells in the spleens of Smad4lox/lox-Mx1Cre mice. The immature myeloid cells in the spleens were demonstrated to originate from the erythroid lineage and additional flow cytometric analysis revealed that these cells were mostly immature erythroblasts. Characterization of the haemolytic anemia in Smad4lox/lox-Mx1Cre mice revealed no surface IgG or IgM coating on red cells, making it unlikely that the haemolytic anemia is autoimmune mediated. Comparison to the haemolytic anemia in the *New Zealand Black* (*NZB)* mouse model, definitively ruled out the possibility that induced Smad4lox/lox-Mx1Cre mice develop an autoimmune mediated haemolytic anemia. This result was surprising, as the previously mentioned TBRIIlox/lox-Mx1Cre mouse showed an autoimmune-mediated inflammatory phenotype and therefore the establishment of an

autoimmune-mediated phenotype in Smad4lox/lox-mx1Cre mice was highly likely. Because autoimmunity was ruled out as the reason for haemolytic anemia in Smad4 deleted mice, the possibility of an intrinsic defect of the erythroid cells was evaluated. The fact that erythropoiesis in Smad4lox/lox-Mx1Cre mice was obviously blocked at the erythroblast stage, implied that the cause for the anemia might be a erythroid maturation defect due to decreased expression of a down stream target gene of Smad-mediated transcription. The GATA-family of transcription factors is known to exert fundamental functions in the differentiation of the erythro-/megakaryocytic lineage. While GATA-1 is believed to exert its function in the earliest stages of hematopoiesis, GATA-2 might be more implicated in the expansion of early progenitors [139,165]. However, the germline deletion of both GATA-1 or GATA-2 resulted in early lethality due to defective embryonic erythropoiesis. Both of these zinc-finger transcription factors are believed to exert overlapping functions in the erythro-/megakaryocytic differentiation [140]. Analysis of GATA-1, GATA-2, GATA-3 and Friend of GATA 1 (FOG-1) mRNA expression by quantitative real-time PCR in bone marrow or purified TER119-positive cells from Smad4lox/lox-Mx1Cre mice revealed decreased levels of GATA-2 in both cell types. The decrease was more pronounced in purified $TER119^+$ cells from bone marrow as well as from the spleens from Smad4 disrupted mice, than in full bone marrow. Two observations further underlined the possibility that the decreased GATA-2 levels might be the cause for the anemia observed in injected Smad4lox/lox-Mx1Cre mice. First, a recently published report demonstrates decreased mRNA levels of GATA-2 in patients with aplastic anemia [166]. Second, experiments using either yolk sac or ES cell derived high-proliferative potential colony-forming cells (HPP-CFCs) from $Smad5^{-/-}$ embryos demonstrated that the disruption of Smad5 led to decreased GATA-2 mRNA levels in erythroid precursors [167] and that these erythroid precursors had a reduced proliferative potential. Together with the notion that Smad5 is able to transmit signals not only from the bone morphogenetic proteins (BMPs), but also from TGF-β1 and TGF-β2, this implicates that abolished GATA-2 function in defective erythropoiesis might originate from an insufficient Smad5 or Smad4 function. As Smad4 is the Co-Smad for all R-Smads, and therefore Smad5 needs Smad4 for effective transcriptional activation of target genes, one might speculate that the induced deletion of Smad5 in bone marrow results in a similar phenotype as described for the Smad4 deletion. A crucial experiment to validate the above-described results and hypothesis is the inducible deletion of Smad5 in a Smad5lox/lox-Mx1Cre mouse.

To restrict the Smad4 deletion exclusively to BM, we set up different bone marrow transplantation models. Smad4lox/lox-Mx1Cre bone marrow was transplanted into lethally

irradiated recipients and Smad4 was disrupted after chimerism reached 80%. Surprisingly, mice transplanted with Smad4lox/lox-Mx1Cre bone marrow appeared phenotypically normal 10 weeks after pIpC-induced Smad4 deletion. Peripheral blood counts from these mice showed no signs of anemia at week 10 post-injection. These results imply that the hematopoietic deficiency in Smad4lox/lox-Mx1Cre mice is not BM cell autonomous and can be compensated by host-derived factors. Besides that, the decreased levels of GATA-2 in $TER119^+$ cells from Smad4lox/lox-Mx1Cre mice are not the reason for anemia in these mice demonstrated by the non-cell autonomous phenotype.

As the expression of the Cre recombinase in Mx1Cre mice is also efficiently detectable in other organs than the bone marrow, such as liver, spleen and kidney, there is good evidence to postulate that the anemia in Smad4lox/lox-Mx1Cre mice results from the deregulation of a factor mainly produced in one of the above-mentioned organs. To test this hypothesis, normal bone marrow from healthy C57BL/6 mice was transplanted into lethally irradiated Smad4lox/lox-Mx1Cre mice and heterozygous controls. At the time of writing this manuscript, the results of this experiment were still pending and therefore cannot be discussed here.

However, looking for such a host-derived factor, which is deregulated upon the targeted disruption of Smad4 in one of the mentioned organs and therefore causes a severe haemolytic anemia in Smad4lox/lox-Mx1Cre mice, resulted in the hypothesis that the major erythroid cytokine, erythropoietin (Epo), might be involved in the establishment of the observed phenotype. Especially one recent report, showing a TGF-β mediated regulation of the *Epo* gene together with the hypoxia inducible factor 1 (HIF-1) (Zermati Y), further underlined a possible involvement of Epo in the anemia phenotype of Smad4lox/lox-Mx1Cre mice. The above-mentioned report demonstrated that a Smad-binding element (SBE) within the 3' Epo enhancer is bound from Smad3/4 via TGF-β activation. Furthermore, physical interaction of Smad3 with HIF-1 could be demonstrated and in vitro studies proved the agonistic action of Smad3/HIF-1 in the induction of Epo expression. Epo production mainly resides in the kidney and therefore Epo expression might be abolished in Smad4lox/lox-Mx1Cre mice due to Cre mediated deletion of Smad4 in the kidney. Normal Epo production in the kidneys of bone marrow transplanted recipient mice should rescue the anemic phenotype seen in Smad4lox/lox-Mx1Cre mice. Unfortunately, at this point I cannot answer this question, because the above-mentioned experiments are still in progress.

A second interesting feature of the transplantation model discussed above is the fact that even after 10 weeks post injection, Smad4lox/lox-Mx1Cre bone marrow transplanted mice did not show any phenotypic manifestations of the wasting syndrome detected in mice with induced disruption of the type II TGF-β receptor. As previously mentioned, mice with induced deletion of TBRII in bone marrow develop an autoimmune-mediated inflammatory phenotype, which is transplantable [7]. A similar phenotype can be found in mice with induced disruption of type I TGF-β receptor (TBRI) as well as in mice deleted for TGF-β1 ligand. All these mouse models generated a similar clinical picture: a multifocal inflammatory disease affecting a multitude of organs. These results clearly substantiated the importance of TGF-β signaling for immune functions and inflammation [7-9]. Induced deletion of Smad4 in Smad4lox/lox-Mx1Cre bone marrow transplanted mice does not show a similar phenotype as the above discussed mouse models for the disruption of TGF-β signaling in bone marrow. Hence, it is valid to postulate that TGF-β signaling in immune responses and inflammation it mediated through a Smad-independent pathway. Smad-independent TGF-β signaling has been described to function through the MAPK pathway [89,91], where direct activation of the extracellular signal-regulated kinase (ERK), c-jun $NH_2$-terminal activated kinase (JNK) and p38 MAPK was shown upon TGF-β signaling. All of these kinases are involved in the direct regulation of the inflammatory cytokine tumor necrosis factor alpha (TNF-α). Therefore, disruption of TGF-β signaling via the MAPK pathway might account for certain aspects in the TBRII, TBRI and TGF-β1 deletion phenotype. The observation of increased TNF-α levels in TGF-β1-null mice fits well into the above-described scenario.

A second Smad-independent pathway potentially involved in the autoimmune-mediated inflammatory phenotype observed in TGF-β signaling deficient mice, is the nuclear factor kappa B pathway (NF-κB). Both, TGF-β1 and BMP4 were shown to activate the TGF-β-activated kinase 1 (TAK1)[168], which can phosphorylate and activate IκB kinase and thereby stimulate NF-κB signaling. As NF-κB signaling is involved in mediating immune responses, a potential deregulation of NF-κB signaling through the induced disruption of the TGF-β pathway might explain some aspects of the autoimmune phenotype in TBRII or TGF-β1 deficient mice.

**Gene-silencing in human hematopoietic stem cells**

Mouse models for the targeted disruption of genes have revealed great insight into the function of many genes *in vivo*. Nevertheless, many knock out approaches in mice to study the function of a particular gene *in vivo* were hampered by the early lethality during embryogenesis due to the germline deletion of the gene. These problems were partially overcome by the introduction of conditional gene deletions in the tissue of interest using the Cre/loxP system. However, the possibility that the gene-deletion phenotype in mice does not reflect the situation in the human system is a major concern of gene targeting experiments in mice. Therefore, a system, which allows gene function studies *in vivo* in human primary cells, is highly desirable. Several studies demonstrating efficient gene-transfer into human hematopoietic stem cells using lentiviral vectors [155,169] opened the possibility to manipulate non-replicating, quiescent hematopoietic progenitors *in vitro* and *in vivo*. In the year 2000 pioneering studies from Tuschl and colleagues [170] demonstrated functional gene-silencing by RNA-interference (RNAi) in mammalian cells. In these studies it could be shown that short double-stranded RNAs, complementary to a specific RNA sequence, can target this RNA for degradation through the so-called RISC complex. First experiments using such double-stranded short RNA-oligomers revealed that the critical length of these functional RNA-oligomers in the vertebrate system should not exceed 30 base-pairs. Longer double-stranded RNAs efficiently induced an anti-viral response in the vertebrate system and therefore RNA-oligomers exceeding this critical length were not useful to silence a target gene by RNA destruction. Additional experiments aimed to design a stable system for the delivery of short interfering RNAs (siRNAs) [171] resulted in a vector-based system for the stable delivery of siRNA into cultured cell lines *in vitro*.

We intended to take advantage of these two systems to create a tool for siRNA delivery into human hematopoietic stem cells. Hence, I designed a lentiviral system for delivery of siRNA into cord blood derived $CD34^+$ cells. Combining the above-mentioned two systems led to the establishment of lentiviral vector harboring an H1-promotor driven expression cassette for short double-stranded hairpin RNAs complementary to the RNA sequence of interest. In a first set of experiments the p53 mRNA was chosen as a target for siRNA mediated gene silencing. Infection of the kidney derived cell line 293-T with a lentiviral construct harboring a p53 siRNA expression cassette, pWPXLp53si, demonstrated efficient p53 gene-silencing. 72 hours after the infection of 293-T cells with the pWPXLp53si lentivirus, p53 protein levels were reduced over 80%. Using the same lentiviral construct for the infection of cord blood

derived human $CD34^+$ hematopoietic stem cells not only revealed high infection efficiencies based on the expression of the reporter gene enhanced green fluorescent protein (EGFP), but also sustained gene-silencing of p53 over time. Measurement of p53 mRNA levels in $CD34^+$ cells infected with pWPXLp53si confirmed the previously obtained results from the infection of the 293-T cell line, where p53 mRNA levels were reduced to 3-10% of the control infected cells. Using long-term culture-initiating cell (LTC-IC) assays I was able to demonstrate that hematopoietic progenitor cells were efficiently silenced for p53 expression over a long period of time. Furthermore, functional studies of $CD34^+$ cells infected with the lentiviral vector for p53 silencing revealed that these cells are more resistant to apoptotic stimuli induced by the cytotoxic drug Etoposide than control-infected cells. These results clearly demonstrate that lentiviral mediated delivery of siRNA into cord blood derived $CD34^+$ is sufficient to induce functional gene-silencing in hematopoietic progenitor cells over time. Infected CD34+ cells were also differentiated into the different lineages of the blood and silencing persisted in fully differentiated cells. The above-discussed approach for functional gene-silencing in human hematopoietic stem/progenitor cells opens several potential applications. First, gene function in human hematopoietic stem cells can be studied *in vivo* by transplanting infected $CD34^+$ cells into immuno-compromised NOD/SCID mice. In this setting it will be possible to evaluate the function of several genes in hematopoiesis. A second application could be the study of transgenic animals, as the lentiviral system has a broad host range and therefore can be used in several animal models. It was recently demonstrated [172] that the infection of mouse oocytes with a lentiviral vector as described above is sufficient to produce transgenic offspring, which is silenced for the siRNA-targeted gene. As the silencing efficiency is dependent on the numbers of integrated proviruses per cell, an above-discussed model for oocyte-infection might result in transgenic animals with different degrees of gene-silencing, which is potentially more reflecting human malignancies due to attenuated gene expression. A third, potentially very ambitious application might be the future use of such a system in patients with hematopoietic diseases, such as leukemias or bone marrow failures. First studies using retroviruses for gene-therapy in children with severe combined immunodeficiencies (SCID) revealed that this application was sufficient to correct the gene-defect in these children. Unfortunately, some of the treated children developed leukemia due to virus-integrations close to the *LMO*2 gene. As the viruses in this clinical trial still possessed a functional viral promotor, the virus-integration close to the T-cell oncogene Lmo2 led to the constitutive expression of this gene and therefore to leukemic transformation of the affected cells. The above-described lentiviral vectors are modified to harbor so-called self-inactivating

long terminal repeats (*SIN*LTRs), which serve as the viral promotors. Upon viral integration these promoters are inactivated and therefore not functional. I therefore believe that the above-discussed technology for lentivirus mediated siRNA delivery into human hematopoietic stem/progenitor cells might be valuable for the treatment of defined hematopoietic malignancies. Nevertheless, potential side effects of the introduced siRNAs and lentiviral vectors have to be proven very seriously.

## *Literature*


***1. Massague J, Chen YG. Controlling TGF-beta signaling. Genes Dev. 2000;14:627-644.***

***2. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor-beta signaling in cancer. J Natl Cancer Inst. 2000;92:1388-1402.***

***3. Kuroda H, Matsunaga T, Terui T, et al. Decrease of Smad4 gene expression in patients with essential thrombocythaemia may cause an escape from suppression of megakaryopoiesis by transforming growth factor-beta1. Br J Haematol. 2004;124:211-220.***

***4. Le Bousse-Kerdiles MC, Chevillard S, Charpentier A, et al. Differential expression of transforming growth factor-beta, basic fibroblast growth factor, and their receptors in CD34+ hematopoietic progenitor cells from patients with myelofibrosis and myeloid metaplasia. Blood. 1996;88:4534-4546.***

***5. Rooke HM, Vitas MR, Crosier PS, Crosier KE. The TGF-beta type II receptor in chronic myeloid leukemia: analysis of microsatellite regions and gene expression. Leukemia. 1999;13:535-541.***

***6. Miller KL, Carlino JA, Ogawa Y, Avis PD, Carroll KG. Alterations in erythropoiesis in TGF-beta 1-treated mice. Exp Hematol. 1992;20:951-956.***

***7. Leveen P, Larsson J, Ehinger M, et al. Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. Blood. 2002;100:560-568.***

***8. Larsson J, Blank U, Helgadottir H, et al. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. Blood. 2003;102:3129-3135.***

***9. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992;359:693-699.***

***10. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. Science. 1995;269:1427-1429.***

***11. Muyrers JP, Zhang Y, Testa G, Stewart AF. Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res. 1999;27:1555-1557.***

***12. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000;100:157-168.***

***13. Moore MA, Metcalf D. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Br J Haematol. 1970;18:279-296.***

***14. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell. 1996;86:897-906.***

***15. Cumano A, Dieterlen-Lievre F, Godin I. The splanchnopleura/AGM region is the prime site for the generation of multipotent hemopoietic precursors, in the mouse embryo. Vaccine. 2000;18:1621-1623.***

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

***17. Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell. 1996;86:47-57.***

***18. Robb L, Elwood NJ, Elefanty AG, et al. The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. Embo J. 1996;15:4123-4129.***

*19. Yamada Y, Warren AJ, Dobson C, Forster A, Pannell R, Rabbitts TH. The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. Proc Natl Acad Sci U S A. 1998;95:3890-3895.*
*20. Hall MA, Curtis DJ, Metcalf D, et al. The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. Proc Natl Acad Sci U S A. 2003;100:992-997.*
*21. Ichikawa M, Asai T, Saito T, et al. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med. 2004;10:299-304.*
*22. McLeod DL, Shreve MM, Axelrad AA. Induction of megakaryocyte colonies with platelet formation in vitro. Nature. 1976;261:492-494.*
*23. Metcalf D, MacDonald HR, Odartchenko N, Sordat B. Growth of mouse megakaryocyte colonies in vitro. Proc Natl Acad Sci U S A. 1975;72:1744-1748.*
*24. Nakeff A, Daniels-McQueen S. In vitro colony assay for a new class of megakaryocyte precursor: colony-forming unit megakaryocyte (CFU-M). Proc Soc Exp Biol Med. 1976;151:587-590.*
*25. Odell TT, Jr., Jackson CW, Friday TJ. Megakaryocytopoiesis in rats with special reference to polyploidy. Blood. 1970;35:775-782.*
*26. Mazur EM, Lindquist DL, de Alarcon PA, Cohen JL. Evaluation of bone marrow megakaryocyte ploidy distributions in persons with normal and abnormal platelet counts. J Lab Clin Med. 1988;111:194-202.*
*27. Kuter DJ, Rosenberg RD. Appearance of a megakaryocyte growth-promoting activity, megapoietin, during acute thrombocytopenia in the rabbit. Blood. 1994;84:1464-1472.*
*28. Jackson CW, Brown LK, Somerville BC, Lyles SA, Look AT. Two-color flow cytometric measurement of DNA distributions of rat megakaryocytes in unfixed, unfractionated marrow cell suspensions. Blood. 1984;63:768-778.*
*29. Penington DG, Olsen TE. Megakaryocytes in states of altered platelet production: cell numbers, size and DNA content. Br J Haematol. 1970;18:447-463.*
*30. Kuter DJ, Rosenberg RD. The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. Blood. 1995;85:2720-2730.*
*31. Ebbe S. Regulation of murine megakaryocyte size and ploidy by non-platelet-dependent mechanisms in radiation-induced megakaryocytopenia. Radiat Res. 1991;127:278-284.*
*32. Ryo R, Yasunaga M, Saigo K, Yamaguchi N. Megakaryocytic leukemia and platelet factor 4. Leuk Lymphoma. 1992;8:327-336.*
*33. Vinci G, Tabilio A, Deschamps JF, et al. Immunological study of in vitro maturation of human megakaryocytes. Br J Haematol. 1984;56:589-605.*
*34. Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. Embo J. 1997;16:3965-3973.*
*35. Tsang AP, Fujiwara Y, Hom DB, Orkin SH. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. Genes Dev. 1998;12:1176-1188.*
*36. Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, et al. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell. 1995;81:695-704.*
*37. Hart A, Melet F, Grossfeld P, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity. 2000;13:167-177.*

***38. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. Science. 1994;265:1445-1447.***
***39. de Sauvage FJ, Carver-Moore K, Luoh SM, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med. 1996;183:651-656.***
***40. Ishibashi T, Burstein SA. Interleukin 3 promotes the differentiation of isolated single megakaryocytes. Blood. 1986;67:1512-1514.***
***41. Kavnoudias H, Jackson H, Ettlinger K, Bertoncello I, McNiece I, Williams N. Interleukin 3 directly stimulates both megakaryocyte progenitor cells and immature megakaryocytes. Exp Hematol. 1992;20:43-46.***
***42. Bruno E, Miller ME, Hoffman R. Interacting cytokines regulate in vitro human megakaryocytopoiesis. Blood. 1989;73:671-677.***
***43. Robinson BE, McGrath HE, Quesenberry PJ. Recombinant murine granulocyte macrophage colony-stimulating factor has megakaryocyte colony-stimulating activity and augments megakaryocyte colony stimulation by interleukin 3. J Clin Invest. 1987;79:1648-1652.***
***44. Quesenberry PJ, Ihle JN, McGrath E. The effect of interleukin 3 and GM-CSA-2 on megakaryocyte and myeloid clonal colony formation. Blood. 1985;65:214-217.***
***45. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood. 1995;85:1719-1726.***
***46. Briddell RA, Bruno E, Cooper RJ, Brandt JE, Hoffman R. Effect of c-kit ligand on in vitro human megakaryocytopoiesis. Blood. 1991;78:2854-2859.***
***47. Briddell RA, Hoffman R. Cytokine regulation of the human burst-forming unit-megakaryocyte. Blood. 1990;76:516-522.***
***48. Bruno E, Briddell RA, Cooper RJ, Hoffman R. Effects of recombinant interleukin 11 on human megakaryocyte progenitor cells. Exp Hematol. 1991;19:378-381.***
***49. Warren MK, Conroy LB, Rose JS. The role of interleukin 6 and interleukin 1 in megakaryocyte development. Exp Hematol. 1989;17:1095-1099.***
***50. Quesenberry PJ, McGrath HE, Williams ME, et al. Multifactor stimulation of megakaryocytopoiesis: effects of interleukin 6. Exp Hematol. 1991;19:35-41.***
***51. Ishibashi T, Miller SL, Burstein SA. Type beta transforming growth factor is a potent inhibitor of murine megakaryocytopoiesis in vitro. Blood. 1987;69:1737-1741.***
***52. Dessypris EN, Gleaton JH, Armstrong OL. Effect of human recombinant erythropoietin on human marrow megakaryocyte colony formation in vitro. Br J Haematol. 1987;65:265-269.***
***53. Gewirtz AM, Calabretta B, Rucinski B, Niewiarowski S, Xu WY. Inhibition of human megakaryocytopoiesis in vitro by platelet factor 4 (PF4) and a synthetic COOH-terminal PF4 peptide. J Clin Invest. 1989;83:1477-1486.***
***54. Sakamaki S, Hirayama Y, Matsunaga T, et al. Transforming growth factor-beta1 (TGF-beta1) induces thrombopoietin from bone marrow stromal cells, which stimulates the expression of TGF-beta receptor on megakaryocytes and, in turn, renders them susceptible to suppression by TGF-beta itself with high specificity. Blood. 1999;94:1961-1970.***
***55. Han ZC, Sensebe L, Abgrall JF, Briere J. Platelet factor 4 inhibits human megakaryocytopoiesis in vitro. Blood. 1990;75:1234-1239.***
***56. Massague J. TGF-beta signal transduction. Annu Rev Biochem. 1998;67:753-791.***
***57. Massague J. How cells read TGF-beta signals. Nat Rev Mol Cell Biol. 2000;1:169-178.***
***58. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet. 2001;29:117-129.***

***59. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science. 2001;291:1304-1351.***
***60. Gentry LE, Lioubin MN, Purchio AF, Marquardt H. Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. Mol Cell Biol. 1988;8:4162-4168.***
***61. Miyazono K, Hellman U, Wernstedt C, Heldin CH. Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. J Biol Chem. 1988;263:6407-6415.***
***62. Wakefield LM, Smith DM, Flanders KC, Sporn MB. Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. J Biol Chem. 1988;263:7646-7654.***
***63. Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. J Biol Chem. 1995;270:4689-4696.***
***64. Taipale J, Miyazono K, Heldin CH, Keski-Oja J. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol. 1994;124:171-181.***
***65. Olofsson A, Ichijo H, Moren A, ten Dijke P, Miyazono K, Heldin CH. Efficient association of an amino-terminally extended form of human latent transforming growth factor-beta binding protein with the extracellular matrix. J Biol Chem. 1995;270:31294-31297.***
***66. Lyons RM, Keski-Oja J, Moses HL. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. J Cell Biol. 1988;106:1659-1665.***
***67. Franzen P, ten Dijke P, Ichijo H, et al. Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. Cell. 1993;75:681-692.***
***68. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF-beta receptor. Nature. 1994;370:341-347.***
***69. Wrana JL. Regulation of Smad activity. Cell. 2000;100:189-192.***
***70. Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-beta signal transduction. J Cell Sci. 2001;114:4359-4369.***
***71. Chen YG, Hata A, Lo RS, et al. Determinants of specificity in TGF-beta signal transduction. Genes Dev. 1998;12:2144-2152.***
***72. Itoh S, Itoh F, Goumans MJ, Ten Dijke P. Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem. 2000;267:6954-6967.***
***73. Wotton D, Lo RS, Lee S, Massague J. A Smad transcriptional corepressor. Cell. 1999;97:29-39.***
***74. Akhurst RJ, Balmain A. Genetic events and the role of TGF beta in epithelial tumour progression. J Pathol. 1999;187:82-90.***
***75. Whitman M. Smads and early developmental signaling by the TGFbeta superfamily. Genes Dev. 1998;12:2445-2462.***
***76. Flanders KC, Kim ES, Roberts AB. Immunohistochemical expression of Smads 1-6 in the 15-day gestation mouse embryo: signaling by BMPs and TGF-betas. Dev Dyn. 2001;220:141-154.***
***77. Li J, Lee GI, Van Doren SR, Walker JC. The FHA domain mediates phosphoprotein interactions. J Cell Sci. 2000;113 Pt 23:4143-4149.***
***78. Shi W, Fan H, Shum L, Derynck R. The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. J Cell Biol. 2000;148:591-602.***

*79. Correia JJ, Chacko BM, Lam SS, Lin K. Sedimentation studies reveal a direct role of phosphorylation in Smad3:Smad4 homo- and hetero-trimerization. Biochemistry. 2001;40:1473-1482.*
*80. Imamura T, Takase M, Nishihara A, et al. Smad6 inhibits signalling by the TGF-beta superfamily. Nature. 1997;389:622-626.*
*81. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell. 1998;95:779-791.*
*82. Miura S, Takeshita T, Asao H, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol. 2000;20:9346-9355.*
*83. Razani B, Zhang XL, Bitzer M, von Gersdorff G, Bottinger EP, Lisanti MP. Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. J Biol Chem. 2001;276:6727-6738.*
*84. Zwaagstra JC, El-Alfy M, O'Connor-McCourt MD. Transforming growth factor (TGF)-beta 1 internalization: modulation by ligand interaction with TGF-beta receptors types I and II and a mechanism that is distinct from clathrin-mediated endocytosis. J Biol Chem. 2001;276:27237-27245.*
*85. Dong C, Li Z, Alvarez R, Jr., Feng XH, Goldschmidt-Clermont PJ. Microtubule binding to Smads may regulate TGF beta activity. Mol Cell. 2000;5:27-34.*
*86. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell. 1997;89:1165-1173.*
*87. Hata A, Lagna G, Massague J, Hemmati-Brivanlou A. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev. 1998;12:186-197.*
*88. Lebrun JJ, Takabe K, Chen Y, Vale W. Roles of pathway-specific and inhibitory Smads in activin receptor signaling. Mol Endocrinol. 1999;13:15-23.*
*89. Kavsak P, Rasmussen RK, Causing CG, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell. 2000;6:1365-1375.*
*90. Hocevar BA, Brown TL, Howe PH. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. Embo J. 1999;18:1345-1356.*
*91. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J Biol Chem. 1999;274:37413-37420.*
*92. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. Embo J. 2002;21:3749-3759.*
*93. Xiao Z, Liu X, Henis YI, Lodish HF. A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. Proc Natl Acad Sci U S A. 2000;97:7853-7858.*
*94. Xiao Z, Watson N, Rodriguez C, Lodish HF. Nucleocytoplasmic shuttling of Smad1 conferred by its nuclear localization and nuclear export signals. J Biol Chem. 2001;276:39404-39410.*
*95. Xu J, Attisano L. Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor beta signaling by targeting Smads to the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A. 2000;97:4820-4825.*
*96. Watanabe M, Masuyama N, Fukuda M, Nishida E. Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. EMBO Rep. 2000;1:176-182.*
*97. Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. Embo J. 2000;19:1745-1754.*

*98. Kim RH, Wang D, Tsang M, et al. A novel smad nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-beta signal transduction. Genes Dev. 2000;14:1605-1616.*
*99. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. 1998;12:2114-2119.*
*100. Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev. 1998;12:2153-2163.*
*101. Luo K, Stroschein SL, Wang W, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev. 1999;13:2196-2206.*
*102. Akiyoshi S, Inoue H, Hanai J, et al. c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J Biol Chem. 1999;274:35269-35277.*
*103. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science. 1995;268:1336-1338.*
*104. Lu SL, Zhang WC, Akiyama Y, Nomizu T, Yuasa Y. Genomic structure of the transforming growth factor beta type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers. Cancer Res. 1996;56:4595-4598.*
*105. Myeroff LL, Parsons R, Kim SJ, et al. A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res. 1995;55:5545-5547.*
*106. Izumoto S, Arita N, Ohnishi T, et al. Microsatellite instability and mutated type II transforming growth factor-beta receptor gene in gliomas. Cancer Lett. 1997;112:251-256.*
*107. Schiemann WP, Pfeifer WM, Levi E, Kadin ME, Lodish HF. A deletion in the gene for transforming growth factor beta type I receptor abolishes growth regulation by transforming growth factor beta in a cutaneous T-cell lymphoma. Blood. 1999;94:2854-2861.*
*108. Goggins M, Shekher M, Turnacioglu K, Yeo CJ, Hruban RH, Kern SE. Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. Cancer Res. 1998;58:5329-5332.*
*109. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science. 1996;271:350-353.*
*110. Woodford-Richens K, Williamson J, Bevan S, et al. Allelic loss at SMAD4 in polyps from juvenile polyposis patients and use of fluorescence in situ hybridization to demonstrate clonal origin of the epithelium. Cancer Res. 2000;60:2477-2482.*
*111. Eppert K, Scherer SW, Ozcelik H, et al. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell. 1996;86:543-552.*
*112. Uchida K, Nagatake M, Osada H, et al. Somatic in vivo alterations of the JV18-1 gene at 18q21 in human lung cancers. Cancer Res. 1996;56:5583-5585.*
*113. Wieser R, Gruber B, Rieder H, Fonatsch C. Mutational analysis of the tumor suppressor Smad2 in acute lymphoid and myeloid leukemia. Leukemia. 1998;12:1114-1118.*
*114. Kurokawa M, Mitani K, Irie K, et al. The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. Nature. 1998;394:92-96.*
*115. Keller JR, McNiece IK, Sill KT, et al. Transforming growth factor beta directly regulates primitive murine hematopoietic cell proliferation. Blood. 1990;75:596-602.*
*116. Ohta M, Greenberger JS, Anklesaria P, Bassols A, Massague J. Two forms of transforming growth factor-beta distinguished by multipotential haematopoietic progenitor cells. Nature. 1987;329:539-541.*

***117. Ploemacher RE, van Soest PL, Boudewijn A. Autocrine transforming growth factor beta 1 blocks colony formation and progenitor cell generation by hemopoietic stem cells stimulated with steel factor. Stem Cells. 1993;11:336-347.***
***118. Li ML, Cardoso AA, Sansilvestri P, et al. Additive effects of steel factor and antisense TGF-beta 1 oligodeoxynucleotide on CD34+ hematopoietic progenitor cells. Leukemia. 1994;8:441-445.***
***119. Eaves CJ, Cashman JD, Kay RJ, et al. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. II. Analysis of positive and negative regulators produced by stromal cells within the adherent layer. Blood. 1991;78:110-117.***
***120. Cashman JD, Eaves AC, Raines EW, Ross R, Eaves CJ. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. I. Stimulatory role of a variety of mesenchymal cell activators and inhibitory role of TGF-beta. Blood. 1990;75:96-101.***
***121. Berthier R, Valiron O, Schweitzer A, Marguerie G. Serum-free medium allows the optimal growth of human megakaryocyte progenitors compared with human plasma supplemented cultures: role of TGF beta. Stem Cells. 1993;11:120-129.***
***122. Jackson H, Williams N, Westcott KR, Green R. Differential effects of transforming growth factor-beta 1 on distinct developmental stages of murine megakaryocytopoiesis. J Cell Physiol. 1994;161:312-318.***
***123. Carlino JA, Higley HR, Creson JR, Avis PD, Ogawa Y, Ellingsworth LR. Transforming growth factor beta 1 systemically modulates granuloid, erythroid, lymphoid, and thrombocytic cells in mice. Exp Hematol. 1992;20:943-950.***
***124. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development. 1995;121:1845-1854.***
***125. Letterio JJ, Geiser AG, Kulkarni AB, et al. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. J Clin Invest. 1996;98:2109-2119.***
***126. Sauer B. Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. Mol Cell Biol. 1987;7:2087-2096.***
***127. Shimshek DR, Kim J, Hubner MR, et al. Codon-improved Cre recombinase (iCre) expression in the mouse. Genesis. 2002;32:19-26.***
***128. Oshima M, Oshima H, Taketo MM. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol. 1996;179:297-302.***
***129. Yang X, Li C, Herrera PL, Deng CX. Generation of Smad4/Dpc4 conditional knockout mice. Genesis. 2002;32:80-81.***
***130. Schedl A, Grimes B, Montoliu L. YAC transfer by microinjection. Methods Mol Biol. 1996;54:293-306.***
***131. Ravid K, Beeler DL, Rabin MS, Ruley HE, Rosenberg RD. Selective targeting of gene products with the megakaryocyte platelet factor 4 promoter. Proc Natl Acad Sci U S A. 1991;88:1521-1525.***
***132. Kufrin D, Eslin DE, Bdeir K, et al. Antithrombotic thrombocytes: ectopic expression of urokinase-type plasminogen activator in platelets. Blood. 2003;102:926-933.***
***133. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999;21:70-71.***
***134. Casanova E, Fehsenfeld S, Mantamadiotis T, et al. A CamKIIalpha iCre BAC allows brain-specific gene inactivation. Genesis. 2001;31:37-42.***
***135. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity. 2000;13:443-451.***

***136. Cashman JD, Eaves CJ, Sarris AH, Eaves AC. MCP-1, not MIP-1alpha, is the endogenous chemokine that cooperates with TGF-beta to inhibit the cycling of primitive normal but not leukemic (CML) progenitors in long-term human marrow cultures. Blood. 1998;92:2338-2344.***

***137. Chacko BM, Qin B, Correia JJ, Lam SS, de Caestecker MP, Lin K. The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. Nat Struct Biol. 2001;8:248-253.***

***138. Larsson J, Goumans MJ, Sjostrand LJ, et al. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. Embo J. 2001;20:1663-1673.***

***139. Shivdasani RA, Orkin SH. The transcriptional control of hematopoiesis. Blood. 1996;87:4025-4039.***

***140. Fujiwara Y, A Chang AN, A Williams AM, A Orkin SH. Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development. Blood. 2004;103:583-585.***

***141. Fuchs O, Simakova O, Klener P, et al. Inhibition of Smad5 in human hematopoietic progenitors blocks erythroid differentiation induced by BMP4. Blood Cells Mol Dis. 2002;28:221-233.***

***142. Kurisaki A, Kose S, Yoneda Y, Heldin CH, Moustakas A. Transforming growth factor-beta induces nuclear import of Smad3 in an importin-beta1 and Ran-dependent manner. Mol Biol Cell. 2001;12:1079-1091.***

***143. Paddison PJ, Hannon GJ. RNA interference: the new somatic cell genetics? Cancer Cell. 2002;2:17-23.***

***144. Shi Y. Mammalian RNAi for the masses. Trends Genet. 2003;19:9-12.***

***145. Uchida N, Sutton RE, Friera AM, et al. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. Proc Natl Acad Sci U S A. 1998;95:11939-11944.***

***146. Case SS, Price MA, Jordan CT, et al. Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. Proc Natl Acad Sci U S A. 1999;96:2988-2993.***

***147. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet. 2000;25:217-222.***

***148. Luther-Wyrsch A CE, Thali M, Buetti E, Nissen C, Surbek D, Holzgreve W, Gratwohl A, Tichelli A, Wodnar-Filipowicz A. Stable transduction with lentiviral vectors and amplification of immature hematopoietic progenitors from cord blood of preterm human fetuses. Hum Gene Ther. 2001;12:377-389.***

***149. Woods NB FC, Mikkola H, Hamaguchi I, Olsson K, Zufferey R, Jacobsen SE, Trono D, Karlsson S. Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. Blood. 2000;96:3725-3733.***

***150. Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A. 2002;99:2140-2145.***

***151. Rubinson DA, Dillon CP, Kwiatkowski AV, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet. 2003;33:401-406.***

***152. Qin XF, An DS, Chen IS, Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci U S A. 2003;100:183-188.***

***153. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science. 2002;296:550-553.***

***154. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol. 1997;15:871-875.***
***155. Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH, Trono D. High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. Blood. 2000;96:3392-3398.***
***156. Kralovics R BA, Teo SS, Coers J, Tichelli A, Van Der Maas AP, Skoda RC. Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders. Blood. 2003;Epub ahead of print.***
***157. Dunkern TR, Wedemeyer I, Baumgartner M, Fritz G, Kaina B. Resistance of p53 knockout cells to doxorubicin is related to reduced formation of DNA strand breaks rather than impaired apoptotic signaling. DNA Repair (Amst). 2003;2:49-60.***
***158. Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell. 1992;70:923-935.***
***159. Dunkern TR WI, Baumgartner M, Fritz G, Kaina B. Resistance of p53 knockout cells to doxorubicin is related to reduced formation of DNA strand breaks rather than impaired apoptotic signaling. DNA Repair. 2003;2:49-60.***
***160. Bassi L CM, Fonti E, Palma de la Pena N, Meschini R, Palitti F. Pifithrin-alpha, an inhibitor of p53, enhances the genetic instability induced by etoposide (VP16) in human lymphoblastoid cells treated in vitro. Mutat Res. 2002;499:163-176.***
***161. Blandino G LA, Oren M. Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene. 1999;18:477-485.***
***162. Vooijs M, Jonkers J, Berns A. A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. EMBO Rep. 2001;2:292-297.***
***163. Cardoso AA, Li ML, Batard P, et al. Release from quiescence of CD34+ CD38- human umbilical cord blood cells reveals their potentiality to engraft adults. Proc Natl Acad Sci U S A. 1993;90:8707-8711.***
***164. Jansen R, Damia G, Usui N, et al. Effects of recombinant transforming growth factor-beta 1 on hematologic recovery after treatment of mice with 5-fluorouracil. J Immunol. 1991;147:3342-3347.***
***165. Fujiwara Y, Chang AN, Williams AM, Orkin SH. Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development. Blood. 2004;103:583-585.***
***166. Fujimaki S, Harigae H, Sugawara T, Takasawa N, Sasaki T, Kaku M. Decreased expression of transcription factor GATA-2 in haematopoietic stem cells in patients with aplastic anaemia. Br J Haematol. 2001;113:52-57.***
***167. Liu B, Sun Y, Jiang F, et al. Disruption of Smad5 gene leads to enhanced proliferation of high-proliferative potential precursors during embryonic hematopoiesis. Blood. 2003;101:124-133.***
***168. Yamaguchi K, Shirakabe K, Shibuya H, et al. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science. 1995;270:2008-2011.***
***169. Woods NB MH, Nilsson E, Olsson K, Trono D, Karlsson S. Lentiviral-mediated gene transfer into haematopoietic stem cells. J Intern Med. 2001;249(4):339-343.***
***170. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 2000;101:25-33.***
***171. Brummelkamp TR BR, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science. 2002;296:550-553.***

***172. Tiscornia G, Singer O, Ikawa M, Verma IM. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci U S A. 2003;100:1844-1848.***

# Curriculum vitae

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## Education

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|---|---|
| 09/2004-today | Post Doc in the laboratory of Prof. Dr. Gerhard Christofori, Institute of Biochemistry and Genetics, Department of Clinical-Biological Sciences, University of Basel, Basel, Switzerland |
| 04/2002-09/2004 | Ph.D.-thesis at the University Hospital Basel, Department Research, Experimental Hematology, Basel under the supervision of Prof. Dr. Radek Skoda. |
| 04/2000-04/2002 | Ph.D.-thesis in the laboratory of Experimental Hematology and Oncology at the German Cancer Research Center Heidelberg, Germany, under the supervision of Prof. Radek Skoda. |
| 10/1996-01/2000 | Diploma in Biology at the Albert-Ludwigs-University Freiburg, Germany. Subjects: Biochemistry, Genetics, Developmental Biology. Optional subject: Clinical chemistry. Thesis work under the supervision of Dr. Gerd Scherer and Prof. Bodo Rak at the Department of Human Genetics. |
| 10/1993-10/1996 | Pre-exam in Biology at the Philipps-University Marburg, Germany. |
| 08/1982-06/1991 | A-level equivalent (Abitur) at the Martin-Luther-Gymnasium Marburg, Germany. |

**Scientific skills**

- Standard methodologies in molecular biology like cloning techniques, PCR, tissue culture, protein biochemistry, RNA/DNA preparation and detection.
- Yeast two-hybrid system for protein-protein interaction, mammalian two-hybrid system
- Real-time PCR and DNA sequencing
- Design and transfection of small interfering RNA oligonucleotides and vector based short hairpin (shRNA) RNA into cell lines and primary cells
- Development of a lenti-viral based system for gene-silencing by RNAi in cell lines and primary cells
- Modification of Bacterial Artificial Chromosomes (BAC) by ET-cloning for the generation of transgenic mouse models
- Experience in culturing and maintenance of primary cells like hematopoietic stem cells
- Linkage analysis by micro-satellite PCR

**Additional skills**

- Training of students and technical staff
- Very good knowledge in word processing (MS Word 2000), graphic and presentation programs (CorelDraw, Canvas, Powerpoint, AdobeIllustrator) and data processing (MS Exel, SciFinder, Endnote, Reference Manager and BLAST)
- English and German (fluent written and spoken), Hungarian, French (school knowledge) and Latin (Latinum)

**References**

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**List of publications**

**T.Schomber**, Hui Hao Shen and Radek C. Skoda: The platelet factor 4-Cre transgenic mouse: a new tool to study megakaryopoiesis *in vivo*. In preparation

**T.Schomber**, C. Kalberer, A.Filipowicz and Radek C. Skoda: Gene silencing by lentivirus-mediated delivery of siRNA in human CD34+ cells. Blood 2004 Jun 15; 103(12): 4511-3

Zhou R, Bonneaud N, Yuan CX, de Santa Barbara P, Boizet B, **Schomber T**, Scherer G, Roeder RG, Poulat F and Berta P: SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. Nucleic Acid Res. 2002 Jul 15;30(14): 3245-52

**Abstracts and Talks**

**T.Schomber,** V.Dalle Carbonare and Radek C. Skoda: The platelet factor 4-Cre mouse: a new tool to study megakaryopoiesis. Meeting of the Swiss Society of Hematology, Lausanne, 2-5.5.2004

**T.Schomber**, C. Kalberer, A.Filipowicz and Radek C. Skoda: Lentiviral gene silencing in human cord blood derived CD34+ cells. 45$^{th}$ American Society of Hematology Meeting, 6-9.12.2003, San Diego, CA

Kralovics R, Schomber T, Skoda RC: Increased expression of the nuclear factor I-B (NFIB) in myoproliferative disorders and its interference with TGF-β responses. Exp. Hematol. 31 (Supplement 1) 231, 2003