Transforming growth factor-β (TGF−β) signaling in hematopoiesis and tumorigenesis

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Summary

Transforming growth factor β (TGF-β) signaling regulates numerous cellular and physiological processes. Dysfunction of components of this signaling pathway leads to a wide range of diseases ranging from malignant hematopoiesis, cardiovascular disease, immunity abnormalities, connective tissue disease, reproductive disorders, metabolic disorders, skeleton and muscular disorders, to developmental defects.

We focused on the role of *Smad4* and *TBRII* genes in the hematopoiesis and studied a conditional *Smad4* knockout mouse. Mice with homozygous *Smad4* deletion ($Smad4^{\Delta/\Delta}$) developed severe anemia 6-8 weeks after induction (mean hemoglobin 70g/L). The anemia was not transplantable, as wild type mice reconstituted with $Smad4^{\Delta/\Delta}$ bone marrow cells had normal peripheral blood counts. In contrast, lethally irradiated $Smad4^{\Delta/\Delta}$ mice transplanted with wild type bone marrow cells developed anemia similar to non-transplanted $Smad4^{\Delta/\Delta}$ mice. Liver iron stores were decreased and blood was present in stool, indicating that the anemia was due to blood loss. Multiple polyps in stomach and colon represent a likely source of the bleeding. We conclude that *Smad4* is not required for adult erythropoiesis and that anemia is solely the consequence of blood loss. Regulation of hepcidin related genes (*Atoh8, Id1* and *Bmp6*) responded to acute bleeding in the absence of *Smad4*, *TBRII* and/or histone deacetylase 1 (*HDAC1*) genes.

$Smad4^{\Delta/\Delta}$ mice did not develop an inflammatory disease typical for mice deficient in *TGF-b* receptors I and II (*TBRI* and *TBRII*), suggesting that suppression of inflammation by TGF-β is Smad4 independent. The same results were obtained when *Smad4* alleles were deleted selectively in hematopoietic cells using the *VavCre* transgenic mice. Mice with a double knockout ($Smad4^{\Delta/\Delta}$ and $TBRII^{\Delta/\Delta}$) did not display the $TBRII^{\Delta/\Delta}$ -driven lethal inflammation suggesting that *Smad4* signaling is required to mediate the inflammatory phenotype. *Smad4/TBRII* was dispensable for the megakaryopoiesis and erythropoiesis. Finally, we confirmed that the Smad4-signaling pathway is required to suppress tumorigenesis in the gastrointestinal tract and loss of Smad4-signaling in hematopoietic cells is sufficient to cause polyp formation in the gut.

General introduction

1. TGF-β superfamily signaling

As an important and pervasive signaling pathway, transforming growth factor β (TGF-β) superfamily signaling pathways regulate a wide range of biological processes at cellular and systematical levels. It dictates not only the single cell's expansion, determination, movement and apoptosis, but also contextual interactions among different cells, tissues and organs, which guide development, immune regulation, tumorigenesis, and wound recovery. Malfunction in these pathways often leads to many kinds of diseases in vertebrates. Detailed studies of these pathways at different levels shed light on the relevant biomarker screens and therapeutic application.

The TGF−β superfamily ligands consists of more than 30 polypeptide growth factors including TGF−βs(1-3), activins (A, B), inhibins (A, B), bone morphogenetic proteins (BMPs 1-9), growth differentiation factors including myostatin, nodal, leftys (1,2), and Mullerian inhibiting substance (MIS). [1,2,3] These members show a similar cysteine knot structure, and are universally expressed. DNA mutations or protein expression abnormality can cause many malfunctions resulting in developmental, metabolic and physiological disorders. [4] [5]

1.1 Basic signaling pathway

Signaling by TGF−β superfamily is mediated through the binding of the ligand to high affinity heterodimeric receptors complexes that consist of seven type I (activin like kinase (ALK) 1-7) and five type II (serine/threonine kinase) subunits. Specific and non-specific ligands bind to different combinations of receptor complexes, forming a complex signaling stimuli. The binding of ligands to heteromeric receptors activates type II receptor kinase to phosphorylate the type I within a glycine and serine-rich domain thereby initiating its kinase function to phosphorylate the downstream messengers in Smad-dependent/independent ways.

The Smad-dependent pathway is facilitated by the membrane-bound scaffold protein Smad anchor for receptor activation (SARA) and results in phosphorylation two receptor-regulated Smad proteins (R-Smad), forming a complex with the common

Smad (Smad4), which translocates into the nucleus to regulate the gene expression both positively and negatively in association with different transcription factors, such as members of the forkhead, homebox, zinc-finger, bHLH, and AP1 families. Under these circumstances, the Smad4 and cytoplasmic preferred R-Smad will accumulate in even distribution in the nucleus. The newly identified R-Smad phophatases in the nucleus are able to release these Smads from nucleus, and end the signaling. Nevertheless, a third class of Smad proteins, the inhibitory Smads (I-Smads), including Smad6 and Smad7, inactivate the receptor complex by degradation via the ubiquitin ligases Smurf1/2 and dephosphrylation via the protein phosphatase I, and interrupt the formation of R-Smad/Smad4 complex.

TGF−β superfamily co-receptors, besides facilitating ligand binding to the signaling receptors, are able to form morphogen gradients during embryonic development, antagonize ligand function, direct receptor localization and internalization, mediate cellular adhesion and orchestrate signaling.[6] For example, the soluble and surface TBRIII impacts on migration and localization of the ligand-receptor complex through interacting with ligands like inhibin,[7,8] BMP,[9] and TGF−β ligands.[10,11,12]

1.2 Noncanonical signaling pathway

TGF−β signals in Smads-independent ways through cross talk with other signaling pathways, such as the mitogen activated protein Kinase (MAPK) pathways, including extracellular-signal-regulated kinase (ERK), p38, and Jun N-terminal kinase (JNK), the phosphoinositide 3-kinase (PI(3)K)/Akt pathway, and the nuclear factor-kappa B (NF-κB) pathway (Figure 1).[13,14] Type II receptors play an important role in these cross-talks, instead of type I receptors.

Furthermore, even in R-Smads-dependent pathways, some factors like TIF1γ[15] and I-kappa-B kinase a (IKKa), as well as a component of the microRNA processing complex DROSHA, p68, are reported to directly bind the R-Smads complex without Smad4 to regulate the downstream genes.[16]

1.3 Smads-dependent signaling pathways

Smads contain a Mad homology (MH)1 and MH2 domain, which are connected by a linker region. MH1 has a nuclear localization signal (NLS), and inhibits the MH2, [17]

the functional domain of Smads. Smads complexes can bind common DNA elements in the promoter region, like GTCT via TGF−β activation,[18] and GCCNC or GRCGNC via BMPs activation.[19]

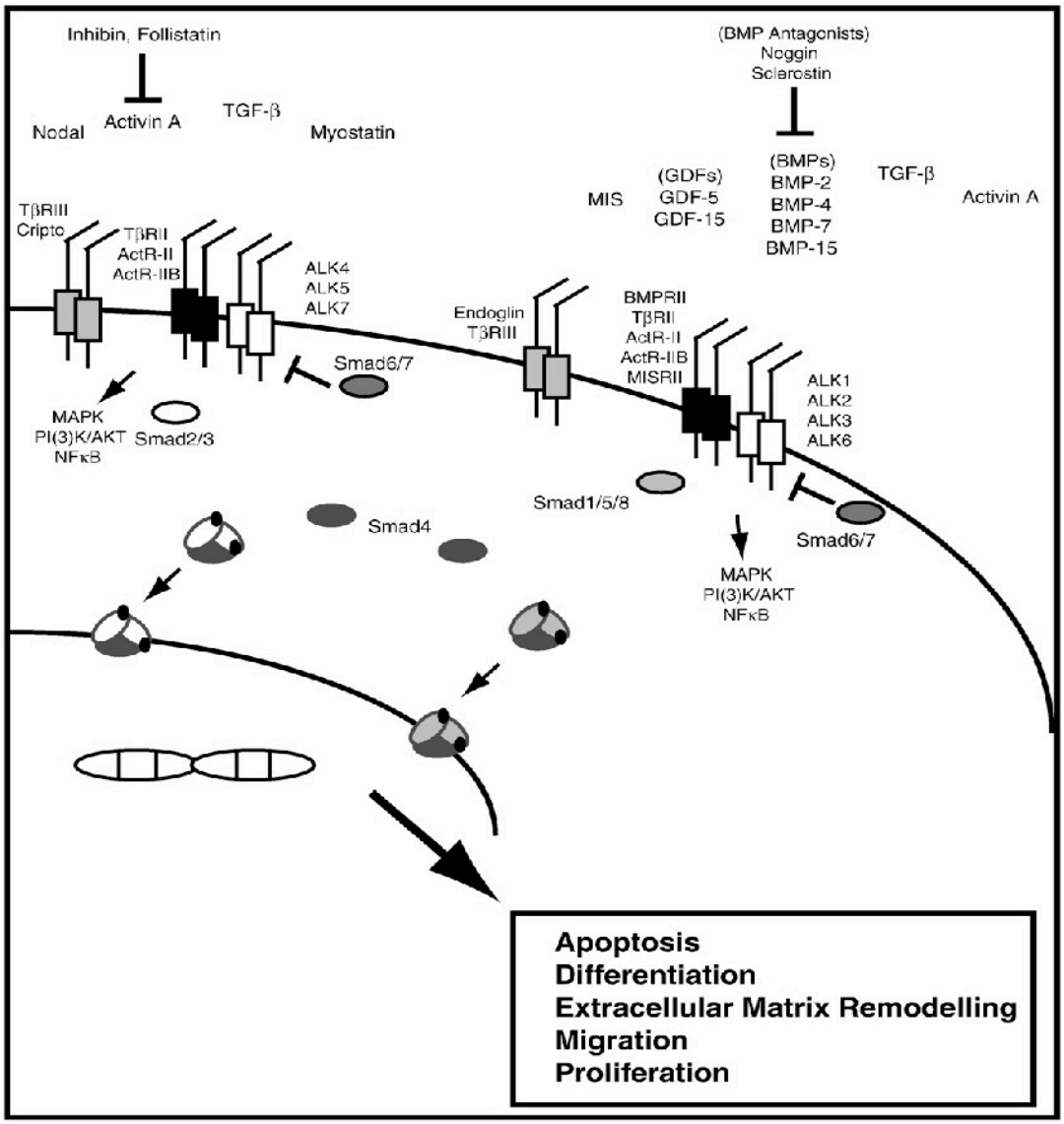


Figure 1. TGF−β superfamily signaling pathways. (cited from Blobe et al., BBA, 1782(2008)197-228)

Smads form a complex with many partners in nucleus upon activation (Table 1)

Under TGF−β signaling, Smad2&3 combined with Smad4 interact with many transcription factors, such as FoxH1, Mix, FoxO, C/EBPβ, or repressor FoxG1, ATF3. The activating protein-1 (Ap-1), NF-κB, runt-related transcription factor-2 (Runx2) and signal protein-1(Sp1) were also reportedly involved in this process(Figure 2). [20,21,22]

Table 1 (Riewed by Hill et al, IJBCB, 2008;(40):383-408,)
Summary of Smad interacting proteins and their reported Smad partner(s)

Smad interacting protein	Class of protein	Reported Smad partner(s)	Mapped Smad-interaction domain
FoxH1a, FoxH1b	Forkhead transcription factor	Smad2/Smad3	MH2 via FM and SIM motifs
Mixer, Milk, Bix3	Mix transcription factor	Smad2/Smad3	MH2 via SIM motif
FoxO	Forkhead transcription factor	Smad2/Smad3, Smad4	MH1
C/EBP	Leucine-zipper transcription factor	Smad3, Smad4	MH1 and MH2
ATF3	Leucine-zipper transcription factor	Smad3	MH2
Schnurri	Zinc ngertranscription/scaffolding factor	Smad1, Smad4	Not described
Runx1, Runx2, Runx3	-Subunits of the PEBP2 transcription factor	Smad1/Smad5/Smad2/Smad3	MH1 and MH2 (of Smad3)
Smurf1	HECT domain E3 ligase	Smad1/Smad5, Smad6/Smad7	PPXY motif within the linker
Smurf2	HECT domain E3 ligase	Smad1/Smad5/Smad2/Smad3, Smad6/Smad7	PPXY motif within the linker, amino terminal domain of Smad7
NEDD4-2	HECT domain E3 ligase	Smad2/Smad3, Smad6/Smad7, Smad1/Smad5 (weak)	PPXY motif within the linker
WWP1/TiuL1	HECT domain E3 ligase	Smad1/Smad5/Smad2/Smad3, Smad6/Smad7	PPXY motif within the linker
Roc1	RING ngerprotein component of the SCF E3 ligase complex	Smad3	MH2
Arkadia	RING ngerE3 ligase	Smad2/Smad3, Smad6/Smad7	MH2 (for Smad7)
UbcH7	E2ñubiquitin conjugating enzyme	Smad7	Amino terminal domain
-TrCP1	F-box protein component of SCF E3 ligase complex	Smad4	Not mapped
Ectodermin	E3 ubiquitin ligase	Smad4, Smad2/Smad3	MH2 domain of Smad2
UCH37	Deubiquitinating enzyme (DUB)	Smad7, Smad2/Smad3 (weak)	Not mapped
Ubc9	E2 SUMO conjugating enzyme	Smad4	MH1
PIASy	SUMO ligase	Smad1/Smad2/Smad3, Smad6/Smad7, Smad4 (weak)	MH2
PIAS3	SUMO ligase	Smad2/Smad3, Smad4 (weak)	MH2
hZimp10	SUMO ligase	Smad3, Smad4	MH2
p300 and CBP	Histone acetyltransferase (HAT) and factor acetyltransferase (FAT)	Smad1/Smad2/Smad3, Smad4, Smad7	MH2
P/CAF	Histone acetyl transferase (HAT)	Smad2/Smad3	MH2
GCN5	Histone acetyl transferase (HAT)	Smad2/Smad3	MH2
HDAC4, HDAC5, HDAC6	Class II histone deacetylases (HDAC)	Smad3 (HDAC4/5), Smad7 (HDAC5/6)	MH2 (for Smad3)
HDAC1, HDAC3	Class I histone deacetylases (HDAC)	Smad7	MH2
ARC105/MED15	Component of the mediator complex	Smad2/Smad3, Smad4	MH2
MSG1	Nuclear factor/co-activator	Smad4	MH2
SRC1	Steroid receptor co-activator	Smad3	Not mapped
Ski	Transcriptional co-repressor	Smad1/Smad2/Smad3, Smad4	MH2
SnoN	Transcriptional co-repressor	Smad2/Smad3, Smad4	MH2
TGIF	Homeodomain co-repressor	Smad2/Smad3, Smad1 (weakly)	Not mapped
Evi-1	Zinc- ngerco-repressor	Smad1/Smad2/Smad3	MH2
Brg1	ATPase component of SWI/SNF chromatin remodelling complex	Smad2/Smad3	MH2 +linker
BAF155, BAF170	Component of the SWI/SNF chromatin remodelling complex	Smad2/Smad3	MH2 +linker

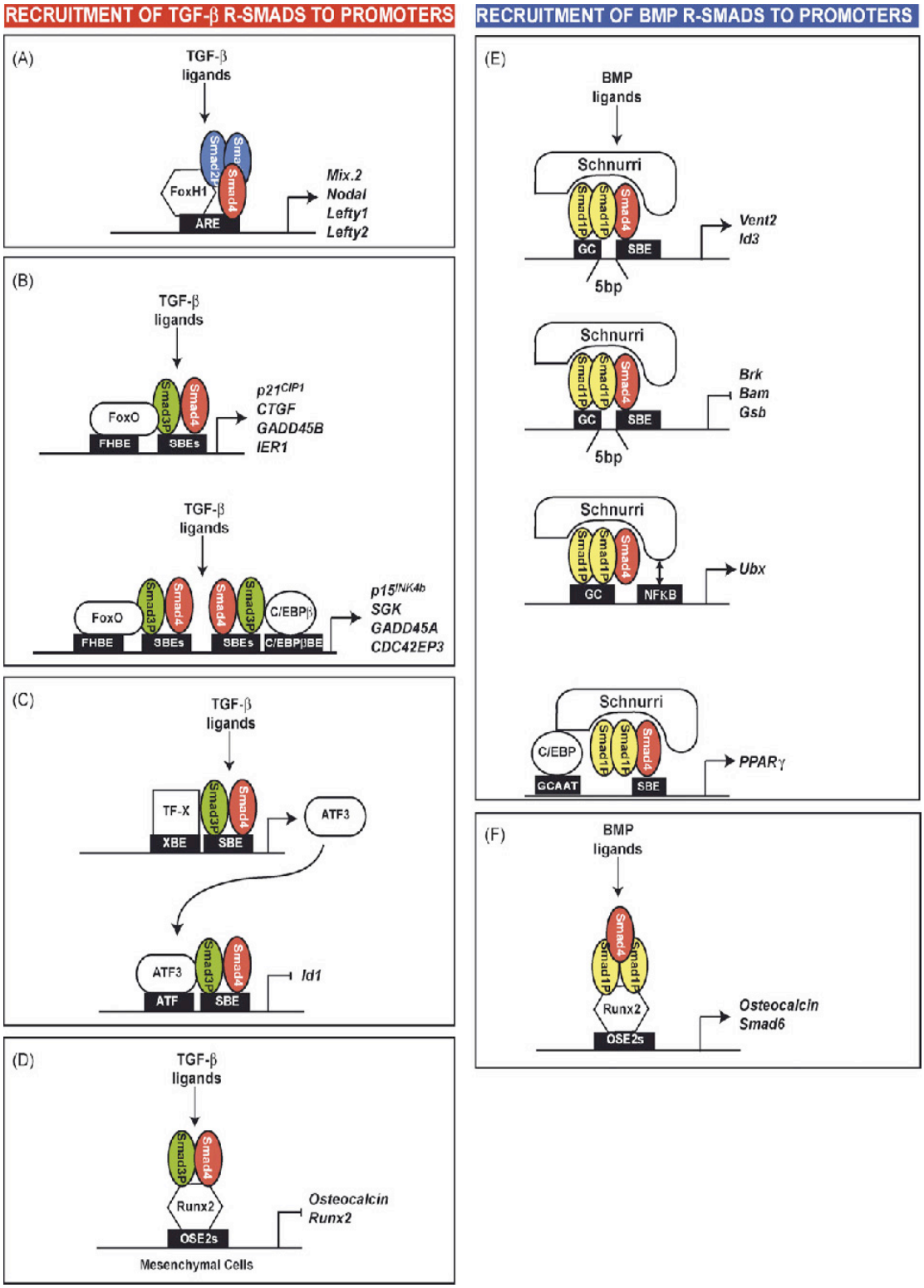


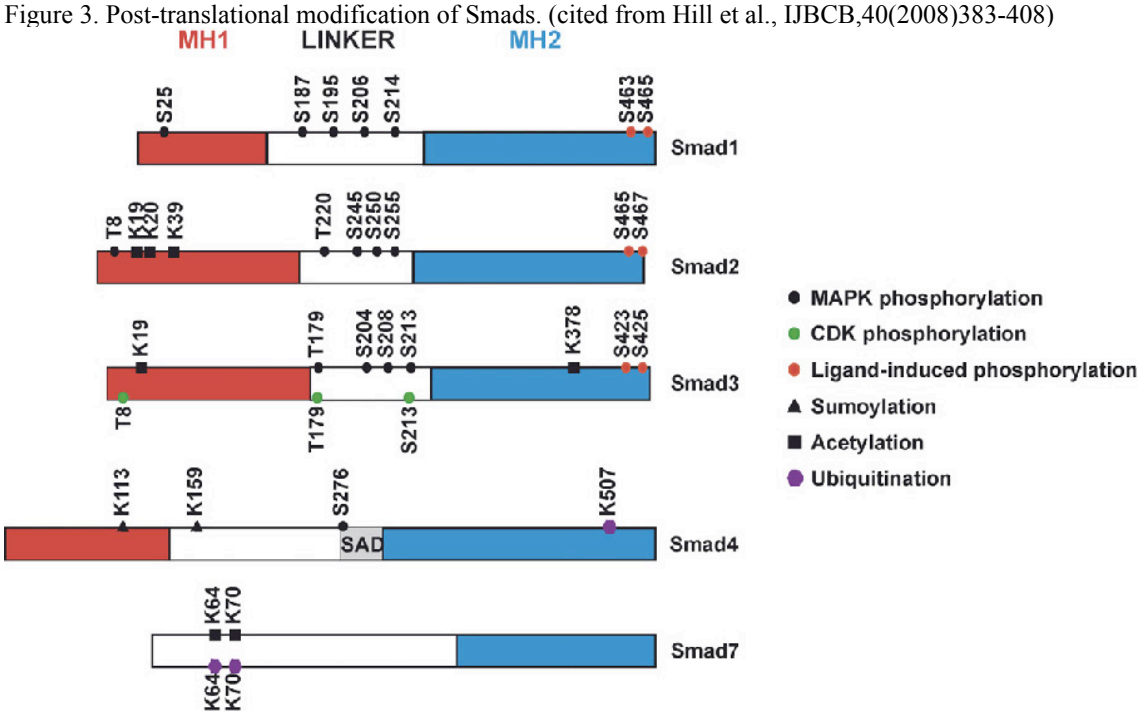
Figure 2. TGF−β and BMP activated the gene regulation. (cited from Hill et al., IJBCB,40(2008)383-408)

Upon activation of BMPs signaling, Smad1-Smad4 complexes recruit co-factor such as Drosophila Schnurri(Shn)-Mad-Medea(Med) complex or polyomavirus enhancer-binding protein2 (PEBP2), to activate or repress genes in a cellular context-dependent way (Figure 2).[23]

1.4 Post-translational modifications of the Smads

Smads are subject to many protein modifications, such as ubiquitilation, sumoylation, acetylation and phosphorylation. These modifications are very important parts of TGF−β signaling.

Figure 3. Post-translational modification of Smads. (cited from Hill et al., IJBCB,40(2008)383-408)



Ubiquitilation

Ubiquitilation is a covalent binding of a 76 amino acid polypeptide called ubiquitin, to mainly lysine residues mainly of target proteins. Ubiquitilation is involved three steps, under the control of enzyme1-3 (E1-3) respectively. E1 is for adenylation of the C-terminus of ubiquitin, E2 for transferring and E3 for final ligation. Because of ubiquitin's own lysine, poly-ubiquitin chains can form and be recognized by proteasome for target protein degradation. Besides protein degradation, ubiquitilation can regulate the substrate trafficking, protein interaction and activity.[24]

Smads are often regulated by ubiquitilation. The Smad ubiquitination regulatory factors (Smurf) E3 ligase, were reported to interact with Smad1and Smad5. Smurf1 impairs nuclear translocation of Smad1by interfering with its binding to Nup214.[25] Smurf2 can ubiquitinate active Smad1 and Smad2 and results in their degradation.[26]

Phosphorylation sites within the C-terminus and the linker region are important for R-Smad-Smurf interaction. The PPXY motif in the linker region is recognized by Smurfs.[27]

Smad3 can be ubiquitinated and degraded through mediation of the regulator of cullins-1(ROC1), which is a component of Skp/cullin/F-box E3 ligase complex.[28]
However, the positive regulation by E3 ligases, like Cbl-b and Itch were observed in TGF−β signaling. Repressor of TGF−β signaling, Sloan-kettering retrovirus proto-oncogene product(Ski) and ski-related novel gene (SnoN), degrade through involvement of Smurf2, anaphase promoting complex(APC) and Arkadia.[29]

I-Smads, Smad6 and Smad7 containing an E3 ligase recognize the motif PPXY, undergo ubiquitin mediated degradation themselves, or mediate ubiquitin-dependent degradation of TGF−β receptor complex and reduce TGF−β signaling.[27]

Like R-Smads, Smad4 can also interact with E3 ligases directly, or indirectly mediated by R-Smad4, but without direct ubiquitination. SCF complex is quite involved in the turn over of Smad4. Another E3 ligase, Ectodermin(Ecto/TIF1γ)[15] competes with Smad4 for binding the Smad2&3 complex to increase the erythroid differentiation. The worthwhile fact is that wild type Smad4 is not poly-ubiquitinated, but mono/oligo-ubiquitinated, while mutants of Smad4 undergo poly-ubiquitination resulting in degradation.

Although E3 ligases mainly impact negatively on TGF−β signaling, positive enhancement is also observed in Smads-independent pathway. Furthermore, the deubiquitinating enzyme ubiquitin C-terminal hydrolase37 (UCH37) can counter-balance with ubiquitination on Smads.[30]

Sumoylation

Small ubiquitin-like modifier (SUMO), a ubiquitin-related polypeptide, is another attractive modification on target protein for signaling. SUMO competes with other modification like acetylation and ubiquitination on lysine residues. Sumoylation of target protein like Smad4, has increases binding affinity for histone deacetylase gene (HDACs).[31] SUMO1 or SUMO E2-conjugating enzyme Ubc9 might be involved in this

process. Unlike the degradation function of ubiquitin, SUMO can interact with Smad4 and change its activity to interact with other transcriptional factors like repressor Daxx,[32] resulting in alter gene regulation. Another example is Protein inhibitors of activated STAT(PIAS) proteins, containing SUMO E3 ligase activity, which are able to interact with Smad1-4 and enhance Smad3/4 transcription by recruiting the co-activator proteins p300 and/or CREB-binding protein(CBP). The fact that Smad4 is regulated via sumoyulation implies that other substrates of sumoylation might exist in the components of TGF−β signaling.[33]

Acetylation

Acetylation is widely reported lysine-specific modification on protein including the histiones and transcriptional factors. Smad7 can be acetylated and de-acetylated by p300 and HDAC respectively. Furthermore, Smad2 and Smad3 are also acetylated by p300 and CBP. These acetylation can re-modify gene expression, Smads complex stability and trafficking.

Phosphorylation

Besides the phosphorylation by type I&II receptors upon TGF−β signaling, MAPKs, CDK and Calmodulin-depentant kinase II (CaMKII) can phosphorylate Smads. Conversely, this phosphates group can be removed by many phosphatases like pyruvate dehydrogenase phosphatase (PDP), RNA polymerase II small X-terminal phosphatases SCP1-3, and PPM2A. The balance of phosphorylation and de-phosphorylation modifies the activities of Smads, and subsequently modulates TGF−β signaling.[34]

Chromatin protein

Naked DNA cannot be bound by Smads complexes. It always needs the help of histone and other chromatin protein. HAT, HDAC, and chromatin remodeling complexes like switching of mating type/sucrose non-fermenting (SWI/SNF), imitation switch (ISWI), nucleosomes remodeling and deacetylase (NuRD) and Ino80 are involved in Smads mediated the regulation.[35] Puzzlingly, important chromatin-modifying enzymes, such as methylation and de-methylation enzymes, are not found to interact with Smads.

1.5 Regulation of TGF−β signaling

As an important and complex signaling pathway, TGF−β superfamily signaling is regulated at all levels, including at the level of ligands, receptors and co-receptors, Smads, as well as autocrine positive and negative feedback loop.

At the ligand level, TGF−β is not functional without cutting off a signal peptide named the latency associated peptide domain (LAP).[36]Many extracellular proteases are involved in this cleavage, such as thrombospondin-1,[37]plasmin, cathepsin D,[38] matrix-metalloproteinases 2 and 9,[39] and furin convertase.[40] It's also reported, upon the LAP's binding to integrins, alphavbeta6 integrin can activate TGF−β1,[41] and alphavbeta8 mediated epithelial homeostasis through MT1-MMP dependent activation of TGF−β1.[42] In contrast to TGF−β, BMPs, secreted in an active form, are regulated by many antagonists, who interact with BMPs directly and inhibit the binding their binding with type I and type II receptors. According to the structure, these antagonists are classified as CAN, twisted gastrulation, and chordin/noggin families.[43] Because of different affinities for BMPs and expression patterns, localization, these antagonists are able to precisely regulated BMPs signaling in a specific time and spatial manner.[44]

At the levels of the receptors, FK506-binding protein 12 (FKBP12) can end signal transmission by blocking the phosphorylation site of type I superfamily receptors from their respective type II receptors.[45,46] By clathrin-mediated endocytosis of the receptors, SARA might enhance TGF−β signaling. However, lipid raft or caveolin-mediated endocytosis results in receptor ubiquitination and degradation. [47]Furthermore, receptor trafficking was also mediated by TGF−β superfamily co-receptors, such as TBRIII and endoglin through their interactions with the scaffolding proteins, GAIP-interacting protein C-terminus (GIPC) and b-arrestin2.[48]
An interesting study reports that MicroRNA miR24 is targeting activin type I receptor ALK4 expression, but regulation of miR24 is still unclear.[49]

At the Smad level, ErbB2/Her2-interacting protein (Erbin) antagonizes phosphorylated R-Smads to make active complex with Smad4 .[29] In addition, PPM1A de-

phosphorylates nuclear phosphorylated R-Smads to recycle them back to the cytoplasm.[50] A new interesting discovery is that p53 interacts with the N-terminal MH1 domain of Smad2, and phosphorylation status of p53 is required to turn on Mix2 expression upon TGF−β activation. This is another example showing the complexity of cross-talking between TGF−β signaling and others signaling networks.[51]

Besides the above mentioned the role of PPM1A in signaling ending, I-Smads including Smad6 and Smad7, whose increased expression upon activation of the signaling, is a negative feedback loop by competing with R-Smads for binding to their respective type I receptors,[52] and degradation of Smads and receptors from recruited ubiquitinase of Smurf1and Smurf2.[53] In addition, TGF−β signaling induced secreted proteins, can also positively (protein acidic rich in cysteine (SPARC)), or negatively (cystatinC and fibulin-5), regulate the signaling.[54,55]

Due to the universal role of TGF−β signaling, dysfunction of the pathway can cause cardiovascular disease, immunity abnormality, connective tissue disease mainly from the disorder of epithelial to mesenchymal transition (EMT), reproductive disorders, metabolic disorders, skeleton and muscular disorders from imbalance of bone homeostasis and muscle dystrophy, development defect due to the break down of asymmetrical distribution of TGF−β signaling especially during embryo development(Table 2&3).[5]

In conclusion, the complexity of TGF−β superfamily signaling exerts a wide range of influences on cellular functions and normal homeostasis in a contextual manner and on varied levels.

	Disease	Superfamily signaling component			
		Antagonists	Ligands	Receptors	Smads
Cardiovascular system	Hereditary Hemorrhagic Telangiectasia			ENG, ACVRL1	SMAD4
	LoeysÖDietz syndrome			TGFBR1, TGFBR2	
	Familial thoracic aortic aneurysm syndrome			TGFBR1, TGFBR2	
	Primary pulmonary hypertension			BMPR2 ACVRL1	
Connective tissue	Marfan syndrome	FBN1		TGFBR1, TGFBR2	
	SphrintzenÖGoldberg syndrome			TGFBR2	
	Furlong syndrome			TGFBR1	
Skeletal and muscular system	CamuratiÖEnglemann disease		TGFB1		
	Fibrodysplasia Ossificans Progressiva			ACVR1	
	HunterÖThompson and Grebe-type chondrodysplasia		GDF5		
	Sclerosteosis	SOST			
	Van Buchem disease	Enhancer for SOST			
	Brachydactyly type C		GDF5		
	Brachydactyly type A2			BMPR1B	
	Symphalangism	NOGGIN	GDF5		
Reproductive system	Premature ovarian failure		BMP15, INHA		
	Persistent M¸llerian duct syndrome		MIS	AMHR2	
Developmental disorders	Situs Ambiguus	LEFTY1	NODAL		
	Cleft palate		TGFB2, TGFB3		
Metabolic disorders	Hereditary hemochromatosis			HJV	
	Juvenile hemochromatosis				
Hereditary cancer	Juvenile polyposis			BMPR1A, ENG	SMAD4
	Hereditary nonpolyposis colorectal cancer			TGFBR1	
	BannayanÖRileyÖRuvalcaba syndrome			BMPR1A	
	Cowden syndrome			BMPR1A	

Table 2. Germ-line mutations in TGF−β superfamily members in human disease (cited from Blobe et al., BBA, 1782(2008)197-228)

	Disease	Superfamily signaling component		
		Ligands	Receptors	Smads
Cardiovascular system	Atherosclerosis	Decreased TGF- 1	TGFBR2 mutations	
	Hypertension	TGFB1 polymorphisms (increased TGF- 1)		
	Dilated Cardiomyopathy	TGFB1 polymorphisms (increased TGF- 1) BMP10 polymorphisms (increased BMP10)		
	Pre-eclampsia		Increased sEnd	
	Restenosis	Increased TGF- 1 levels		
Skeletal and muscular system	Osteoporosis	TGFB1 polymorphisms (increased TGF- 1)		
Cancer	Breast cancer	TGFB1 polymorphisms (increased TGF- 1) TGFB2 polymorphisms (increased TGF- 2) Increased BMP-4 Decreased BMP-7	TGFBR1 polymorphisms; decreased T RIII, T RII, T RI; increased ALK6	
	Colon cancer	TGFB1 polymorphisms (increased TGF- 1) increased BMP	TGFBR2 mutations	SMAD2, SMAD4 mutations
	Lung cancer		TGFBR2 mutations, decreased T RII	SMAD2, SMAD3 mutations
	Pancreatic cancer	Increased TGF- 1, TGF- 2	TGFBR2 mutations, decreased T RIII, T RII, T RI	SMAD4 mutation
	Prostate cancer	TGFB1 polymorphisms (increased TGF- 1) increased GDF-15	Decreased T RIII	Increased Smad3, Smad4 nuclear localization

Table 3. Alterations in TGF−β superfamily members in sporadic human disease (cited from Blobe et al., BBA, 1782(2008)197-228)

2. TGF−β signaling and Hematopoiesis

2.1 Hematopoiesis

Hematopoiesis is a process to generate all the kinds of blood cells. It is a complex interplay between the hematopoietic stem cells(HSC) and their environment, which determine the fate of blood cells to stay quiescent, or proliferate, differentiate, self-renew, or apoptosis. HSCs, under control of a variety of growth factors, divide into two lineages, the lymphoid one giving rise to B,T and NK cells, the myeloid one differentiating to red blood cells(RBC), platelets, monocytes and granulocyte. It starts in the aorta-mesonephros-gonad region (AGM), the extra-embryonic yolk sac(YS), the placenta, the thymus and the fetal liver(FL) of the embryo, and settle down in bone marrow(BM) after birth. By the mutated estrogen receptor labeling system under control of Runx-Cre, the cells can be traced to show the progress of embryonic hematopoiesis. Together with many other observations in vitro culture and in vivo transplantation experiments, we know that the YS is the starting site of embryonic hematopoiesis, and mesodermal precursors produce both endothelial cells and hematopoietic cells in the YS. Primitive erythrocytes, primitive myeloid cells and some definitive myeloid progenitors and adult HSC were produced in this stage. The planceta and the FL can provide an appropriate environment for HSC maturation and/or expansion.[56,57]

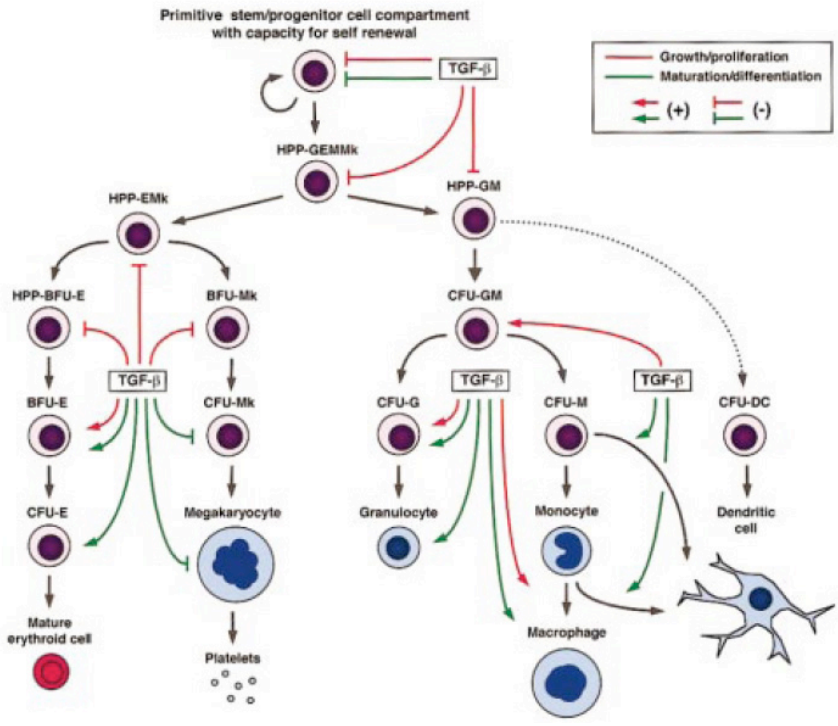


Figure 4. Regulatory effects of TGF−β on the growth/ proliferation and maturation/differentiation of developmentally distinct hematopoietic cells. (cited from Hatzfeld et al., Blood, 2000 (96) 2022-37)

In adult BM, HSCs remain relatively quiescent without overt injury, blood loss or exotic stimulus like irradiation or chemotherapy. HSCs undergo asymmetric division giving rise to a new cell for differentiation, and on keeps in the pluripotent status. How to distinguish these two daughter cells is still under investigation. Other stem cell studies might shed a light on that. The muscle stem cell would keep template strand DNA in the undifferentiated daughter cell, and give the newly synthesized DNA strand for differentiated one. The epigenetic markers on the template DNA strand might contain it original potency characteristics.[58] This process continues during the lifespan under precise modulation and cross-talking of different signaling pathways. The differentiated cells become more functional specific and vanish in the end with a lifespan from days to the whole life.

Notch, Wnt, TGF−β and Sonic hedgehog (Shh) signaling pathways are involved in this delicate and large-scale cell production. Notch signaling is active in HSCs and is down regulated when HSCs differentiate.[59] Wnt signaling regulates hematopoiesis through the pathways of Wnt/beta-catenin, Wnt/Ca^{2+}, the planar cell polarity(PCP) and Wnt/G protein. Shh is involved through the its influence on Stat3 and CDK.[60] These signaling are triggered by blood cells themselves in autocrine or paracrine ways, extracelluar matrix(ECM) components, nutrients and many other chemicals from the different microenvironments for hematopoiesis. So the hematopoiesis is a thorough collaboration of different types of blood cells and environment.

TGF−β signaling is deeply associated in numerous steps of the hematopoiesis. Many in vitro studies suggest that TGF−β inhibits the cell cycling of the most primitive hematopoietic cells. In vivo, TGF−β protects HSCs from chemotherapeutic drug, like 5-fluorouracil(5-FU).[61] Administration of TGF−β in mice modulates hematopoietic development in a lineage-specific manner. [62] TGF−β, TBRII deficient BM can cause significant autoimmune disease.[63,64] Smad4 deficient BM had HSCs renewal defect.[65] Smad1 expands the hemangioblast population between embryo day2 and day2.25.[66] Overexpression of Smad7 to block the entire TGF−β signaling in murine HSCs causes increased self-renewal in vivo.[67] Disruption of Smad5 gene leads to enhanced proliferation of high-proliferative potential precursors during embryonic hematopoiesis.[68]

TGF−β also plays a critical role in thymic T cell development and peripheral T cell homeostasis, tolerance to self antigens, and T cell differentiation during the immune response. TGF−β signaling promotes the CD8+ T cell differentiation in by studying TBRII deficient $Rag2^{-/-}$ mice.[69] TBRI deletion will delay the natural CD4+Fox3+ regulatory T(nTreg) cells differentiation. TBRII deletion can reduce the number of natural killer T (NKT) cells. The loss of naïve T cells and hypereactivation of T cell is observed in T cell specific TBR deletion mice. Differentiation from naïve T cells to Th1&2 cells was inhibited by TGF−β. nTreg cells differentiation was slow down in the same mice.[70] Induced Treg(iTreg) cells differentiation are accelerated under overexpression of TGF−β1. Th17 including regulatory (rTh17) and effector (iTh17) cells, as an critical player in innate immunity, are positively regulated by TGF−β signaling together with IL6 trigged signaling.[71,72]

Many studies reveal the defects of TGF−β signaling in hematopoietic malignancies. In acute myeloid leukemia(AML), acute promyelocytic leukemia(APL) was from t(15;17), forming the fusion protein PML-RARα. It binds the Smad/SARA/TBRI/TBRII complex, thereby dampening TGF−β signaling.[73] Some polymorphisms in TBRI and frameshifts in Smad4 are found in AML patient. In chronic myeloid leukemia(CML), Evi-1, a repressor of TGF−β signaling via binding to Smad3, is elevated.[74] In childhood T-cell acute lymphocytic leukemia (ALL), Smad3 protein is absent or significantly decreased. Smad3 deficient mice, only when combined with the loss $p27^{kip1}$, develop T-cell leukemia.[75]

In myeloproliferative diseases, including polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis with myeloid metaplasia(MMM), an activating JAK2 mutation play a major role in most cases.[76,77] However, TGF−β signaling still has complex role. In ET, PV and MMM patients, decreased the TBRII has been observed, suggesting this deregulation confer TGF−β resistance in the diseases. In TPO-induced MMM phenotype in mice, the lack of TGF−β and dominant negative TBRII expression in bone marrow can prevent the development of MMM.[78] In Chronic lymphocytic leukemia (CLL), decrease of TBR and increase of an antagonist of TBR, TBRIII, are detected.[79]

2.2. TGF−β signaling and Iron homeostasis

Iron is a trace metal for the survival of most forms of life, from animals, plants to most bacterial species. Iron is also essential for a immune system. But, together with oxygen, iron can constitute free oxygen radicals with oxidative stress, result in cell and tissue damage. Therefore, it is important to control this necessary but potentially toxic substance at a moderate (non-toxic) level. Iron homeostasis is a complex balance between toxicity of iron overload and iron deficiency. It involved in transport, uptake, utilization, and storage of iron at the transcriptional and translational level via many signaling pathways.[80]

Ferritin and transferrin sequester iron to keep it nonreactive. Transferrin receptor 1 (TRF1) is expressed on a wide range of rapidly dividing cells, activated lymphocytes and erythroid precursors. The ferri-reductase STEAP3 reduced Fe^{3+} to Fe^{2+}. The divalent metal transporter 1 (DMT1) move Fe^{2+} across the membrane.[81] Ferroportin is a transmembrane protein in enterocytes in the duodenum, hepatocytes, macrophages, that release iron from the inside of the cell. Most of iron related gene mRNA has a iron responsive elements (IREs) in the UTR region forming a stable RNA hairpins with a characteristic secondary structure. These IREs are recognized by iron regulatory proteins (IREBPs) to regulate the mRNA transcription or degradation. [82]

Hepcidin, a 25 amino acid peptide made mainly in liver, binds to ferroportin, resulting in tyrosine phosphorylation, internalization, and ubiquitin-mediated degradation. Hepcidin deficient mice develop hemochromatosis, which has iron overload in many organs, whereas overexpression of hepcidin causes anemia in human and mouse. Hepcidin gene responds to the signaling of IL6/STAT3, or BMP/Smad4. [83] Loss of function mutants in Smad4, hemojuvelin, HFE, or TFR2 in BMP/Smad4 will also lead to hemochromatosis similar to hepcidin deficiency. Hepcidin production is regulated by iron, erythropoietic activity, hypoxia and inflammation, and its levels in turn modulate plasma iron content.[84]

3. TGF−β signaling and tumorigenesis

Tumorigenesis consists of several steps including cell transformation, evasion of immune surveillance, expansion and metastasis. TGF−β signaling has a numerous

impact on these steps. It can impact cell cytostasis, differentiation, apoptosis, suppression of tumorigenic inflammation and stroma-derived mitogens, induce evasion of immune surveillance, autocrine mitogen production and motility, increase the EMT, myofibroblast mobilization, enhance extravasation, osteoclast mobilization and secretion of metastasis-related cytokines and proteases.

TGF−β inhibits cells through CDK inhibitor and suppression of c-Myc.[85] For example, loss of function of mutants in components of TGF−β signaling, might not directly trigger the premalignant progression, but render cell highly sensitivity to neoplasia. TGF−β promotes cell differentiation to prevent abnormal proliferation. It also modulates cell senescence through the regulation of the inhibitor of Differentiation/DNA bind protein (Id).[86,87,88] Deletion of Smad4 in T cell stimulates the occurrence of polyps in mouse intestine. Dominant negative TBRII in stromal cells get a high level of hepatocyte growth factor (HGF) that leads to hyperplasia of the adjacent epithelia. [89] Tumor derived TGF−β also induces hypomethylation of platelet-derived growth factor B(PDGF-B), a common mitogen found in many cancer patients. This autocrine TGF−β can inhibit the CD8+CTLs through the repression of production of cytolytic factors including the pre-forming protein perforin, the caspase-activating secreted factor granzymes A and B, and the proapoptotic cytokines Fas-ligand and IFN-γ.[90] A high level of TGF−β1 was found in many cancer metastasis.[91] Active TBRI can enhance the metastasis in mouse mammary tumors induced by ErbB2/HER2,[92] while dominant negative TBRII can prevent metastasis of human prostate cancer cells when implanted in the mouse prostate,[93] but also enhance metastasis of mouse prostate tumors caused by SV40 large T antigen.[94]

This multi-step intervention is pleiotropic, coordinative and context dependent involving in many regulation levels from epigenetics mark on DNA to complex cross-talking with other signaling pathways.

Results

Part I: Normal erythropoiesis but severe polyposis and bleeding anemia in *Smad4* deficient mice

A part of the results presented in this section was published in: Pan, D., Schomber, T., P. Kalberer, C.P., Terracciano, L.M., Hafen, K., Krenger, W., Hao-Shen, H., Deng, C., Skoda, R.C. Blood. 2007;110: 3049-3055,

Summary

The tumor suppressor Smad4 mediates signaling by transforming growth factor-beta (TGF−β) superfamily of ligands. Previous studies showed that several TGF−β family members exert important functions in hematopoiesis. Here, we studied the role of Smad4 in adult murine hematopoiesis using the inducible Mx-Cre/loxP system. Mice with homozygous *Smad4* deletion ($Smad4^{\Delta/\Delta}$) developed severe anemia 6-8 weeks after induction (mean hemoglobin 70g/L). The anemia was not transplantable, as wild type mice reconstituted with $Smad4^{\Delta/\Delta}$ bone marrow cells had normal peripheral blood counts. These mice did not develop an inflammatory disease typical for mice deficient in *TGF−β* receptors I and II. The same results were obtained when *Smad4* alleles were deleted selectively in hematopoietic cells using the *VavCre* transgenic mice. In contrast, lethally irradiated $Smad4^{\Delta/\Delta}$ mice transplanted with wild type bone marrow cells developed anemia similar to non-transplanted $Smad4^{\Delta/\Delta}$ mice. Liver iron stores were decreased and blood was present in stool, indicating that the anemia was due to blood loss. Multiple polyps in stomach and colon represent a likely source of the bleeding. We conclude that *Smad4* is not required for adult erythropoiesis and that anemia is solely the consequence of blood loss.

Mice with a MxCre inducible double knockout ($Smad4^{\Delta/\Delta}$ and $TBRII^{\Delta/\Delta}$) did not display the $TBRII^{\Delta/\Delta}$ -driven lethal inflammation suggesting that *Smad4* signaling is required to mediate the inflammatory phenotype. Furthermore, *Smad4/TBRII* was dispensable for megakaryopoiesis and erythropoiesis.

Introduction

Smad4 is necessary for signaling by both the TGF−β and the BMP families of ligands. *Smad4*$^{-/-}$ mice can be expected to show severe defects in hematopoiesis. However, *Smad4*$^{-/-}$ mice die during embryogenesis before the onset of hematopoiesis. To directly investigate the role of Smad4 in hematopoiesis, we crossed mice with a conditional Smad4 knockout allele (*Smad4*$^{fl/fl}$), and a strain containing a Cre-recombinase gene controlled by the interferon-inducible Mx1 promoter (*Mx-Cre*). The Mx-Cre inducible mouse was widely used in studies of hematopoiesis and showed high efficiency of recombination in bone marrow and other tissues. Upon induction of Mx-Cre expression, the conditional *Smad4* alleles (fl/fl) recombined to yield dysfunctional Smad4 alleles (Δ/Δ) and these mice developed severe anemia by 6-8 weeks after induction. To inactivate the *Smad4* conditional allele in hematopoietic cells only, we crossed the *Smad4* mice to the *VavCre* strain, which expresses Cre selectively in hematopoietic cells. We show that erythropoiesis was not directly affected by the loss of Smad4. Rather, anemia is the consequence of blood loss from polyps that rapidly form in the stomach and colon of these mice.

TGF−β signaling through the TBRII is mediated by Smad4, but a Smad4-independent pathway also exists. Furthermore, Smad4 also mediates the signals from other type II receptors.[23] We investigated the *Smad4*/*TBRII* pathway in hematopoiesis by transplanting Mx-Cre induced *Smad4*/*TBRII* double excised bone marrow cells into wild type BL6 recipients. No weight loss and inflammatory symptoms were observed, whereas lethal inflammation caused by single *TBRII* deletion bone marrows was showed in other reports.[63 64]

To study more linage-specific impact of gene expression in hematopoiesis, a mouse strain containing platelet factor 4 (Pf4) promoters driven Cre (*Pf4-Cre*) was developed in our lab, [95] which can direct expression of Cre recombinase to megakaryocytes and precursors. This new strain using a bacterial artificial chromosome (BAC) clone containing the entire mouse Pf4 promoter, can avoid position effect variegation compared to old relative short promoter transgene means. We crossed this *Pf4-Cre*

strain with *TBRII*$^{fl/fl}$ and *Smad4*$^{fl/fl}$ to obtain the *Pf4-Cre;TBR*$^{fl/fl}$*;Smad4*$^{fl/fl}$ strain. This megakaryocyte-specific TBR and Smad4 double deficient strain shows normal platelets.

Results

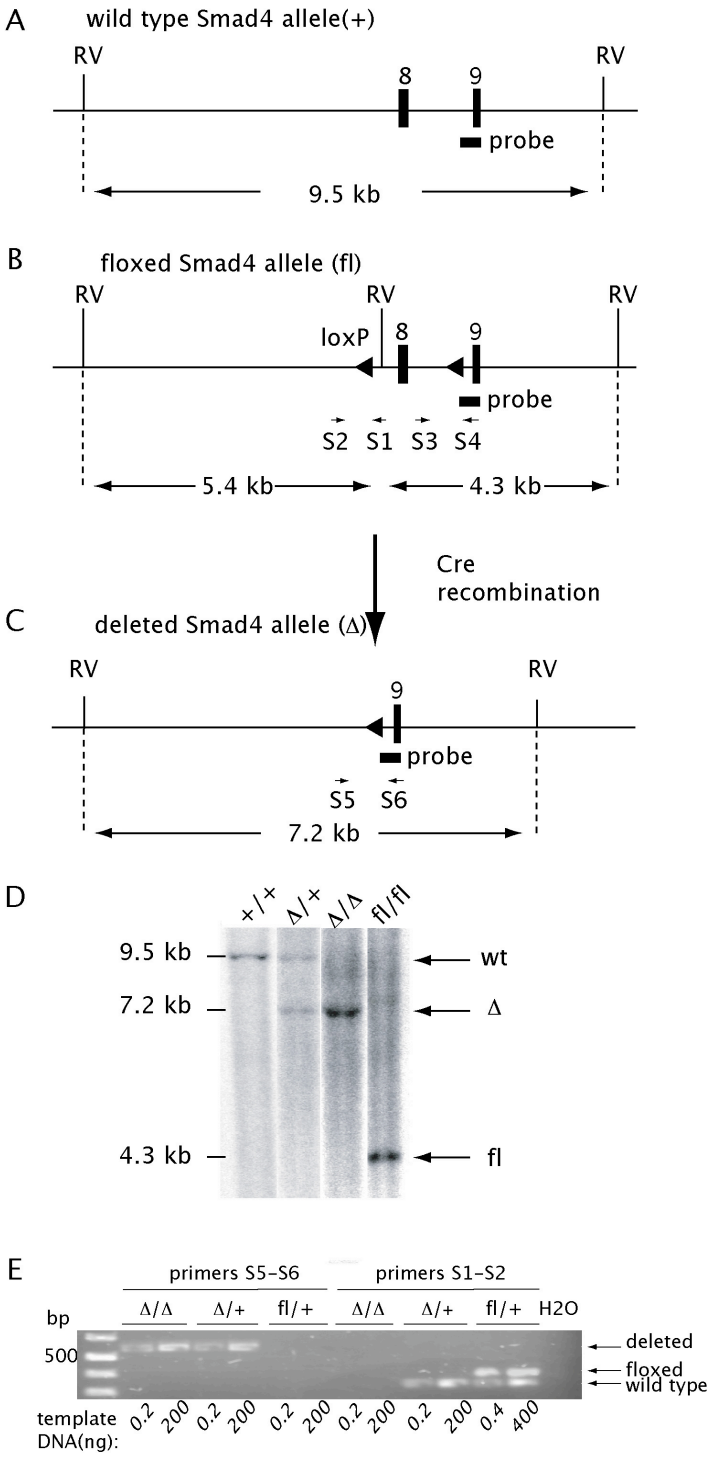


Figure 1

Genomic structure of Smad4 conditional knockout allele. The probe used for Southern blot is indicated by a thick horizontal line. A) The wild type allele of Smad4 with EcoRV sites located in introns 7 and 9 is shown. B) The conditional allele (fl) with insertion of two loxP sites (triangles) and an additional EcoRV site. C) The deleted allele after Cre recombination (Δ). S1-S5, position of primers used for genotyping. D) Southern blot of DNA from bone marrow digested with EcoRV is shown. Arrows indicate the positions of the wild type allele (wt), the floxed conditional allele (fl), and the deleted allele after Cre recombination (Δ).

Mice with induced deletion of Smad4 develop severe anemia

Four weeks after Mx-Cre induced excision of *Smad4*$^{fl/fl}$, the resulting *Smad4*$^{\Delta/\Delta}$ mice developed anemia and after 8 weeks the hemoglobin concentration and hematocrit were decreased to 30% of normal values (Figure 2A). The mean corpuscular volume (MCV)

was unchanged, but the reticulocyte count was strongly increased. The white blood count and platelet levels remained unchanged. The *Smad4*$^{\Delta/\Delta}$ alleles were detectable by PCR in 39/40 bone marrow derived colonies (not shown). The apparent half-life of erythrocytes from *Smad4*$^{\Delta/\Delta}$ mice was reduced to 10-12 days, while the half-life in *Smad4*$^{fl/fl}$ control mice was 23 days(Figure 2B). A direct antiglobulin test showed no evidence for IgM or IgG surface antibodies on erythrocytes from *Smad4*$^{\Delta/\Delta}$ mice, arguing against autoimmune-antibody-meditated hemolytic anemia (not shown). The apparent reduction of the erythrocyte half-life in circulation can be explained by compensatory increase in regeneration, marked by massive reticulocytosis (Figure 2) and increased erythropoietin serum levels (>5000 pg/ml, n=5; normal range 50-200 pg/ml, n=4).

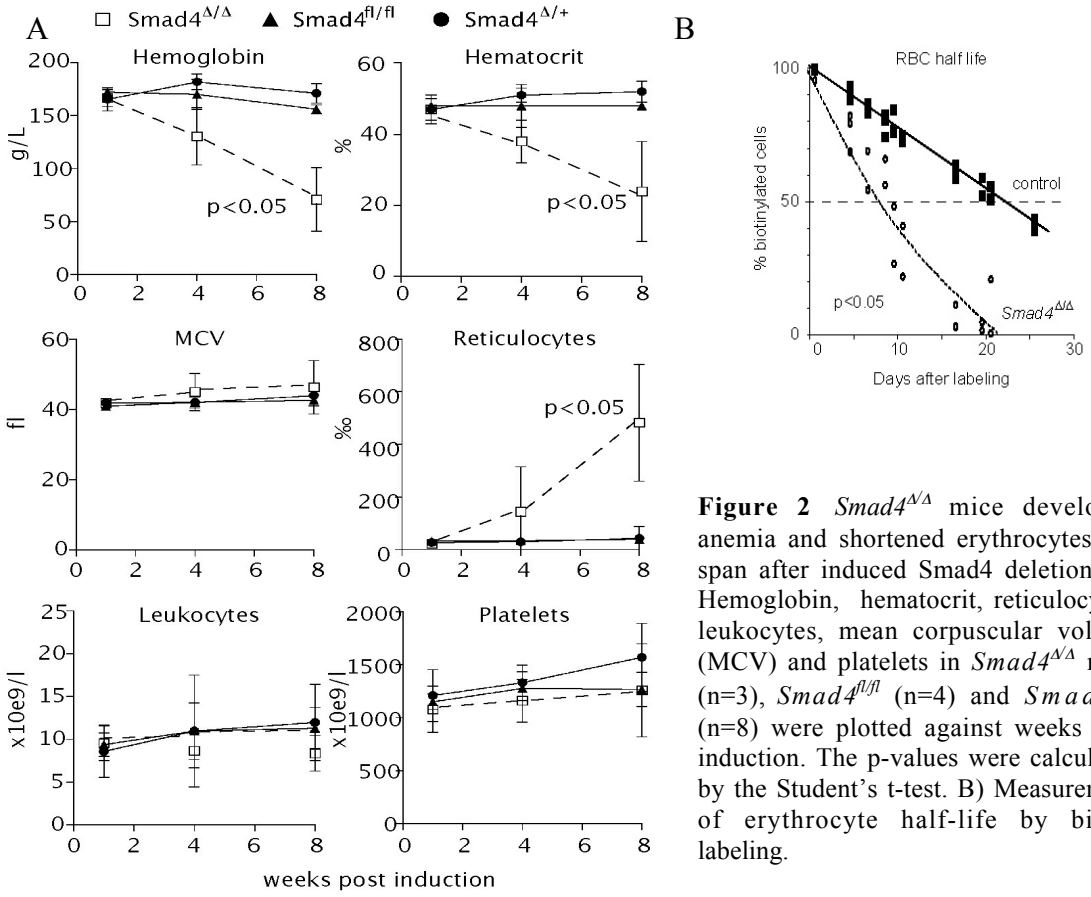


Figure 2 *Smad4*$^{\Delta/\Delta}$ mice developed anemia and shortened erythrocytes life span after induced Smad4 deletion. A) Hemoglobin, hematocrit, reticulocytes, leukocytes, mean corpuscular volume (MCV) and platelets in *Smad4*$^{\Delta/\Delta}$ mice (n=3), *Smad4*$^{fl/fl}$ (n=4) and *Smad4*$^{\Delta/+}$ (n=8) were plotted against weeks post induction. The p-values were calculated by the Student's t-test. B) Measurement of erythrocyte half-life by biotin labeling.

Smad4 is dispensable for adult murine erythropoiesis

To determine whether the observed anemia was cell-autonomous, bone marrow cells from *Mx-Cre;Smad4*$^{fl/fl}$ mice and controls were transplanted into lethally irradiated recipient mice. Eight weeks after bone marrow transplantation, the mice were sacrificed

and peripheral blood counts were performed (Figure 3). The red blood cell parameters as well as white blood counts and platelets remained normal (Figure 3). The recipients of *Smad4*$^{\Delta/\Delta}$ bone marrow cells did not show any signs of inflammatory disease typical for knockouts of the TGF−β receptors (i.e. absence of weight loss, leukocytosis, signs of inflammation of the eyes and upon autopsy absence of organ damage). Chimerism of recipient mice was determined in peripheral blood by assessing the ratio of CD45.2 donor cells to CD45.1 recipient cells by flow cytometry. Both groups of mice displayed a ratio of donor to recipient cells of greater than 100:1 (Table 2). No differences in B cells (B220), T cells (CD3), and myeloid cells (Gr-1) were detected in bone marrow. Deletion of the floxed Smad4 alleles was found by PCR in DNA from peripheral blood cells of donor *Smad4*$^{\Delta/\Delta}$ mice (not shown). Thus, anemia was not transplantable with Smad4-deficient bone marrow cells, indicating that Smad4 is dispensable for adult murine erythropoiesis.

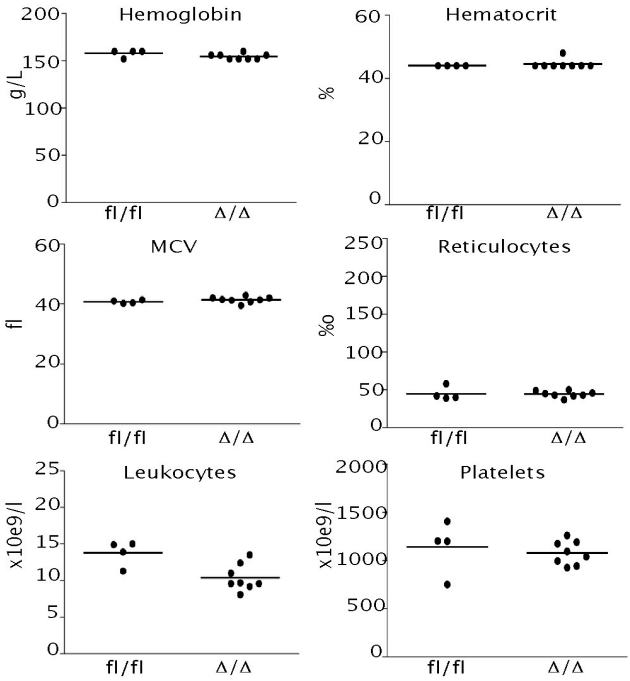


Figure 3 Transplantation of *Smad4*$^{\Delta/\Delta}$ bone marrow cells into wild type recipient mice did not lead to the development of anemia. Hemoglobin, hematocrit, mean corpuscular volume (MCV), reticulocytes, leukocytes and platelets remained stable in control and experimental group. Eight recipients of *Smad4*$^{\Delta/\Delta}$ bone marrow cells and 4 recipients of Smad4$^{fl/fl}$ controls were analyzed.

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To confirm this observation in a system not depending on transplantation, we generated mice with a hematopoietic specific deletion of Smad4. The *VavCre* strain has been shown to excise loxP target sequences in hematopoietic cells only.[96] The resulting *VavCre;Smad4*$^{fl/fl}$ mice had normal blood counts and showed no symptoms of inflammation (Figure 4). We also confirmed practically complete excision of *Smad4* in

peripheral blood cells of these mice by PCR (Figure 1E). These results implied that host factors might be causing the anemia phenotype.

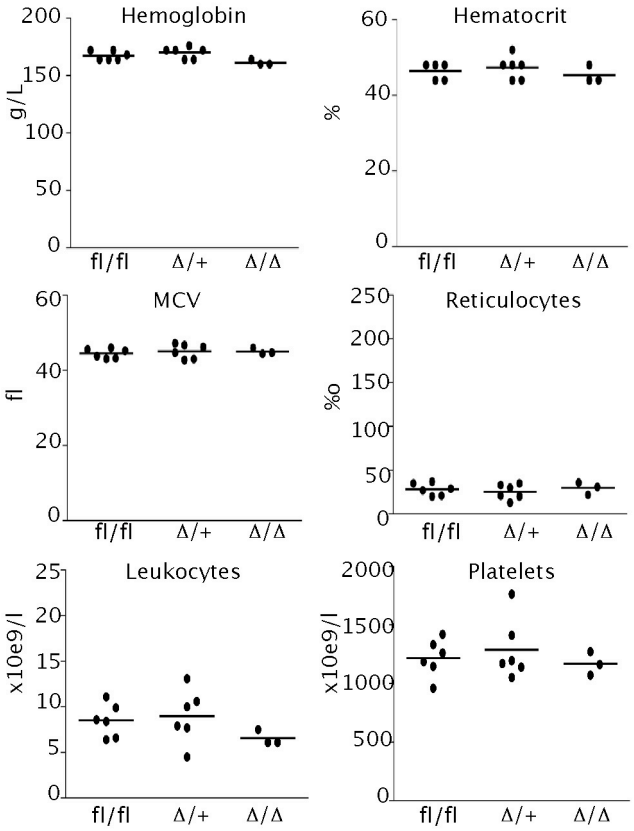

Figure 4 *VavCre;Smad4*$^{fl/fl}$ mice did not develop anemia. Peripheral blood parameters were determined in 10 weeks old mice. *VavCre;Smad4*$^{fl/fl}$ (Δ/Δ), *VavCre;Smad4*$^{fl/+}$ (Δ/+) and *Smad4*$^{fl/fl}$ (fl/fl). Dots represent the values of individual mice, horizontal lines indicate the mean.

Table 2 Hematopoietic lineage distribution in bone marrow of wild type recipient mice transplanted with *Smad4*$^{\Delta/\Delta}$ or *Smad4*$^{fl/fl}$ bone marrow

	Smad4$^{\Delta/\Delta}$	*Smad4*$^{fl/fl}$
	(n=4)	(n=4)
CD45.2+	87.4 ± 3.3	91.1 ± 3.2
CD45.1+	0.24 ± 0.1	0.50 ± 0.4
B220+	8.4 ± 2.1	5.8 ± 0.2
CD3	2.5 ± 0.9	2.4 ± 0.6
Gr1	14.8 ± 1.2	14.4 ± 1.3

Donor cells (CD45.2+), recipient cells (CD45.1+)

Anemia of *Smad4*$^{\Delta\Delta}$ mice is non cell-autonomous

To determine if anemia is caused by the host environment, bone marrow from wild type C57BL/6J mice was transplanted into lethally irradiated *Mx-Cre;Smad4*$^{fl/fl}$ and *Smad4*$^{fl/fl}$ control mice. From week 2-4 after pIpC-induced deletion of Smad4, recipients began developing anemia (Figure 5). Interindividual differences in the severity of anemia were observed in *Smad4*$^{\Delta/\Delta}$ mice. The control *Smad4*$^{fl/fl}$ recipient mice remained healthy

without any changes in blood parameters. These results demonstrate that anemia of *Smad4*$^{\Delta/\Delta}$ mice is caused by alterations outside of the hematopoietic system.

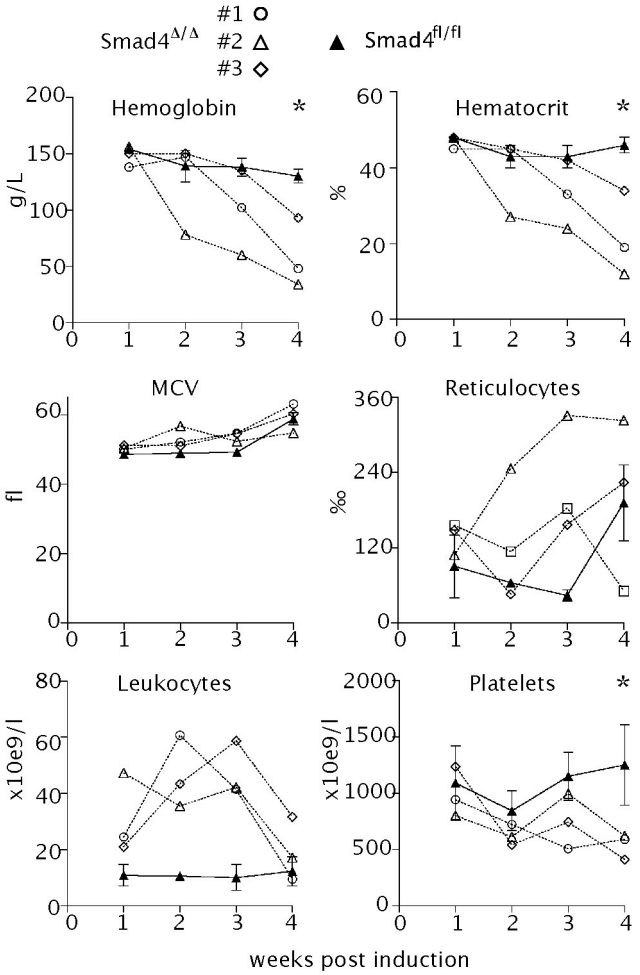

Figure 5 Transplantation of wild type bone marrow into *Smad4*$^{\Delta/\Delta}$ recipients leads to anemia. Hemoglobin, hematocrit, mean corpuscular volume (MCV), reticulocytes, leukocytes and platelets are shown. Three individual Smad4$^{\Delta/\Delta}$ recipient mice (#1, #2, and #3 with open symbols) developed anemia with individual differences in severity and kinetics. Asterisks denotes significant differences at 4 weeks ($p \leq 0.03$; Student's t-test). The values for the Smad4$^{fl/fl}$ control recipient mice (filled triangles) are shown as the mean of 6 mice with standard deviation.

Smad4$^{\Delta/\Delta}$ mice show severe iron deficiency

The presence of hypochromic erythrocytes at later stages of anemia in *Smad4*$^{\Delta/\Delta}$ *mice* (Figure 6A) suggested that iron deficiency can be involved in the pathogenesis of anemia. Liver iron in *Smad4*$^{\Delta/\Delta}$ mice 4 weeks after pIpC induction was decreased to 23% (2.6±0.3 μmol/g, n=6), as compared to liver iron of *Smad4*$^{fl/fl}$ mice (11.5±4.0 µmol/g, n=6) or wild type C57BL/6J (16.6±4.7 µmol/g, n=3). No differences in plasma transferrin (Tf), determined by ELISA specific for mouse Tf, were found between Smad4$^{\Delta/\Delta}$ mice (1.9 ± 0.2 µg/l, n=6) and *Smad4*$^{fl/fl}$ (1.9 ± 0.9 µg/l, n=10) or wild type BL6 mice (1.7 ± 0.08 µg/l, n=3). We determined the expression of genes that are involved in iron metabolism by quantitative PCR (Figure 6B and 5C). The efficiency of

Cre-mediated excision in the livers of Smad4$^{\Delta/\Delta}$ mice measured by Southern blot was ranging 66-96% (not shown) and the expression of Smad4 mRNAs was severely

Table 1 Sequences of primers used for quantitative RT-PCR

Gene	Forward primer	Reverse primer
Smad4	GTTCAGGTAGGAGAGACGTTTAAGGT	CCTTTACATTCCAACTGCACTCCT
Hepc	CCTATCTCCATCAACAGATG	AACAGATACCACACTGGGAA
Fpn	AAGGATTGACCAGCTAACCAACA	CAGCCAATGACTGGAGAACCA
Dcytb	GCAGCGGGCTCGAGTTTA	TTCCAGGTCCATGGCAGTCT
Dmt1	AACCAACAAGCAGGTGGTTGA	CTTTGTAGATGTCCACAGCCA
Transferrin	TTGTGCCATCCCATCACAAC	CTAGTGTCCGATGCCTTCACC
Hephaestin	TTGTCTCATGAAGAACATTACAGCAC	CATATGGCAATCAAAGCAGAAGA
Hfe	CTGAAAGGGTGGGACTACATGTTC	GGACACCACTCCCAACTTCGT
Tfr1	CAGAAAGTTCCTCAGCTCAACCA	GTTCAATTCAACGTCATGGGTAAG
Tfr2	AGCTGGGACGGAGGTGACTT	TCCAGGCTCACGTACACAACA
Sft	CTGTGCTCATTGAAGAGGACCTT	TCTGGTTGCTTTCTCAGTCACG

Smad4, small mutants (C. elegans) and mothers against decapentaplegic homolog 4 (Drosophila); *Hepc*, hepcidin; 1 *Fpn*, *ferroportin*; *Dcytb*, cytochrome b reductase 1; *Dmt1*, divalent metal transporter 1; *Tf*, transferrin; Heph, hephaestin; *Hfe*, major histocompatibility complex class I-like protein; *Tfr1*, transferrin receptor 1; *Tfr2*, transferrin receptor 2; *Sft*, stimulator of Fe transport

reduced (Figure 6B). To assure that only the full length mRNA was measured, the forward primer (Table 1) used for the quantification of the full length *Smad4* mRNA was placed in exon 8, which is deleted by Cre-mediated excision. We also determined the expression of genes involved in the regulation of iron metabolism. Hepcidin (*Hepc*) mRNA was severely decreased, whereas the expression of divalent metal transporter 1 (*Dmt1*), cytochrome b reductase 1 (*Dcytb*), ferroportin 1 (*Fpn*), and transferrin (*Tf*) remained unchanged (Figure 6B). In the duodenum, the floxed Smad4 allele was excised only by 4-8% (not shown) and Smad4 mRNA was just merely decreased, while *Dmt1* and *Dcytb* increased 5-50 fold, and transferrin receptor 2 (*Tfr2*) increased 2-20 fold (Figure 6C). Other iron related genes, such as major histocompatibility complex class I-like protein (*Hfe*), transferrin receptor 1 (*Tfr1*), hephaestin (*Heph*), and stimulator of Fe transport (*Sft*) showed no significant changes in expression (Figure 6C). Taken together, these changes fit well with a state of increased iron uptake and demand.

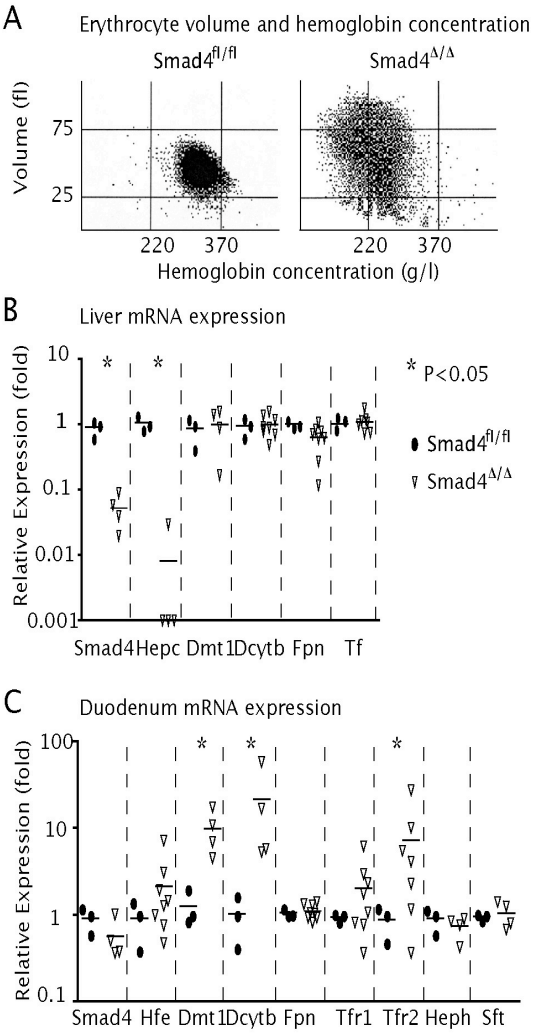

Figure 6 *Smad4*$^{\Delta/\Delta}$ mice display severe iron deficiency anemia. A) Hypochromic erythrocytes in Smad4$^{\Delta/\Delta}$ mice. Cell volume was plotted against hemoglobin concentration. Left panel, normal control. Right panel shows hypochromic red blood cells (<220 g/L) and volume increase (> 75 fl) due to reticulocytosis in *Smad4*$^{\Delta/\Delta}$. B) In liver, *Smad4* and hepcidin (*Hepc*) expression are almost abrogated, and ferroportin 1 (*Fpn*) is slightly decreased. Cytochrome b reductase 1 (*Dcytb*), divalent metal transporter 1 (*Dmt1*) and transferrin (*Tf*) were unchanged. C) In Duodenum, Smad4, Fpn, hephaestin (*Heph*), major histocompatibility complex class I-like protein (*Hfe*), transferrin receptor 1 (*Tfr1*) and stimulator of Fe transport (*Sft*) were unchanged, and Dmt1, Dcytb, and transferrin receptor 2 (*Tfr2*) were dramatically increased. *Smad4*$^{fl/fl}$ littermates were chosen as controls. The p-values were calculated by Student's t-test.

Polyps in stomach and colon cause blood loss in *Smad4*$^{\Delta/\Delta}$ mice

Histopathology of the GI tract of *Smad4*$^{\Delta/\Delta}$ mice revealed polyps in stomach and colon (Figure 7), but not in the small intestine. Most of them were histologically characterized by a branching architecture reminiscent of hyperplastic lesions, mostly with foci of low- and/or high-grade dysplasia. Moreover, "serrated" aspects of the polyps were also detected focally. Additionally, colon polyps frequently displayed cystic changes. None of the gastric or colon polyps fulfilled the criteria of "juvenile polyps", as they lacked the typical histological features of such lesions, e.g. prominent stroma overgrowth. To show that iron deficiency in *Smad4*$^{\Delta/\Delta}$ mice is due to GI bleeding, we collected stool over several weeks and determined the presence of heme by the hemoccult assay (Figure 8). Bleeding was detectable in all mice, but with variable onset and duration. In some mice, bleeding started 17 days after pIpC induction of *Smad4* deletion, whereas in others the onset was delayed until 31 days. At the time of the first detectable bleeding

the mice did not yet display severe anemia and the severity of anemia did not correlate with the time of onset of bleeding.

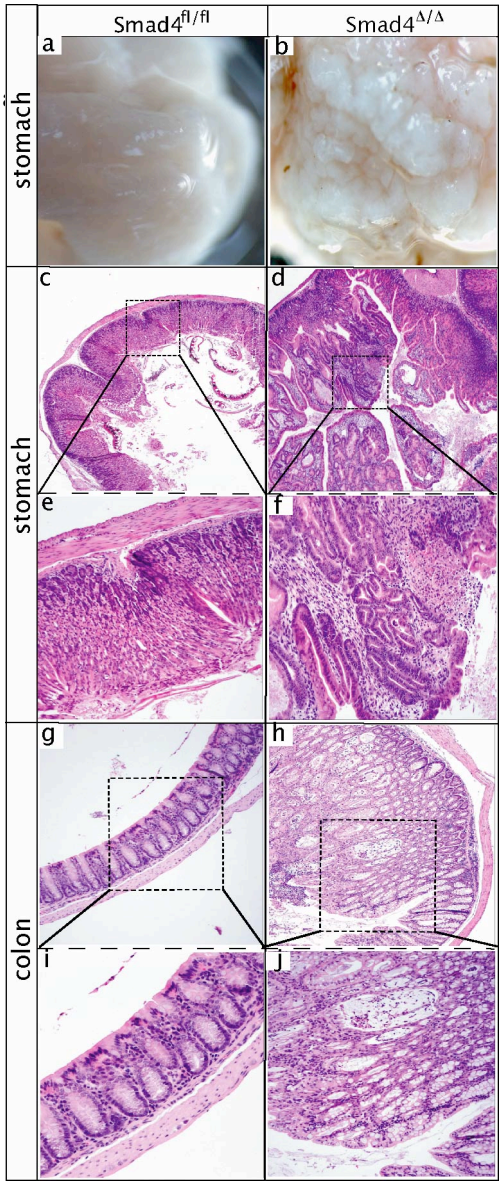

Figure 7 Stomach and colon polyp formation in *Smad4*$^{\Delta/\Delta}$ mice. Left panel shows a *Smad4*$^{fl/fl}$ control mouse, right panel shows a Smad4$^{\Delta/\Delta}$ mouse. Gross macroscopy of stomach (a, b), histological hematoxylin-eosin staining of stomach (c, d, e, f) and colon (g, h, i, j) with magnified view of the boxed

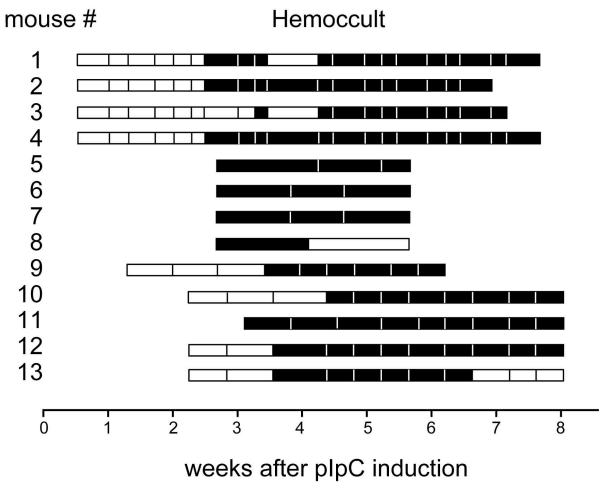

Figure 8 Fecal occult blood test in *Smad4*$^{\Delta/\Delta}$ mice. A total of 13 mice were analyzed (numbered in y-axis). Time in weeks after first pIpC injection is shown on the x-axis. Horizontal bars represent the duration of the stool collection; empty box, negative hemoccult tests; filled boxes, positive hemoccult tests

Smad4 deficiency increase adult T cells proliferative capacity, but allows normal T cell development and has no effects on B cells development

Even though no difference in T cells (CD3) was found in Smad4 deficient donor bone marrow cells in normal recipients, in peripheral blood, higher percentage of CD3 positive cells was observed compared to the controls (45.8±5.7% vs 23.0±1.3%, $p<0.05$, n=4 respectively) and B cell remain unchanged (46.9±5.8% vs 37.8±2.8%, $p>0.05$, n=4 respectively). Furthermore, *Vav-Cre;Smad4*$^{fl/fl}$ mice also display the similar phenotype (Figure 9). To examine whether this T cells difference is coming from abnormal T cells development, *Vav-Cre;Smad4*$^{fl/fl}$ mice at the age of 6 weeks were sacrificed to collect thymus cells for flow cytometry analysis of CD25,CD44 and Lin- markers. The results showed no difference in TN1-4 stages, four T cells development steps. This implies a role of Smad4 in expansion of T cells instead of development.

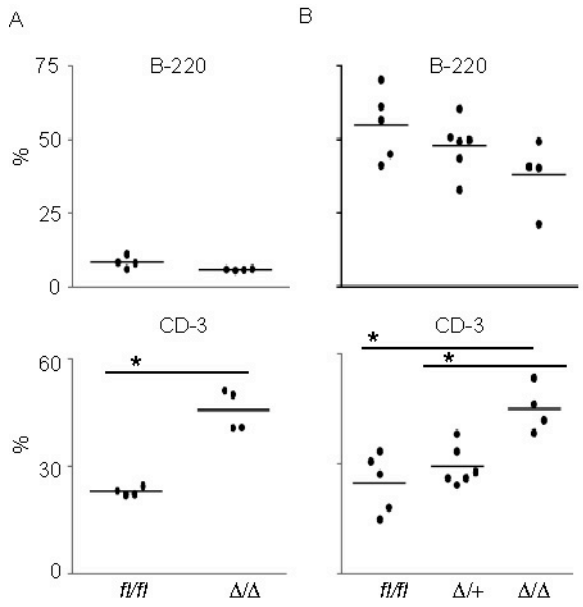

Figure 9. Smad4 deficiency increased T cells in peripheral blood. B cells and T cells lineage were performed by flow cytomety analysis. A) Wild type BL recipient mice reconstituted with bone marrow cell from *Smad4*$^{\Delta/\Delta}$ (n=4), or with *Smad4*$^{fl/fl}$ (n=4) background were analyzed 10 weeks after transplantation. B) *VavCre;Smad4*$^{fl/fl}$ (Δ/Δ), *VavCre;Smad4*$^{fl/+}$ (Δ/+) and *Smad4*$^{fl/fl}$ (fl/fl) mice in 10-week-old. * means statistical significance. The p-values were calculated by Student's t-test.

Mice bone marrows with Mx-Cre induced *Smad4/TBRII* double deletion cause no inflammatory symptom in normal BL6 recipients

To achieve double deletion of Smad4 and TBR in hematopoiesis, bone marrow cells from pIpC-induced *Mx-Cre;TBR*$^{fl/fl}$*;Smad4*$^{fl/fl}$ and controls were transplanted into lethally irradiated recipient mice. Eight weeks after bone marrow transplantation, mice were subject to phlebotomy to perform the peripheral blood counts. Successful transplantation was confirmed by analyzing the ratio of CD45.2 donor cells and CD45.1 recipient cells with flow cytometry, and excision of Smad4 and TBR was confirmed by PCR from peripheral blood samples.

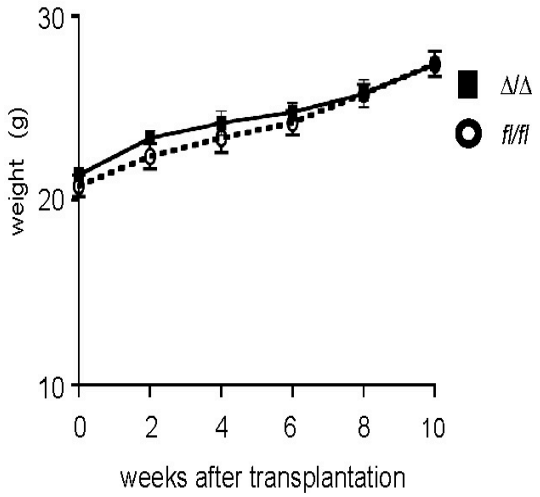

Figure 10. Transplantation of *Smad4*$^{\Delta/\Delta}$*;TBRII*$^{\Delta/\Delta}$ bone marrow cells into wild type recipient mice did not lead to mice show normal growth rate. The weight of wild type BL recipient mice reconstituted with bone marrow cell from *Smad4*$^{\Delta/\Delta}$ */TBRII*$^{\Delta/\Delta}$ (Δ/Δ, n=5), or with *Smad4*$^{fl/fl}$/ *TBRII*$^{fl/fl}$ (fl/fl, n=5) background were monitored up to 10 weeks after transplantation.

These recipient mice had no weight loss (Figure 10) and showed no symptom as described in another report with single TBRII-deficient bone marrow cells transplantation. The blood counts in these mice were also normal (Figure 11).

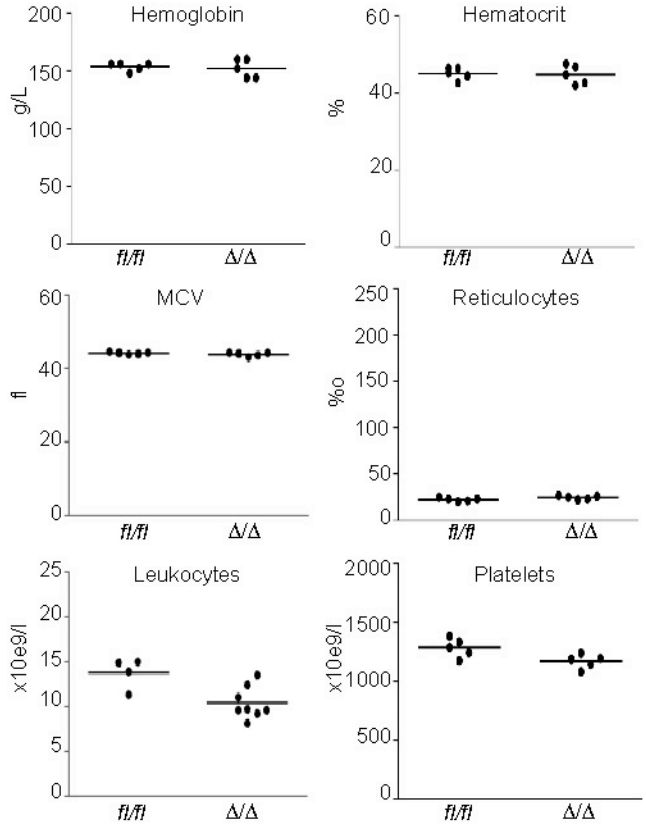

Figure 11. Transplantation of *Smad4*$^{\Delta/\Delta}$*/TBRII*$^{\Delta/\Delta}$ bone marrow cells into wild type recipient mice did not lead to any inflammation symptoms. Hemoglobin, hematocrit, mean corpuscular volume (MCV), reticulocytes, leukocytes and platelets remained stable in control and experimental group. Five recipients of *Smad4*$^{\Delta/\Delta}$ */TBRII*$^{\Delta/\Delta}$ bone marrow cells and 5 recipients of *Smad4*$^{fl/fl}$*/ TBRII*$^{fl/fl}$ controls were analyzed.

Double deletion of *Smad4/TBRII* in megakaryocytes has no effect on circulating platelet counts

Because the above study of double deletion of Smad4 and TBR in hematopoiesis was performed in artificial environment due to transplantation, and *Mx-Cre;TBR$^{fl/fl}$;Smad4$^{fl/fl}$* mice developed lethal symptom in normal physiological condition after deletion induction, we used *Pf4-Cre;TBR$^{fl/fl}$;Smad4$^{fl/fl}$* mice to study megakaryopoiesis under natural physiological condition. The *Pf4-Cre;TBR$^{fl/fl}$;Smad4$^{fl/fl}$* mice with megakarycyte-specific excision did not show any alterations in circulating platelet counts (Figure 12), in agreement with the above results obtained by transplantation of with pIpC-induced *Mx-Cre;TBR$^{fl/fl}$;Smad4$^{fl/fl}$* double gene deletion bone marrow cells into normal recipients.

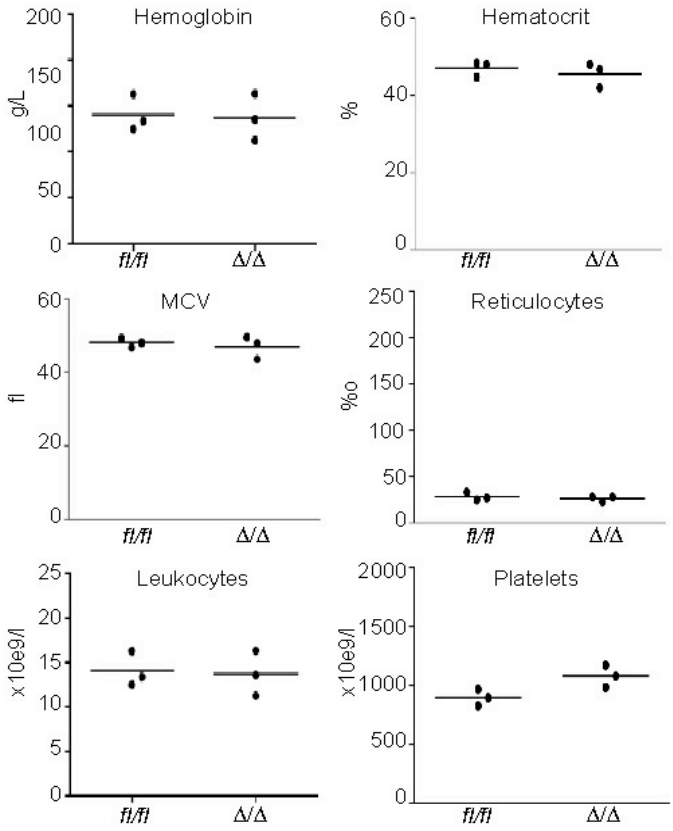

Figure 12. Double deletion of Smad4/TBRII in megakaryocyte has no effect on circulating platelet counts. Peripheral blood parameters were determined in 6 weeks old mice. *Pf4Cre;Smad4$^{fl/fl}$/TBRII$^{Δ/Δ}$* (Δ/Δ), and *Smad4$^{fl/fl}$/ TBRII$^{fl/fl}$*(fl/fl)

Discussion

Our results from transplantation of *Smad4*$^{\Delta/\Delta}$ bone marrow into wild type recipients and deletion of Smad4 selectively in hematopoietic cells in *VavCre;Smad4*$^{fl/fl}$ mice demonstrate that Smad4 signaling is dispensable for adult erythropoiesis in vivo (Figure 3 and 4). The red blood cell parameters in peripheral blood were normal. Our study is consistent with the results of an in vitro study that examined cultured human CD34+ hematopoietic stem/progenitor cells under shRNA mediated Smad4 knock-down.[15] We also found normal megakaryopoiesis in our study, arguing that the decrease in circulating platelet counts and increase in megakaryocyte numbers in mice injected with TGF−β protein in vivo is mediated by Smad4-independent signaling.[97] The white blood counts of neutrophil, basophil and eosinophil granulocytes as well as monocytes and lymphocytes were normal. The severe anemia observed in *Smad4*$^{\Delta/\Delta}$ mice is caused by blood loss.

TGF−β signaling plays an important role in immune surveillance. By producing the immunosuppressive cytokine TGF−β, tumors may escape from immune surveillance via inhibiting the expression of cytolytic genes.[90] TGF−β receptor II (TBR II) dominant-negative approaches led to autoimmune inflammatory disease and spontaneous T-cell activation.[98] Mice with TBR II deletion in bone marrow showed not only increased CD8+ proliferation in vivo, but also developed a lethal inflammatory disease,[69,99] and the TBR II deficient cells of hematopoietic origin, most likely T cells, can induce multifocal inflammatory disease in a dominant way. On the contrary, our *Smad4*$^{\Delta/\Delta}$ bone marrow recipients and *VavCre;Smad4*$^{fl/fl}$ mice were healthy and did not show any signs of inflammatory disease. This result implies that inflammation is not mediated through TBRII-Smad4 pathway. It remains to be determined, whether this effect is mediated by the traditional TGF−β pathway with an alternative downstream mediator like TIF1, or by crosstalk with other signaling through MAPK, JNK, PI3K, or other mediators. Furthermore, these two strains both show higher T cells number in peripheral blood, but TN1-4 flow cytometry analysis shows normal T cells development. This observation is consistent with a previous report showing that TBRII deficient thymocytes develop normally but demonstrating increased CD8+ proliferation in vivo,[69] which implies that this enhanced proliferative capacity might be due to TBRII/SMAD4 signal blockage.

An interesting finding is that double deletion of TBRII and Smad4 did not cause any symptom of inflammation, which is in contrast to multi-focal lethal inflammation caused by single deletion of TBRII. Due to complex cross-talking with other signaling pathways in the TGF−β signaling network, that whether Smad4 suppressed gene is required for *TBBRII* deficient-mediated inflammation symptom still remain unclear.

By using mice with megakaryocyte-specific Cre expression, we can investigate the role of TBRII and Smad4 in magakaryopoiesis. Previous reports showed the inhibitory effect of TGF−β1 on CFU-Megs in vitro,[95] decreased platelets with TGF−β1 administration in mouse, and lower mRNA expression of TGF−β1 and its receptors in patients with myeloproliferative disorders. Single deletion of TBRII (Tibor Schomber's PhD thesis, Basel University) or Smad4 has been proven to have no effect on megakaryopoiesis. Our double deletion experiment in *Pf4-Cre* transgenic line is firmly in agreement with this observation. Together with transplantation with Mx-Cre induced double deletion bone marrows, the potential role of TBRII-Smad4 pathway in megakaryopoiesis was excluded.

Smad4 is a tumor suppressor gene, and Smad4 mutations are frequently detected in pancreatic cancer, colon cancer, gastric polyps and adenocarcinomas.[100-104] Mice heterozygous for the Smad4 knockout appear normal, but develop gastrointestinal polyps after a long latency with loss of heterozygosity in the epithelial cells.[105,106] Our *Smad4*$^{\Delta/\Delta}$ mice, which have complete excision in bone marrow as well as partially in stomach (10-50%), started bleeding around day 20 after pIpC induction (Figure 8), and quickly developed a severe anemia and significant polyps in the GI tract without formation of real tumors before they needed to be sacrificed. Ablation of TGF−β by T-cell specific over-expression of dominant negative TBR II can accelerate azoxymethane induced colon carcinogenesis through increased T-cell production of IL6.[107] Recently, it was reported that T cell specific deletion of Smad4 leads to spontaneous epithelial tumors throughout the GI tract. Mice in this study displayed weight loss starting 3 month after birth and eventually developed clinical features of systemic illness. In contrast, mice with epithelial-specific deletion of Smad4 remained free of polyps, suggesting that polyp formation and tumorigenesis are induced by Smad4 deficient T cells.[70] Our *Mx-Cre;Smad4*$^{\Delta/\Delta}$ mice developed polyps with a much faster kinetics and

since they suffered from severe bleeding anemia, we can not examine tumor progression. When *Mx-Cre* induced *Smad4*$^{\Delta/\Delta}$ mice were used as recipients for the transplantation of normal bone marrow cells, we observed rapid evolution of anemia (Figure 5), which against that the dispensable role of Smad4 in polyps onset. [70] However, we cannot exclude that some host T cells deficient for Smad4 remained functional in these mice.

Recently, shRNA mediated knockdown of bone morphogenic protein type II receptor (*Bmpr2*), another member of the TGF−β receptor family, resulted in severe mucosal hemorrhage, gastrointestinal hyperplasia and blood loss.[66] This phenotype was related to vascular dysmorphogenesis. Other studies suggested that malformed vessels are present in polyps of *SMAD4-/-* juvenile polyposis.[108]In contrast, no malformations of blood vessels were observed in polyps from our *Smad4*$^{\Delta/\Delta}$ mice.

Our *Smad4*$^{\Delta/\Delta}$ mice showed an almost complete loss of hepcidin mRNA expression in liver. This can be due to two mechanisms. One, liver-specific Smad4 deletion results in markedly decreased hepcidin transcription,[108] and second, increased erythropoiesis due to anemia suppresses the production of hepcidin.[109] In our mice, both mechanisms are likely to be contributing to the strongly suppressed hepcidin expression.

Our results demonstrate that Smad4 is dispensable for adult erythropoiesis and suggests that polyp formation in mice with homozygous deletion of Smad4 is not entirely T cell dependent. Same results were recently reported by Karlsson and colleagues.[65] The *Smad4*$^{\Delta/\Delta}$ mice can be used to further study polyp formation and the interplay between the inflammatory response and alterations in the epithelial cells and the stroma of the GI tract.

Part II: A pilot study of TGF−β signaling and iron homeostasis

(Dejing Pan and Radek Skoda, unpublished data)

Summary

Iron is a critical factor for the oxygen-transport protein hemoglobin in red blood cells and complex of the electron transport chain in mitochondria. Hepcidin, a peptide synthesized in liver, regulates the iron flux of cellular iron through interacting with ferrportin. Unlike the up-regulation of hepcidin, its down-regulation was not well studied. After phlebotomy, hepcidin expression drops rapidly. We examined the change in expression of the iron metabolism related genes in the background of blocked expression of *Smad4*, *Smad4*/*TBRII*, and *Smad4*/*HDAC1*, which were specifically induced in the liver. After phlebotomy, in *Smad4* deficient liver, *Atoh8* and *Id1* were down-regulated, and *Bmp6* and *Smad7* slightly decrease. In *Smad4*/*TBRII* deficient liver, *Atoh8*, *Id1* and *Smad7* were down-regulated, and *Bmp6* does not show significant decrease. In *Smad4*/*HDAC1* deficient liver, the expression *Atoh8, Id1*, *Bmp6* and *Smad7* were significantly down-regulated. *Tmprss6* and *Hjv* mRNA remain unaltered in all the mice strains.

Introduction

Iron homeostasis consists of a complex regulatory network that regulates proteins involved in transport, uptake, utilization, and storage of iron, at the transcriptional and translational level via many signaling pathways. Due to toxic effects of free iron radicals, the iron homeostasis has to be systemically controlled by stringent balance of intestinal iron uptake, and iron efflux of macrophages and hepatocytes.

Hepcidin plays a central role in directing the use and storage of iron. It works through binding to ferrportin, a cell surface iron exporter in enterocytes, macrophages and hepatocytes, leading to its internalization and degradation. The circulating iron pool is one tenth of the daily requirements, thus demanding a high turnover rate. Loss of ferrportin from the cell surface inhibits iron release and causes failure to meet the need of rapid iron cycling, thus resulting in iron deficiency. [110]

Hepcidin normally functions to reduce plasma iron levels and increased level of hepcidin can cause systemic anemia.[84]

Hepcidin simulation has been reported in many reports. The BMP/Smad4 signaling has been demonstrated to be an important signaling pathway for hepcidin expression and several of its components have been associated with it. Hemojuvelin (*Hjv*), as a BMP co-receptor, binds BMP receptor and helps activate *hepcidin* expression.[83]A liver-specific deletion of *Smad4* gene in mice results in decreased *hepcidin* expression and causes hemochromatosis, an iron overload of organs.[111]

However, the pathway to down-regulate *hepcidin* expression was not well known.

Gene expression of *Atoh8*, *Bmp6*, *Id1*, and *Smad7* has been reported to positively correlate with *hepcidin* level and to iron burden caused by iron-enriched diets in a long time schedule. *Tmprss6* is a trans-membrane serine protease and is a newly discovered protein involved in the *hepcidin* repression. A recent report shows that HDAC inhibition increased *hepcidin* expression in HCV replicating cells.[115] Due to close interaction with Smads, whether *HDAC1* participates in the hepcidin expression was investigated here.

We wanted to find out how these above-mentioned genes would react to a sharp reduction of *hepcidin*, as in the case of anemia or acute bleeding, in partially defective TGF−β signaling backgrounds. After phlebotomy, erythropoiesis was highly activated and hepcidin expression was rapidly decreased. Using pIpC-induced *Mx-Cre* mice, in which high excision of loxp target genes occurs in the liver, we were investigating the possible effects of *Smad4*, *Smad4*/*TBRII*, and *Smad4*/*HDAC1* deficiency in the situation of decreased hepcidin level. In these different signaling backgrounds, we examined the *Atoh8*, *Bmp6*, *Id1*, *Smad7*, *Tmprss6* and *Hjv* expression.

Results

After phlebotomy, all mice showed splenomagly and decreased *hepcidin* expression

Mice of desired genotypes were bled 300 ul/day on two consecutive days and humanely sacrificed 30 hours after the last bleeding. All mice examined showed a spleen weight of around 200-400 mg, higher than the normal average weight of around 100 mg. This enlarged spleen, termed splenomagly, is a sign of increased erythropoietic activity. In adult mice, spleen, besides the bone marrow, also functions in erythropoiesis. In human, the equivalent organ is liver. Erythropoietic activity is a prerequisite for the down-regulation of hepcidin in anemia status. In all the mice that underwent phlebotomy, *hepcidin* was sharply down-regulated as expected (Figure 13-15).

Smad4 deficiency does not prevent the drop of *Atoh8* and *Id1* mRNA level, causes a slight decrease in *Bmp6*, *Smad7*, *Hjv* and *Tmprss6* expression after phlebotomy

Expression was determined by real-time PCR using the following primers (Table 3).

Table 3 Sequences of primers used for quantitative RT-PCR [113]

Gene	Forward primer	Reward primer
Hepc	AAGCAGGGCAGACATTGCGAT	CAGGATGTGGCTCTAGGCTATGT
Hjv	TCAGAGTAATGCTTGACCTC	CCGGTTCTTCCCAGATGATG
Atoh8	CACCATCAGCGCAGCCTTC	CCATAGGAGTAGCACGGCACC
Id1	ACCCTGAACGGCGAGATCA	TCGTCGGCTGGAACACATG
Bmp6	ATGGCAGGACTGGATCATTGC	CCATCACAGTAGTTGGCAGCG-
Smad7	GCAGGCTGTCCAGATGCTGT	GATCCCCAGGCTCCAGAAGA
Tmprss6	CAGTGTGAACGACATAGTCG	ATAGCTGTAGCGATAACAGC

The results are shown in Figure 13. *Smad4* expression level was used as an indicator for deletion showed the relative efficiency of excision in all mouse strains in this paper. As shown on Figure 13, the efficiency of *Smad4* down-regulation induced by pIpC in liver was demonstrated by real time PCR. As expected, hepcidin gene expression was decreased in to 1/20 of its original level after phlebotomy. Expression profiles of these 6 iron-related genes in *Smad4* deficient and $Smad4^{fl/fl}$ mice after phlebotomy were similar,

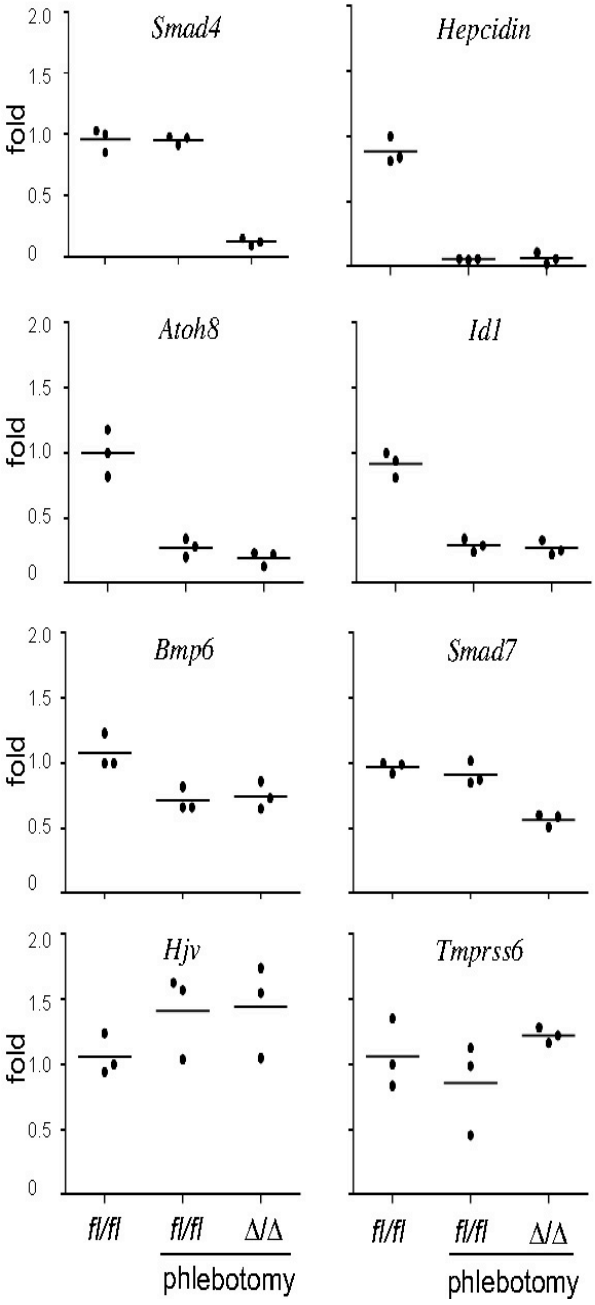

Figure 13. Iron-related gene expression in $Smad4^{\Delta/\Delta}$ liver. $Smad4^{fl/fl}$ and $Smad4^{\Delta/\Delta}$ mice were performed phlebotomy. $Smad4^{fl/fl}$ mice without phlebotomy were chosen as controls.

showing no statistical difference. Compared with mice undergoing no phlebotomy, they all showed 3 to 5 folds decrease of *Atho8* and *Id1*, 15% to 35% decrease of *Bmp6* and no significant change of *Tmprss6* and *Hjv*. In $Smad^{fl/fl}$ mice, *Smad7* expression level was stable, whereas in *Smad4* deficient mice, *Smad7* expression was reduced by 40% to 50%.

***Smad4/HDAC1* deficient mice have a lower *Atoh8, Bmp6, Id1* and *Smad7* mRNAs level and show unchanged *Hjv* and *Tmprss6* expressions after phlebotomy**

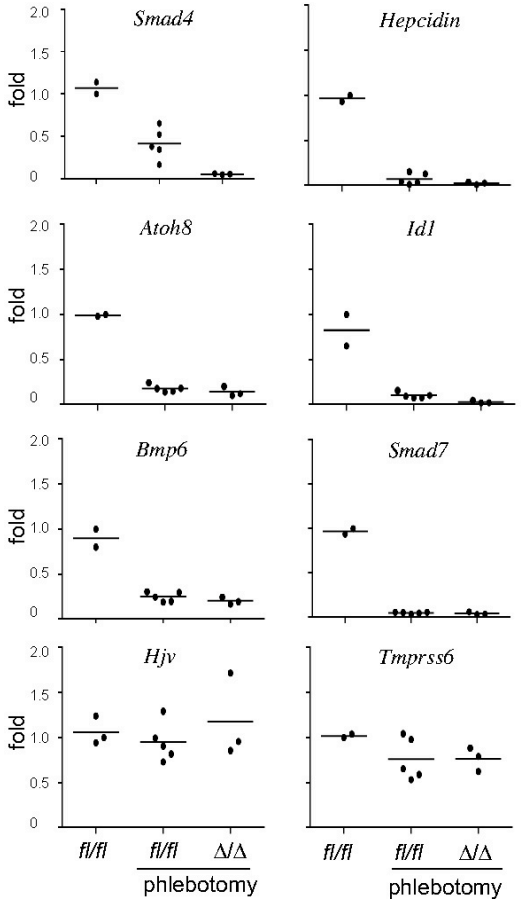

Figure 14.
Iron-related gene expression in $Smad4^{\Delta/\Delta}/HDAC1^{\Delta/\Delta}$ mice liver. $Smad4^{\Delta/\Delta}/HDAC1^{\Delta/\Delta}$ and $Smad4^{fl/fl}/HDAC1^{fl/fl}$ mice were performed phlebotomy. $Smad4^{fl/fl}/HDAC1^{fl/fl}$ mice without phlebotomy were chosen as controls.

To explore whether *HDAC1* is involved with iron metabolism, we try to use this double deletion of *Smad4/HDAC1* to study its effects on these genes expression. As shown is Figure 14, in *Smad4/HDAC1* deficient mice, *Atoh8*, *Bmp6*, *Smad7* and *Id1* mRNAs reduced by 70-95%. *Hjv* and *Tmprss6* did not give a significant change as expected.

Smad4/TBRII deficient mice do not prevent the drop of _Atoh8_, _Id1_ and _Smad7_ mRNAs level and show a slight decrease of _Bmp6_ and normal level of _Hjv_ and _Tmprss6_ expressions after phlebotomy

As shown in Figure 15, *Smad4/TBRII* deficient mice can not reverse the drop down of *Atoh8*, *Id1*, *Bmp6* and *Smad7* when hepcidin expression level was decreased. *Tmprss6* had a slightly decrease by 25% to 37%. Hjv did not give a significant decrease.

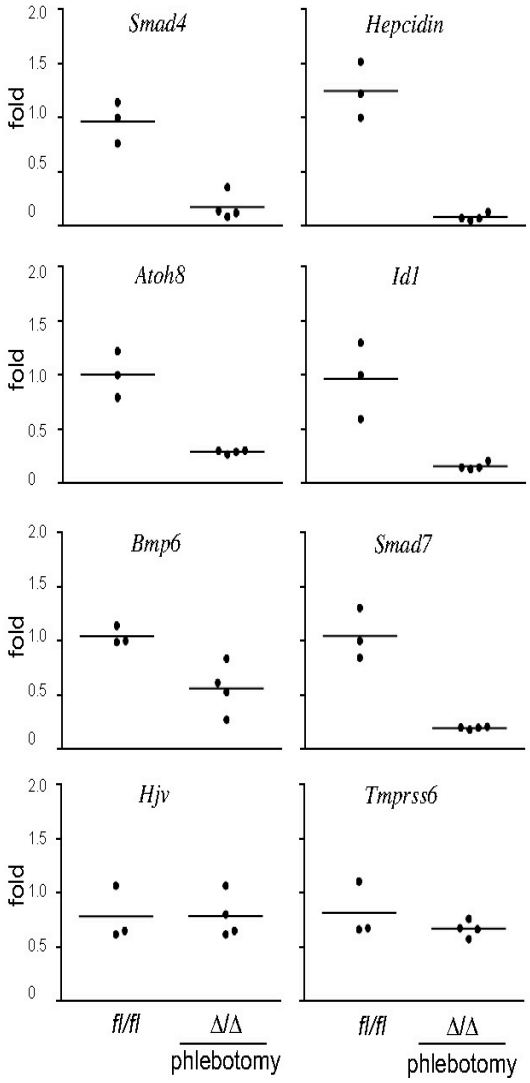

Figure 15.
Iron-related gene expression in $Smad4^{\Delta/\Delta}/TBRII^{\Delta/\Delta}$ mice liver. $Smad4^{\Delta/\Delta}/ TBRII^{\Delta/\Delta}$ mice were performed phlebotomy. $Smad4^{fl/fl}/TBRII^{fl/fl}$ mice without phlebotomy were chosen as controls.

Discussion:

Anemia, bleeding, chemotherapy, irradiation and bone marrow transplantation in patients decrease the hepcidin expression, increasing the intestinal iron absorption, iron release from macrophages and hepatocytes, in order to maintain sufficient iron supply for physiological needs such as erythropoiesis. Some inflammatory responses stimulate the hepcidin expression and lead to anemia, while blockage of hepcidin in humans causes different types of hemochromatosis. IL6-Jak-Stats might be responsible for enhanced expression of hepcidin during inflammation.[112] BMP/Smad4 pathway is crucial for the regulation of the basal expression of hepcidin, as showed in many reports. The sensor pathway responsible for detecting acute iron loss in the body remains uncertain.

BMP/Smad4 pathway is part of TGF−β signaling. To examine if other components of the TGF−β signaling pathway could be involved in this acute iron loss situation, we used mice carrying in the liver inducible down-regulation of genes such as *Smad4*, *TBRII*, and *HDAC1* (a nuclear Smad4 co-factor), to study the change in gene expression of some reported potential iron-related regulators, under the condition of phlebotomy that mimics a physiological hepcidin drop.

By studying mice undergoing phlebotomy, we noticed even *hepcidin* expression decreased 10 to 20 folds, but still remained around 10 fold to Rpl 19 house keep gene, a calibrator in real time PCR. Interestingly, Smad4 deficiency did not exacerbate this reduction, while liver-specific *Smad4* deletion using *Alb-Cre* strain with 90% reduction on *Smad4* with similar efficiency in our system, will cause more than 100 fold decrease without any exotic stress. This implies that Smad4 might be important for the basal level regulation of hepcidin, but it does not effectively impact acute down-regulation of hepcidin. Smad4/TBRII, and Smad4/HDAC1 double deficiency were shown to exert no effects on this regulation, either.

We examined *Atoh8*, *Id1*, *Bmp6* and *Smad7* based on a new report of Kautz et al, [113] which showed the long term effect of iron-burden on gene expression profiles. Atoh8 and Id1 are clearly down-regulated in all mice undergoing phlebotomy. *Bmp6* and

Smad7 did not always show the tendency of reduction, but based on the standard of this paper on fold change >1.5 fold, our results, to some extent, are in agreement with the report on *Bmp6* and *Smad7*.

Tmprss6, a trans-membrane protein, has recently been identified to be a hepcidine-suppression regulator, and it might become a promising candidate of iron sensor. [114]. Tmprss6 defect causes hepcidin to increase and over-expression of *Tmprss6* suppresses the activation of the hepcidin promoter. The *Tmprss6* expression level was measured in liver of all three strains of *Smad4*, *Smad4/TBRII*, and *Smad4/HDAC1*, but no expected significant increase was observed in *Tmprss6* mRNA levels.

Hemojuvelin, identified as a BMP co-factor, coordinates the BMP signaling, positively regulating hepcidine. Unfortunately, no expected decrease in expression or any difference was found in this experiment, either.

In these acute hepcidine down-regulation experiments, the effects of *Smad4, Smad4/TBRII* and *Smad4/HDAC1* related signaling pathways were not observed. It demonstrates that Smad4 possibly acts on maintaining the basal expression level of hepdicin instead of functioning as a real-time sensor. Changes in Atoh8, Id1 and Bmp6 expression were confirmed. Tmprss6 and Hjv expression seemed not to be regulated as expected.

Part III: TGF−β signaling in tumorgenesis

(Dejing Pan and Radek Skoda, unpublished data)

Summary

Smad4 is a central mediator of TGF−β signaling pathway, thus regarded as a tumor suppressor gene to prevent tumorgenesis. *Smad4* mutation was found in hereditary juvenile polyposis, and sporadic colon and lung cancers. T cells specific Smad4 deletion causes the spontaneous epithelial cancers throughout the gastrointestinal tract in mice, whereas epithelial specific deletion of the Smad4 does not. We monitored the wild-type mice reconstituted with *Smad4* deletion bone marrow cells and observed a shorter life span compared to the controls with health bone marrow cells. The similar results were obtained when Smad4 alleles were deleted selectively in hematopoietic cells using the *VavCre* transgenic mice. Furthermore, these *VavCre;Smad4* mice could be rescued by wild-type bone marrow cells reconstitution, whereas the control mice would significantly manifest a symptom of weight loss. Pathology study of *VavCre;Smad4* has revealed the presence of the polyps in rectum and cecum.

Introduction

Smad4 is mediating the major signals from the upstream active receptors complex in TGF−β signaling, even though there are a lot of cross-talkings with other signaling pathways without the involvement of Smad4. The germ-line mutations in three TGF−β signaling components were found in juvenile Polyposis patients, with 20% containing *Smad4* mutations, 20% containing *BMPR1A* mutations, and a small subset of patients containing *ENG* mutations. In sporadic colorectal cancers, 16-25% patients were identified to carry a *Smad4* mutation.[116,117]

Kim et al., demonstrate that when *Smad4* was deleted in T cells using *Lck-Cre* or *CD4-Cre* transgenic mice, there was a significant increase in the occurrence of gastrointestinal tumors. However, the epithelial specific deletion using *MMTV-Cre* or transthyretin-Cre line, did not lead to epithelial carcinoma anywhere in the gastrointestinal tract.[70] Here we used our model to verify these observations.

Results

Wild type recipient mice reconstituted with bone marrows containing induced *Smad4* deletion had a shorter life span

We transplanted pIpC induced *Mx-Cre;Smad4*$^{fl/fl}$ bone marrow cells, which are Smad4-deleted, into 5 wild type BL6 mice. Around 500 days after transplantation, only one mouse was alive, whereas 4 of the control mice, reconstituted with *Smad4*$^{fl/fl}$ bone marrow cells containing wild-type *Smad4*, were alive at day 460 and 3 alive 500 days after transplantation (Figure 16).

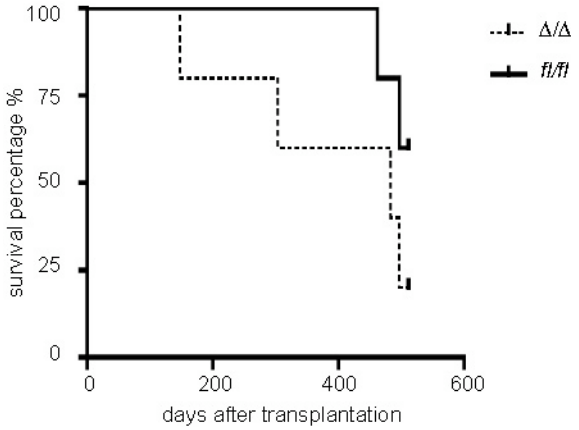

Figure 16. Survival curve of wild type BL6 mice (n=5) reconstituted with *Smad4*$^{\Delta/\Delta}$ bone marrows or bone marrow from control mice (fl/fl) transplanted into BL6 mice (n=4).

Mice with hematopoietic-cell- specific deletion of *Smad4* also show a sharply shortened life span

VavCre;Smad4$^{fl/fl}$ mice carry deletion of Smad4 selectively in hematopoietic cells. The mice and their littermate controls were monitored up to more than one year (Figure 17). Only 3 *VavCre;Smad4*$^{fl/fl}$ mice were still alive out of a total of 26, whereas 33 littermate control mice survived among a total number of 35. *VavCre;Smad4*$^{fl/fl}$ mice started to die

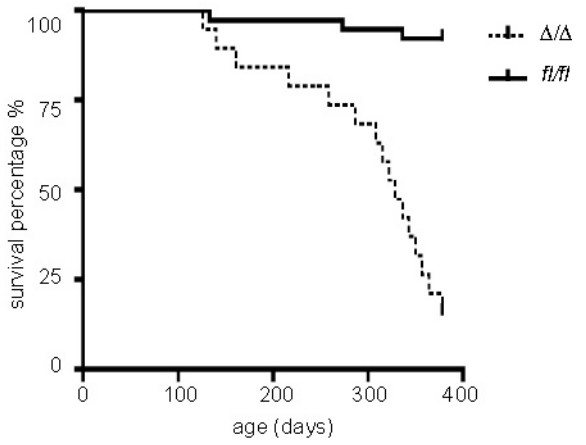

Figure 17. Survival curve of *VavCre;Smad4*$^{fl/fl}$ mice (Δ/Δ; n=26) and *Smad4*$^{fl/fl}$ (*fl/fl*; n=35) mice that were chosen as controls.

at 18 weeks. Around 50% to 60% of *VavCre;Smad4*$^{fl/fl}$ mice were positive in fecal occult blood test, and some examined mice showed splenomegaly, which may indicate internal bleeding in gastrointestinal tracts and a demand of compensational erythropoiesis. Furthermore, *VavCre;Smad4*$^{fl/fl}$ mice appeared smaller than their control littermates.

VavCre;Smad4$^{fl/fl}$ mice reconstituted with wild type BL6 bone marrow cells reverse their growth defect

If the shortened lifespan of *VavCre;Smad4*$^{fl/fl}$ mice is caused by the Smad4-deficient hematopoietic cells, bone marrow cells from healthy wild type mice should rescue this phenotype. To test this hypothesis, we transplanted bone marrow cells from wild type BL6 mice into *VavCre;Smad4*$^{fl/fl}$ mice. As a control, we transplanted *VavCre;Smad4*$^{fl/fl}$ bone marrow cells into *VavCre;Smad4*$^{fl/fl}$ mice, and *VavCre;Smad4*$^{fl/fl}$ bone marrow cells into *Smad4*$^{fl/fl}$ mice. The follow up is too short to determine the effects on survival. However, we noticed that *VavCre;Smad4*$^{fl/fl}$ recipient mice transplanted with Smad4 deficient bone marrow showed weight loss (Figure 18), whereas *Smad4*$^{fl/fl}$ recipients that received Smad4 deficient bone marrow or *VavCre;Smad4*$^{fl/fl}$ mice that received healthy bone marrow showed normal weight gain after transplantation.

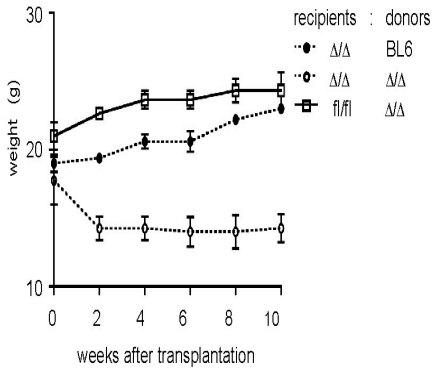

Figure 18 *VavCre;Smad4*$^{fl/fl}$ mice reconstituted with normal wild type BL6 bone marrow cells reversed its growth defect. Lethally irradiated *VavCre;Smad4*$^{fl/fl}$ recipients (Δ/Δ) reconstituted with bone marrows from wild type BL6 (n=5) or *VavCre;Smad4*$^{fl/fl}$ donors (n=4) and *Smad4*$^{fl/fl}$ recipients (fl/fl, n=3) reconstituted with bone marrows *VavCre;Smad4*$^{fl/fl}$ donors were monitored on weight up to 10 weeks after transplantation

The reason for the rapid weight loss in *VavCre;Smad4*$^{fl/fl}$ mice that received bone marrow cells from *VavCre;Smad4*$^{fl/fl}$ mice is unclear, but could be related to the smaller size of older *VavCre;Smad4*$^{fl/fl}$ mice. Interestingly, the presence of recipient T cells in *Smad4*$^{fl/fl}$ recipients that received Smad4 deficient bone marrow appears to counteract this effect.

Polyps formed in the rectum and the cecum in *VavCre;Smad4$^{fl/fl}$* mice

As shown in Figure 19, histopathology of the gastrointestinal tract of *VavCre;Smad4$^{fl/fl}$* mice revealed polyps in rectum and cecum. Most of them were histologically characterized by a branching architecture reminiscent of hyperplastic lesions, mostly with foci of low- and/or high-grade dysplasia. We classified these lesions as mixed hyperplastic/adenomatous polyps. Moreover, "serrated" aspects of the polyps were also detected focally.

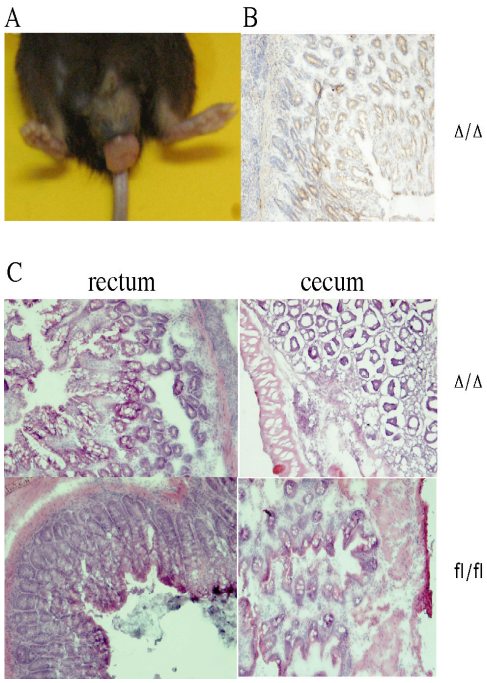

Figure 19. Polyps in rectum and cecum in *VavCre;Smad4$^{fl/fl}$* mice. A) a typical rectum polyps in *VavCre;Smad4$^{fl/fl}$* mice. B) Smad4 immunohistochemical staining showed the positive signal in polyps tissue.C) HE staining showed the polyps morphology in rectum and cecum. *VavCre;Smad4$^{fl/fl}$* mice (Δ/Δ), *Smad4$^{fl/fl}$* mice (fl/fl).

To exclude the possibility that this polyposis could be resulted from Smad4 deletions in the epithelial cells, immunohistochemical stainings through sections of rectum and cecum using rabbit anti-Smad4 antibody convincingly demonstrated the normal presence of Smad4 in these polyp cells. In this way, we came to the conclusion that the polyposis phenotype was predominantly caused by Smad4-deficient hematopoietic cells.

Discussion

We have demonstrated that Smad4-dependent signaling in hematopoietic cells is required for tumor suppression in the intestinal environment. In our experiments, *VavCre;Smad4*$^{fl/fl}$ mice manifested a phenotype of shortened lifespan, potential internal bleeding, and polyp formation in rectum and cecum. Immunohistochemical staining of Smad4 excluded the possibility that *Smad4* deletion in polyp cells themselves could cause the polyp formation. Thus, it points to the possibility that Smad4 in hematopoietic cells may normally act to suppress polyposis. In agreement with this hypothesis, lethally irradiated *VavCre;Smad4*$^{fl/fl}$ mice reconstituted with normal bone marrow cells display perfect health, whereas the same lethally irradiated mice reconstituted with bone marrow cells taken from *VavCre;Smad4*$^{fl/fl}$ mice clearly displayed weight loss. This weight loss is not due to the failure of reconstitution, since the lethally irradiated *Smad4*$^{fl/fl}$ mice reconstituted with exactly the same *VavCre;Smad4*$^{fl/fl}$ bone marrow cells showed no similar weigh losing phenotype. In general, bone marrow cells extracted from one donor mouse are enough for transplanting 10 to 20 recipient mice.

Even with a shorter life-span compared with controls, the lethally irradiated wild type BL6 mice reconstituted with Smad4 deficient bone marrow still show a longer lifespan than *VavCre;Smad4*$^{fl/fl}$ mice. One possible explanation is that in lethally irradiated mice, there could still exist some surviving irradiation-resistant functional T cells, especially in thymus. In general, T cells have a longer lifespan than other hematopoietic cell lineages; they can even survive through the entire lifespan of adult. These T cell survivors might still function partially to maintain the suppression of tumorigenesis. The observation that *Smad4*$^{fl/fl}$ mice reconstituted with bone marrow cells from *VavCre;Smad4*$^{fl/fl}$ mice show normal growth progress is quite in line with this hypothesis. On the contrary, because *VavCre;Smad4*$^{fl/fl}$ mice do not contain Smad4 in any hematopoietic cell lineage, so there will be no functional surviving T cells or other potential candidate cells that maintain the normal suppression of tumorigenesis.

In conclusion, we have demonstrated that Smad4-mediated TGF−β signaling in hematopoietic cells plays an important role in the suppression of tumorigenesis in the epithelial cells of gastrointestinal tracts.

Perspective

In the results part I, the studying of TGF−β signaling and hematopoiesis, we noticed the wild type mice reconstituted with *Smad4/TBRII* deficient bone marrow cells from pIpC-induced *Mx-Cre;Smad4*$^{fl/fl}$*;TBRII*$^{fl/fl}$ mice did not show the symptom of inflammation, which was opposite to the observation of recipient mice with *TBRII* deficient bone marrow cells. Why the additional *Smad4* defect could rescue the *TBRII* deficiency mediated inflammation needs more stringent evidences. Several experiments could be performed in the future:

1) The *Smad4/TBRII* deficient hematopoietic lineage cells, especially immune related lineage cells, could be isolated and their RNAs can be extracted to detect the expression profile for figuring out potential candidate genes responsible for this inhibition.

2) At protein level, the plasma could be separated to study cytokine alteration by cytokine protein arrays, and to explore the possible soluble factors involved with the inhibition of inflammation. Cell surface markers' alteration needs to be studied by flow cytometry analysis.

3) Competitive bone marrows transplantation together with *TBRII* deficient bone marrows could be performed to determine if this inhibition comes from the hematopoietic cells' mutual interaction.

In the results part II, we studied several iron-related gene alteration responding to the acute bleeding with *Smad4, Smad4/TBRII and Smad4/HDAC1* deficiency in liver.

We noticed all of these deficiencies are not responsible for the iron-related gene alteration, or only in the baseline regulation. The examined iron-related genes would respond to acute bleeding as they did in the chronic alteration of iron-burden situation. However, all of these experiments are performed in the mRNA level, thus, in the future, their corresponding protein products and related cell surface markers need to be analyzed responding to this acute environmental stress.

In the last results part, we demonstrate the suppression of tumorigenesis in gastrointestinal tracts required the Smad4-dependent signaling pathway. The clear mechanism in this suppression needs to be carefully discerned.

1) In future, the circulating soluble factors, related immune cytokine and cell surface markers' alteration need to be studied by proteomics methods or flow cytometry analysis.

2) Due to this immune related phenotype, the factors of genetic background need to be concerned carefully. We used SNP analysis to check the genetic background of Smad4 mice showing the strong mixture and S129 and BL6. To continue more stringent studies in the immune system, purer genetic background is required. So that the backcross of *Smad4* mice with wild type BL6 mice must be performed more than 5 generations.

Materials and Methods

Mice

All mice used in this study were kept under specific pathogen-free conditions and in accordance to Swiss federal regulations. The *Smad4*$^{fl/fl}$ mice (Figure 1) were crossed with the *Mx-Cre* mice, which contain Cre recombinase under control of the interferon inducible Mx1 promoter. [118,119] *TBRII*$^{fl/fl}$ mouse was kindly provided by Dr.J.Roes from the University of college London. *Pf4-Cre* mice are from our own lab.[95] *VavCre* mice were kindly provided by Dr. Dimitris Kioussis. [96] *HDAC1*$^{fl/fl}$ mice were kindly provided by Prof. Patrick Matthias in Fredrich Miescher Institute in Basel. Smad4 mice were genotyped using the primers S1:ACTTTACAGGATGATGGTTA and S2:GGTCAAGCAGATTACAGCAA that yield a 360 bp fragment for the floxed Smad4 allele (*Smad4*^{fl}) and 310bp for the wild type Smad4 allele (Smad4^{+}) and in parallel also with the primers S3:GGGCAGCGTAGCATATAAGA and S4:GACCCAAACGTCACCTTCAC that produce a 450 bp fragment for *Smad4*^{fl} and 390bp for Smad4^{+}. The alleles carrying the Cre-induced deletion of Smad4 (*Smad4*$^{\Delta}$) were detected with primer S5:TCCCACATTCCTCTTAGTTTTGA and primer S6: CCAGCTTCTCTGTCCAGGTAGTA yielding a 500 bp fragment for *Smad4*$^{\Delta}$. The efficiency of the Cre-mediated excision was assessed by Southern blot using a probe generated by the primers CTCGAGTAGGTTAACAAGG and CTTTATATACGCGCTTGGG located in intron 8 and exon 9 of the floxed Smad4 allele (Figure 1). Genotyping of Mx-Cre mice was performed with the primers AGGTGTAGAGAAGGCACTTAGC and CTAATCGCCATCTTCCAGCAGG that amplify a 300 bp fragment. Mx-Cre expression was induced by the intraperitoneal injection of 300µg polyinosine-polycytosine (pIpC) 3 times every 2 days. Genotyping of VavCre mice was performed with the primers CTCTGACAGATGCCAGGACA and TGATTTCAGGGATGGACACA that give 500 bp fragment. The primers for genotyping of *TBRII*$^{fl/fl}$ was TGTAATCGTTGCACTCTTCCATGT and AGATAAACGAAGCCGGTGCA, and for Pf4 mice is CCCATACAGCACACCTTTTG and TGCACAGTCAGCAGGTT

Blood analysis

Blood was collected from the tail vein or by cardiac puncture and blood counts were determined by the Advia 120 Hematology Analyzer using the "Multispecies Software" (Bayer, Leverkusen, Germany). The concentration of transferrin in mouse blood plasma was determined by Mouse Serum Transferrin ELISA Kit (ADI alpha diagnostic, TX, USA). Direct antiglobulin test (DAT) was used for detecting IgG/IgM autoantibodies on the membrane of erythrocytes. Phycoerythrin (PE) or fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG/IgM was from Pharmingen BD, Belgium. Blood from New Zealand Black (*NZB)* mice served as positive controls and *Smad4*$^{fl/fl}$ as negative controls for DAT. Serum erythropoietin levels were measured with the Quantikine Mouse/Rat Epo Immunoassay kit (R&D Systems, Abingdon, UK).

Red blood cell half-life measurement

Mouse blood cells were labeled by intravenous injection of 3mg NHS-X-Biotin (Sigma, Germany). For analysis, capillary blood obtained by tail puncture was diluted in 3.8% sodium citrate, spun down by 180 g, resuspended in 500ul PBS-FACS buffer (0.5%BSA, 0.02%NaN_3) and incubated 30 min at 4°C with PE-conjugated streptavidin (Becton Dickinson, Germany). The cells were centrifuged and the pellet was resuspended in 1ml PBS-FACS buffer for flow cytometric analysis on a FACSCalibur (Becton Dickinson, Germany) to determine the fraction of labeled RBCs remaining.

Quantitative RT-PCR

Total RNA was isolated from tissue using Trizol (Peqlab, Erlangen, Germany) and reverse transcribed after random hexamer priming using the Omniscript RT kit (Qiagen, Germany). Quantitative measurement of gene expression was carried out with SYBR Green PCR master mix on an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The mouse *RPL19* mRNA assessed with the primers ATCCGCAAGCCTGTGACTGT and TCGGGCCAGGGTGTTTTT was used for normalization.[120] Relative expression values were calculated by the ΔΔCT method using one bone marrow sample as a calibrator that was set to the value of 1.[121,122] The primer pairs used for quantification of specific mRNAs are listed in Table 1 and were in part described previously.[123-125]

Liver iron analysis

Total iron in the liver tissues was determined by flame atomic absorption spectroscopy under alkaline conditions on a Varian SpectrAA220 spectrometer (Varian, Switzerland) following solubilization by tetramethylammonium hydroxide.[126]

Feces analysis

The feces of mice were collected over several weeks with sampling intervals of 3.5 days. The presence of blood in feces was detected by Hemoccult-R (Beckman Coulter, Germany).

Flow cytometry

FITC- and PE-conjugated monoclonal antibodies against TER119, B220, Mac-1, GR-1, CD45.1, CD45.2, CD25, CD4, CD11b, CD11c, Mc1.1, TCRb, TCRg-epsilon, CD19 and mouse IgG&M (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, organs were grinded and cells were filtered through a 40µm nylon mesh.

Bone marrow transplantation

Bone marrow cells from femurs and tibiae were filtered through a 40µm mesh and 3 $x10^6$ cells in 200µl HBSS (1X Hank's Balanced Salt solution with 20 mM Hepes buffer) were injected into the tail vein of lethally irradiated (1100 cGy in two doses, separated by 3 hours) 7-10 week old recipient mice. *Mx-Cre;Smad4*$^{fl/fl}$ and control mice received pIpC 10 days before being used as bone marrow donors. C57BL/6SJL-Ptprc^{a}Pep3^b/BoyJ (B6.CD45.1) mice were used as the recipients. In the converse experiment, B6.CD45.1 wild type donor bone marrow cells were transplanted into irradiated *Mx-Cre;Smad4*$^{fl/fl}$,*MxCreTBR*$^{fl/fl}$*;Smad4*$^{fl/fl}$, or control mice. The recipient mice received pIpC 5 weeks after transplantation. Chimerism of transplanted mice was analyzed by flow cytometric analysis of CD45.1 and CD45.2 positive cells.

Histology

Stomach, small intestine and colon were isolated, fixed in 4% formalin and paraffin embedded and 4µm-sections were stained with Hematoxylin and Eosin (H&E).

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PUBLICATIONS

Normal erythropoiesis but severe polyposis and bleeding anemia in *Smad4*-deficient mice

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The tumor suppressor *Smad4* mediates signaling by the transforming growth factor beta (TGF-β) superfamily of ligands. Previous studies showed that several TGF-β family members exert important functions in hematopoiesis. Here, we studied the role of *Smad4* in adult murine hematopoiesis using the inducible Mx-Cre/loxP system. Mice with homozygous *Smad4* deletion (*Smad4*$^{\Delta/\Delta}$) developed severe anemia 6 to 8 weeks after induction (mean hemoglobin level 70 g/L). The anemia was not transplantable, as wild-type mice reconstituted with *Smad4*$^{\Delta/\Delta}$ bone marrow cells had normal peripheral blood counts. These mice did not develop an inflammatory disease typical for mice deficient in *TGF*-β receptors I and II, suggesting that the suppression of inflammation by TGF-β is Smad4 independent. The same results were obtained when *Smad4* alleles were deleted selectively in hematopoietic cells using the *VavCre* transgenic mice. In contrast, lethally irradiated Smad4$^{\Delta/\Delta}$ mice that received wild-type bone marrow cells developed anemia similar to *Smad4*$^{\Delta/\Delta}$ mice that did not receive a transplant. Liver iron stores were decreased and blood was present in stool, indicating that the anemia was due to blood loss. Multiple polyps in stomach and colon represent a likely source of the bleeding. We conclude that *Smad4* is not required for adult erythropoiesis and that anemia is solely the consequence of blood loss. (Blood. 2007;110:3049-3055)

Introduction

The members of the transforming growth factor beta (TGF-β) superfamily of ligands modulate cell proliferation, differentiation, apoptosis, adhesion, and cell migration.[1] These ligands, including TGF-β, activins, and bone morphogenetic proteins (BMPs), bind cell-surface receptors, classified as type I and II receptors, that contain an intracellular serine/threonine protein kinase domain. Upon ligand activation, the type II and type I receptors form an active ligand-receptor complex that phosphorylates members of the Small mutants (*Caenorhabditis elegans*) and mothers against the decapentaplegic homolog (Smad) family of proteins. The Smad family members that directly interact with the receptors are called receptor Smads (R-Smad). The type I receptors for TGF-β, activin, nodal, and myostatin phosphorylate R-Smad2 and 3, whereas the BMPs phosphorylate R-Smad1, 5, and 8. The R-Smads associate with Smad4, also called common partner Smad (co-Smad), and as a complex enter the nucleus to regulate transcription. Smad6 and Smad7 TGF-β inhibit signaling through multiple mechanisms and are called inhibitory Smads (I-Smad).[2]

Hematopoiesis is a tightly balanced process consisting of cell self-renewal, differentiation, and apoptosis of hematopoietic cells. The effects of TGF-β signaling on hematopoiesis are cell and context specific. TGF-β1 has an inhibitory function in early expansion of committed hematopoietic precursors,[3] and BMP4 is implicated in mesoderm induction and hematopoietic commitment during embryogenesis.[4] Mice deficient for *TGF*-β1 die 3 to 4 weeks after birth due to an inflammatory syndrome,[5,6] whereas the knockouts of the *TGF*-β receptors I and II are embryonically lethal during midgestation.[7,8] Cells taken from embryos deficient for *TGF*-β receptors I and II display an increase in erythroid colony-forming cells, consistent with an inhibitory effect of TGF-β in early expansion of committed hematopoietic precursors.[8] *Smad1*$^{-/-}$ and *Smad5*$^{-/-}$ mice showed defects of hematopoietic and vascular development.[9,10] *Smad1* expression is sufficient to expand the number of cells that commit to the hemangioblast fate.[11] *Smad5* is dispensable for hematopoiesis in the adult mouse.[12] Overexpression of *Smad7* promotes self-renewal capacity of hematopoietic stem cells (HSCs) in vivo.[13]

Since Smad4 is necessary for signaling by both the TGF-β and the BMP families of ligands, *Smad4*$^{-/-}$ mice could be expected to show severe defects in hematopoiesis. However, *Smad4*$^{-/-}$ mice die during embryogenesis before the onset of hematopoiesis.[14,15] To directly investigate the role of *Smad4* in hematopoiesis, we crossed mice with a conditional *Smad4* knockout allele (*Smad4*$^{fl/fl}$)[16] and a strain containing a *Cre*-recombinase gene controlled by the interferon-inducible *Mx1* promoter (*Mx-Cre*).[17] The *Mx-Cre*–inducible mouse was widely used in studies of hematopoiesis and showed high efficiency of recombination in bone marrow.[17,18] Upon induction of *Mx-Cre* expression, the conditional *Smad4*$^{fl/fl}$ alleles (*fl/fl*) recombined to yield dysfunctional *Smad4* alleles (Δ/Δ) and these mice developed severe anemia by 6 to 8 weeks after

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induction. To inactivate the *Smad4*$^{fl/fl}$ conditional allele in hematopoietic cells only, we crossed the *Smad4* mice to the *VavCre* strain, which expresses *Cre* selectively in hematopoietic cells under the control of the *Vav* promoter.[19] We show that erythropoiesis was not directly affected by the loss of *Smad4*. Rather, anemia is the consequence of blood loss from polyps that rapidly form in the stomach and colon of these mice.

Materials and methods

Mice

All mice used in this study were kept under specific pathogen-free conditions and in accordance with Swiss federal regulations. The *Smad4*$^{fl/fl}$ mice (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) were crossed with the *Mx-Cre* mice, which contain Cre recombinase under control of the interferon-inducible *Mx1* promoter.[16,17] *Smad4* mice were genotyped using the primers S1 (ACTTTACAGGATGATGGTTA) and S2 (GGTCAAGCAGATTACAGCAA), which yield a 360-bp fragment for the floxed *Smad4* allele (*Smad4*^{fl}) and 310-bp fragment for the wild-type *Smad4* allele (*Smad4*$^{+}$), and also in parallel with the primers S3 (GGGCAGCGTAGCATATAAGA) and S4 (GACCCAAACGTCACCTTCAC), which produce a 450-bp fragment for *Smad4*^{fl} and a 390-bp fragment for *Smad4*$^{+}$. The alleles carrying the *Cre*-induced deletion of *Smad4* (*Smad4*$^{\Delta}$) were detected with primer S5 (TCCCACATTCCTCTTAGTTTTGA) and primer S6 (CCAGCTTCTCTGTCCAGGTAGTA), yielding a 500-bp fragment for *Smad4*$^{\Delta}$. The efficiency of the Cre-mediated excision was assessed by Southern blot using a probe generated by the primers CTCGAGTAGGTTAACAAGG and CTTTATATACGCGCTTGGG located in intron 8 and exon 9 of the floxed *Smad4* allele (Figure S1). Genotyping of *Mx-Cre* mice was performed with the primers AGGTGTAGAGAAGGCACTTAGC and CTAATCGCCATCTTCCAGCAGG, which amplify a 300-bp fragment. *Mx-Cre* expression was induced by the intraperitoneal injection of 300 μg polyinosine-polycytosine (pIpC) 3 times every 2 days. *VavCre* mice were kindly provided by Dr Dimitris Kioussis.[19] These mice were crossed to obtain *VavCre;Smad4*$^{fl/fl}$ mice. Genotyping of *VavCre* mice was performed with the primers CTCTGACAGATGCCAGGACA and TGATTTCAGGGATGGACACA, yielding a 500-bp fragment.

Blood analysis

Blood was collected from the tail vein or by cardiac puncture and blood counts were determined by the Advia 120 Hematology Analyzer using the Multispecies Software, version 2.3.01-MS (Bayer, Leverkusen, Germany). The concentration of transferrin in mouse blood plasma was determined by a mouse serum transferrin enzyme-linked immunosorbent assay (ELISA) kit (ADI Alpha Diagnostic, San Antonio, TX). Direct antiglobulin test (DAT) was used for detecting IgG/IgM autoantibodies on the membrane of erythrocytes. Phycoerythrin (PE)– or fluorescein isothiocyanate (FITC)–labeled goat anti–mouse IgG/IgM was from Pharmingen BD (Erembodegen, Belgium). Blood from New Zealand Black (*NZB*) mice served as positive controls and *Smad4*$^{fl/fl}$ as negative controls for DAT. Serum erythropoietin levels were measured with the Quantikine Mouse/Rat Epo Immunoassay kit (R&D Systems, Abingdon, United Kingdom).

Red blood cell half-life measurement

Mouse blood cells were labeled by intravenous injection of 3 mg NHS-X-Biotin (Sigma, Deisenhofen, Germany). For analysis, capillary blood obtained by tail puncture was diluted in 3.8% sodium citrate, spun down by 180*g,* resuspended in 500 μL PBS-FACS buffer (0.5% BSA, 0.02% NaN_3), and incubated 30 minutes at 4°C with PE-conjugated streptavidin (Becton Dickinson, Heidelberg, Germany). The cells were centrifuged and the pellet was resuspended in 1 mL PBS-FACS buffer for flow-cytometric analysis on a FACSCalibur (Becton Dickinson) to determine the fraction of labeled red blood cells (RBCs) remaining.

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissue using Trizol (Peqlab, Erlangen, Germany) and reverse transcribed after random hexamer priming using the Omniscript RT kit (Qiagen, Hilden, Germany). Quantitative measurement of gene expression was carried out with SYBR Green PCR master mix on an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The mouse *RPL19* mRNA assessed with the primers ATCCGCAAGCCTGTGACTGT and TCGGGCCAGGGTGTTTTT was used for normalization.[20] Relative expression values were calculated by the delta-delta cycle threshold (ΔΔCT) method using 1 bone marrow sample as a calibrator that was set to the value of 1.[21,22] The primer pairs used for quantification of specific mRNAs are listed in Table 1 and were in part described previously.[23-25]

Liver iron analysis

Total iron in the liver tissues was determined by flame atomic absorption spectroscopy under alkaline conditions on a Varian SpectrAA220 spectrometer (Varian, Zug, Switzerland) following solubilization by tetramethylammonium hydroxide.[26]

Feces analysis

The feces of mice were collected over several weeks with sampling intervals of 3.5 days. The presence of blood in feces was detected by Hemoccult-R (Beckman Coulter, Krefeld, Germany).

Flow cytometry

FITC- and PE-conjugated monoclonal antibodies against TER119, B220, Mac-1, GR-1, CD45.1, CD45.2, and mouse IgG and IgM (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, organs were grinded and cells were filtered through 70-μm nylon mesh.

Bone marrow transplantation

Bone marrow cells from femora and tibiae were filtered through 40-μm mesh, and 3×10^6 cells in 200 μL HBSS (1 × Hanks balanced salt solution with 20 mM Hepes buffer) were injected into the tail vein of lethally irradiated (1100 cGy in 2 doses, separated by 3 hours) 7- to 10-week-old recipient mice. *Mx-Cre;Smad4*$^{fl/fl}$ and control mice received pIpC 10 days before being used as bone marrow donors. C57BL/6SJL-Ptprc^{a}Pep3^{b}/BoyJ (B6.CD45.1) mice were used as the recipients. In the converse experiment, B6.CD45.1 wild-type donor bone marrow cells were transplanted into irradiated *Mx-Cre;Smad4*$^{fl/fl}$ or control mice. The recipient mice received

Table 1. Sequences of primers used for quantitative RT-PCR

Gene	Forward primer	Reverse primer
Smad4	GTTCAGGTAGGAGAGACGTTTAAGGT	CCTTTACATTCCAACTGCACTCCT
Hepc	CCTATCTCCATCAACAGATG	AACAGATACCACACTGGGAA
Fpn	AAGGATTGACCAGCTAACCAACA	CAGCCAATGACTGGAGAACCA
Dcytb	GCAGCGGGCTCGAGTTTA	TTCCAGGTCCATGGCAGTCT
Dmt1	AACCAACAAGCAGGTGGTTGA	CTTTGTAGATGTCCACAGCCA
Tf	TTGTGCCATCCCATCACAAC	CTAGTGTCCGATGCCTTCACC
Heph	TTGTCTCATGAAGAACATTACAGCAC	CATATGGCAATCAAAGCAGAAGA
Hfe	CTGAAAGGGTGGGACTACATGTTC	GGACACCACTCCCAACTTCGT
Tfr1	CAGAAAGTTCCTCAGCTCAACCA	GTTCAATTCAACGTCATGGGTAAG
Tfr2	AGCTGGGACGGAGGTGACTT	TCCAGGCTCACGTACACAACA
Sft	CTGTGCTCATTGAAGAGGACCTT	TCTGGTTGCTTTCTCAGTCACG

Smad4 indicates small mutants (*C elegans*) and mothers against decapentaplegic homolog 4 (Drosophila); *Hepc,* hepcidin; *Fpn,* ferroportin; *Dcytb,* cytochrome b reductase 1; *Dmt1,* divalent metal transporter 1; *Tf,* transferrin; *Heph,* hephaestin; *Hfe,* major histocompatibility complex class I–like protein; *Tfr1,* transferrin receptor 1; *Tfr2,* transferrin receptor 2; and *Sft,* stimulator of Fe transport.

pIpC 5 weeks after transplantation. Chimerism of recipient mice was analyzed by flow-cytometric analysis of CD45.1$^+$ and CD45.2$^+$ cells.

Histology

Stomach, small intestine, and colon were isolated, fixed in 4% formalin, and paraffin embedded, and 4-μm sections were stained with hematoxylin and eosin (H&E). The images were viewed and captured with a Leica DMZ75 microscope (Leica, Wetzlar, Germany) and Leica DFC480 R2 camera with 1.0× plan objective, and Zeiss AX10 microscope (Carl Zeiss, Oberkochen, Germany) and Axio CamHR camera objective Plan-Neofluar 2.5×/0.075, Plan-Apochromat 5×/0.16, and Plan-Apochromat 1.0×/0.45. Images were acquired using Leica FireCam software version 1.5 and Axio CamHR version 4.6. Brightness/contrast and color balance were adjusted using Adobe Photoshop Elements 2.0 (Adobe Systems, San Jose, CA).

Results

Mice with induced deletion of Smad4 develop severe anemia

Four weeks after *Mx-Cre*–induced excision of *Smad4*$^{fl/fl}$, the resulting *Smad4*$^{\Delta/\Delta}$ mice developed anemia and after 8 weeks the hemoglobin concentration and hematocrit were decreased to 30% of normal values (Figure 1). The mean corpuscular volume (MCV) was unchanged, but the reticulocyte count was strongly increased. The white blood count and platelet levels remained unchanged. The *Smad4*$^{\Delta/\Delta}$ alleles were detectable by PCR in 39 of 40 bone marrow–derived colonies (not shown). The apparent half-life of erythrocytes from *Smad4*$^{\Delta/\Delta}$ mice was reduced to 10 to 12 days,

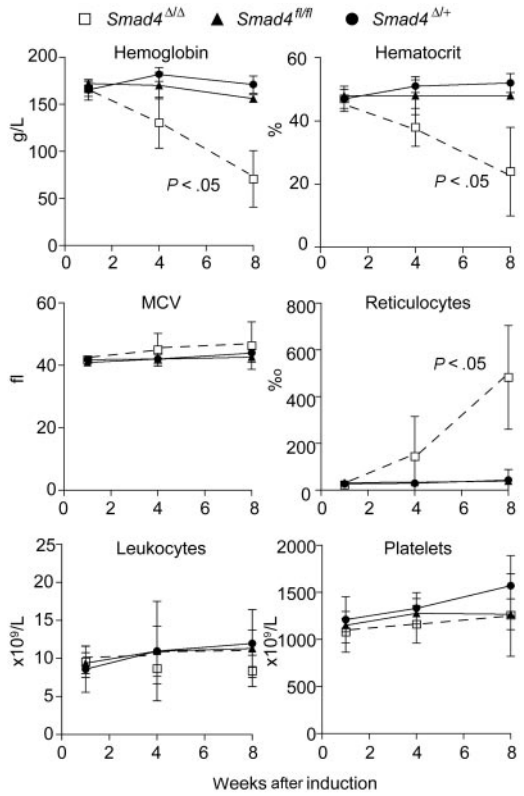

Figure 1. Smad4$^{\Delta/\Delta}$ mice developed anemia after induced *Smad4* deletion. Hemoglobin level, hematocrit, reticulocyte count, leukocyte count, mean corpuscular volume (MCV), and platelet count in *Smad4*$^{\Delta/\Delta}$ (n = 3), *Smad4*$^{fl/fl}$ (n = 4), and *Smad4*$^{\Delta/+}$ (n = 8) mice were plotted against weeks after induction. The *P* values were calculated by Student *t* test. The error bars indicate standard deviation.

whereas the half-life in *Smad4*$^{fl/fl}$ control mice was 23 days (data not shown). A direct antiglobulin test showed no evidence for IgM or IgG surface antibodies on erythrocytes from *Smad4*$^{\Delta/\Delta}$ mice, arguing against autoimmune antibody–meditated hemolytic anemia (not shown). The apparent reduction of the erythrocyte half-life in circulation can be explained by compensatory increase in regeneration, marked by massive reticulocytosis (Figure 1) and increased erythropoietin serum levels (> 5000 pg/mL, n = 5; normal range 50-200 pg/mL, n = 4).

Smad4 is dispensable for adult murine erythropoiesis

To determine whether the observed anemia was cell autonomous, bone marrow cells from pIpC-induced *Mx-Cre;Smad4*$^{fl/fl}$ mice and controls were transplanted into lethally irradiated recipient mice. Eight weeks after bone marrow transplantation, the mice were killed and peripheral blood counts were performed (Figure 2). The red blood cell parameters as well as white blood cell counts and platelet levels remained normal (Figure 2). The recipients of *Smad4*$^{\Delta/\Delta}$ bone marrow cells did not show any signs of inflammatory disease typical for knockouts of the *TGF*-β receptors (ie, absence of weight loss, leukocytosis, signs of inflammation of the eyes, and upon autopsy absence of organ damage). Chimerism of recipient mice was determined in peripheral blood by assessing the ratio of CD45.2 donor cells to CD45.1 recipient cells by flow cytometry. Both groups of mice displayed a ratio of donor to recipient cells of greater than 100:1 (Table 2). No differences in B cells (B220), T cells (CD3), or myeloid cells (Gr-1) were detected in bone marrow. Deletion of the floxed *Smad4* alleles was found by PCR in DNA from peripheral blood cells of the recipient mice (not shown). Thus, anemia was not transplantable with *Smad4*-deficient bone marrow cells, indicating that *Smad4* is dispensable for adult murine erythropoiesis.

To confirm this observation in a system not depending on transplantation, we generated mice with a hematopoietic-specific deletion of *Smad4*. The *VavCre* strain has been shown to excise *loxP* target sequences in hematopoietic cells only.[19] The resulting *VavCre;Smad4*$^{fl/fl}$ mice had normal blood counts and showed no symptoms of inflammation (Figure 3). We also confirmed complete excision of *Smad4* in peripheral blood cells of these mice by PCR (Figure S1E). These results implied that host factors might be causing the anemia phenotype.

Anemia of *Smad4*$^{\Delta/\Delta}$ mice is non–cell-autonomous

To determine if anemia is caused by the host environment, bone marrow from wild-type C57BL/6J mice was transplanted into lethally irradiated *Mx-Cre;Smad4*$^{fl/fl}$ and *Smad4*$^{fl/fl}$ control mice. From weeks 2 to 4 after pIpC-induced deletion of *Smad4*, recipients began developing anemia (Figure 4). Interindividual differences in the severity of anemia were observed in *Smad4*$^{\Delta/\Delta}$ mice. The control *Smad4*$^{fl/fl}$ recipient mice remained healthy without any changes in blood parameters. These results demonstrate that anemia of *Smad4*$^{\Delta/\Delta}$ mice is caused by alterations outside of the hematopoietic system.

Smad4$^{\Delta/\Delta}$ mice show severe iron deficiency

The presence of hypochromic erythrocytes at later stages of anemia in *Smad4*$^{\Delta/\Delta}$ mice (Figure 5A) suggested that iron deficiency could be involved in the pathogenesis of anemia. Liver iron in *Smad4*$^{\Delta/\Delta}$ mice 4 weeks after pIpC induction was decreased to 23% (2.6 ± 0.3 μmol/g, n = 6) compared with liver iron of *Smad4*$^{fl/fl}$ mice

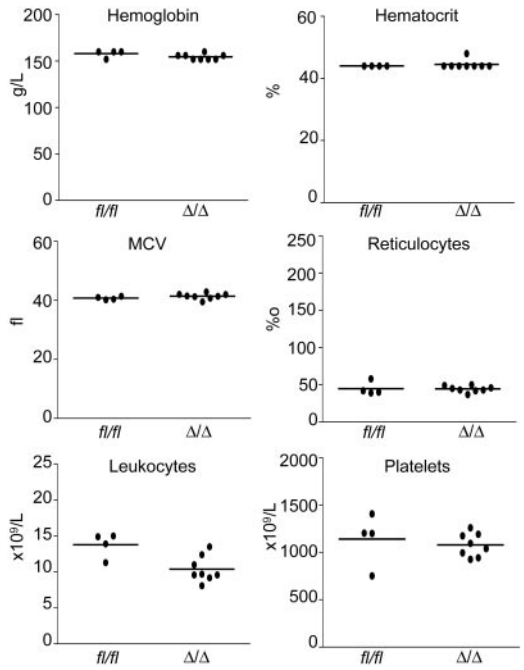

Figure 2. Transplantation of *Smad4*$^{\Delta/\Delta}$ bone marrow cells into wild-type recipient mice did not lead to the development of anemia. Hemoglobin level, hematocrit, MCV, reticulocyte count, leukocyte count, and platelet count remained stable in the control and experimental groups. Eight recipients of *Smad4*$^{\Delta/\Delta}$ bone marrow cells and 4 recipients of *Smad4*$^{fl/fl}$ controls were analyzed 8 weeks after transplantation. Dots represent the values of individual mice; horizontal lines indicate the mean.

(11.5 ± 4.0 μmol/g, n = 6) or wild-type C57BL/6J mice (16.6 ± 4.7 μmol/g, n = 3). No differences in plasma transferrin (Tf), determined by ELISA specific for mouse Tf, were found between *Smad4*$^{\Delta/\Delta}$ mice (1.9 ± 0.2 μg/L, n = 6) and *Smad4*$^{fl/fl}$ (1.9 ± 0.9 μg/L, n = 10) or wild-type BL6 mice (1.7 ± 0.08 μg/L, n = 3). We determined the expression of genes that are involved in iron metabolism by quantitative PCR (Figure 5B-C). The efficiency of Cre-mediated excision in the livers of *Smad4*$^{\Delta/\Delta}$ mice measured by Southern blot was ranging from 66% to 96% (not shown) and the expression of *Smad4* mRNA was severely reduced (Figure 5B). To assure that only the full-length mRNA was measured, the forward primer (Table 1) used for the quantification of the full-length *Smad4* mRNA was placed in exon 8, which is deleted by Cre-mediated excision. We also determined the expression of genes involved in the regulation of iron metabolism. Hepcidin (*Hepc*) mRNA was severely decreased, whereas the expression of divalent metal transporter 1 (*Dmt1*), cytochrome b reductase 1 (*Dcytb*), ferroportin 1 (*Fpn*), and transferrin (*Tf*) remained unchanged (Figure 5B). In the duodenum, the floxed *Smad4* allele was excised by only 4% to 8% (not shown) and *Smad4* mRNA was just merely decreased, whereas *Dmt1* and *Dcytb* increased 5- to 50-fold and transferrin receptor 2 (*Tfr2*) increased 2- to 20-fold (Figure 5C). Other iron-related genes, such as major histocompatibility complex class I–like protein (*Hfe*), transferrin receptor 1 (*Tfr1*), hephaestin (*Heph*), and stimulator of Fe transport (*Sft*) showed no significant changes in expression (Figure 5C). Taken together, these changes fit well with a state of increased iron uptake and demand.

Table 2. Hematopoietic lineage distribution in bone marrow of wild-type recipient mice that received *Smad4*$^{\Delta/\Delta}$ or *Smad4*$^{fl/fl}$ bone marrow

	Smad4$^{\Delta/\Delta}$	*Smad4*$^{fl/fl}$
n	4	4
CD45.2$^+$	87.4 ± 3.3	91.1 ± 3.2
CD45.1$^+$	0.24 ± 0.1	0.50 ± 0.4
B220$^+$	8.4 ± 2.1	5.8 ± 0.2
CD3	2.5 ± 0.9	2.4 ± 0.6
Gr1	14.8 ± 1.2	14.4 ± 1.3

Donor cells (CD45.2$^+$), recipient cells (CD45.1$^+$).

Polyps in stomach and colon cause blood loss in *Smad4*$^{\Delta/\Delta}$ mice

Histopathology of the gastrointestinal (GI) tract of *Smad4*$^{\Delta/\Delta}$ mice revealed polyps in stomach and colon (Figure 6) but less frequently in the small intestine. Most of them were histologically characterized by a branching architecture reminiscent of hyperplastic lesions, mostly with foci of low- and/or high-grade dysplasia. We classified these lesions as mixed hyperplastic/adenomatous polyps. Moreover, "serrated" aspects of the polyps were also detected focally. Additionally, colon polyps frequently displayed cystic changes. None of the gastric or colon polyps fulfilled the criteria of "juvenile polyps," as they lacked the typical histologic features of such lesions (eg, prominent stroma overgrowth).

To show that iron deficiency in *Smad4*$^{\Delta/\Delta}$ mice is due to GI bleeding, we collected stool over several weeks and determined the presence of heme by the hemoccult assay (Figure 7). Bleeding was detectable in all mice but with variable onset and duration. In some mice, bleeding started 17 days after pIpC induction of *Smad4* deletion, whereas in others the onset was delayed until 31 days. At the time of the first detectable bleeding, the mice did not yet display severe anemia and the severity of anemia did not correlate with the time of onset of bleeding.

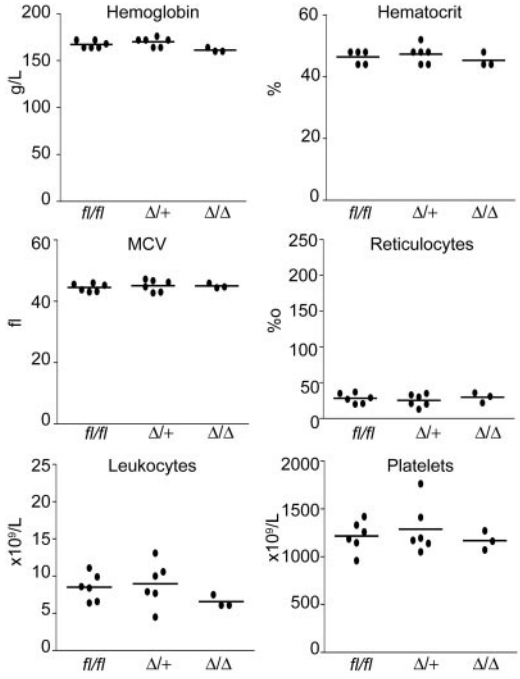

Figure 3. Absence of anemia in *VavCre;Smad4*$^{fl/fl}$ mice. Peripheral blood parameters were determined in 10-week-old mice. *VavCre;Smad4*$^{fl/fl}$ (Δ/Δ), *VavCre;Smad4*$^{fl/+}$ (Δ/+), and *Smad4*$^{fl/fl}$ (fl/fl). Dots represent the values of individual mice; horizontal lines indicate the mean.

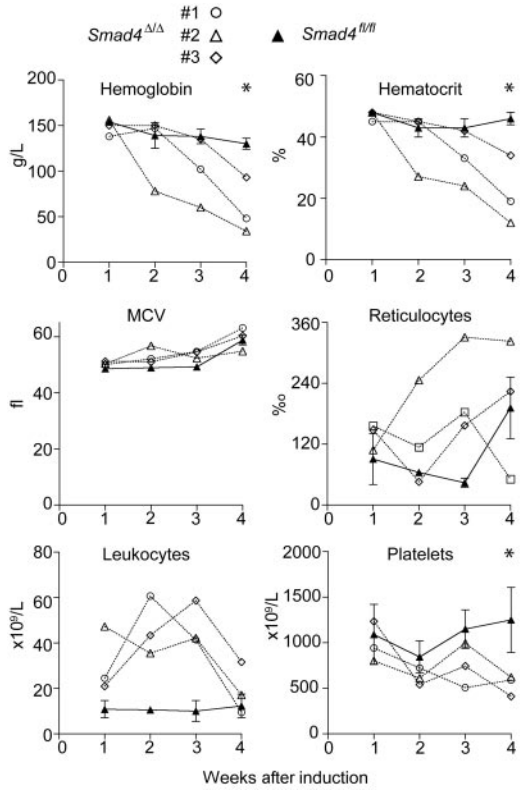

Figure 4. Transplantation of wild-type bone marrow into *Smad4*$^{\Delta/\Delta}$ recipients resulted in anemia. Hemoglobin level, hematocrit, MCV, reticulocyte count, leukocyte count, and platelet count are shown. Three individual *Smad4*$^{\Delta/\Delta}$ recipient mice (#1 [○], #2 [△], and #3 [◇]) developed anemia with individual differences in severity and kinetics. *Significant differences at 4 weeks ($P \leq .03$; Student *t* test). The values for the *Smad4*$^{fl/fl}$ control recipient mice (▲) are shown as the mean of 6 mice with standard deviation. The error bars indicate standard deviation.

Discussion

Our results from transplantation of *Smad4*$^{\Delta/\Delta}$ bone marrow into wild-type recipients and deletion of *Smad4* selectively in hematopoietic cells in *VavCre;Smad4*$^{fl/fl}$ mice demonstrate that *Smad4* signaling is dispensable for adult erythropoiesis in vivo (Figures 2,3). The red blood cell parameters in peripheral blood were normal. Our study is consistent with the results of an in vitro study that examined cultured human CD34$^+$ hematopoietic stem/progenitor cells under shRNA-mediated *Smad4* knock-down.[27] We also found normal megakaryopoiesis in our study, arguing that the decrease in circulating platelet counts and increase in megakaryocyte numbers in mice injected with TGF-β protein in vivo are mediated by Smad4-independent signaling.[28] The white blood cell counts of neutrophil, basophil, and eosinophil granulocytes as well as monocytes and lymphocytes were normal. The severe anemia observed in *Smad4*$^{\Delta/\Delta}$ mice is caused by blood loss.

TGF-β signaling plays an important role in immune surveillance. By producing the immunosuppressive cytokine TGF-β, tumors may escape from immune surveillance via inhibiting the expression of cytolytic genes.[29] TGF-β receptor II (*TBRII*) dominant-negative approaches led to autoimmune inflammatory disease and spontaneous T-cell activation.[30] Mice with *TBRII* deletion in bone marrow not only showed increased CD8$^+$ proliferation in vivo but also developed a lethal inflammatory disease,[18,31] and the *TBRII*-deficient cells of hematopoietic origin, most likely T cells, could induce multifocal inflammatory disease in a dominant way. On the contrary, our *Smad4*$^{\Delta/\Delta}$ bone marrow recipients and *VavCre;Smad4*$^{fl/fl}$ mice were healthy and did not show any signs of inflammatory disease. This result implies that T-cell–mediated suppression of inflammation via TGF-β signaling is Smad4 independent. It remains to be determined whether this effect is mediated by the traditional TGF-β pathway with an alternative downstream mediator like TIF1 or by crosstalk with other signaling through MAPK, JNK, PI3K, or other mediators.

Smad4 is a tumor suppressor gene, and *Smad4* mutations are frequently detected in pancreatic cancer, colon cancer, gastric polyps, and adenocarcinomas.[32-37] Mice heterozygous for the *Smad4* knockout appear normal but develop gastrointestinal polyps after a long latency with loss of heterozygosity in the epithelial cells.[38,39] Our *Smad4*$^{\Delta/\Delta}$ mice, which have complete excision in bone marrow as well as partially in stomach (10%-50%), started bleeding around day 20 after pIpC induction (Figure 7) and quickly developed a severe anemia and significant polyps in the GI tract without formation of real tumors before they needed to be killed. Ablation of TGF-β by T-cell–specific overexpression of dominant-negative TBRII could

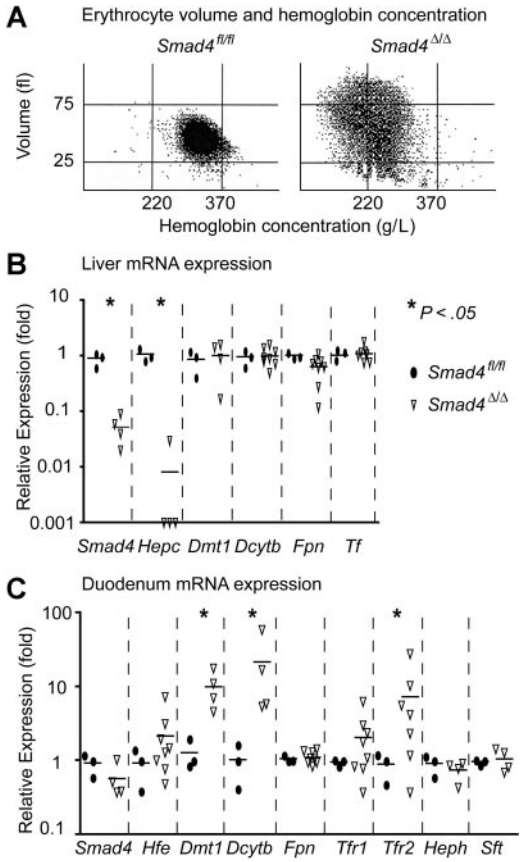

Figure 5. Smad4$^{\Delta/\Delta}$ mice display severe iron deficiency anemia. (A) Hypochromic erythrocytes in *Smad4*$^{\Delta/\Delta}$ mice. Cell volume was plotted against hemoglobin concentration. (Left) Normal control. (Right) Hypochromic red blood cells ($<$ 220 g/L) and volume increase ($>$ 75 fl) due to reticulocytosis in *Smad4*$^{\Delta/\Delta}$. (B) In liver, *Smad4* and hepcidin (*Hepc*) expression are almost abrogated, and ferroportin 1 (*Fpn*) is slightly decreased. Cytochrome b reductase 1 (*Dcytb*), divalent metal transporter 1 (*Dmt1*), and transferrin (*Tf*) were unchanged. (C) In duodenum, *Smad4*, *Fpn*, hephaestin (*Heph*), major histocompatibility complex class I–like protein (*Hfe*), transferrin receptor 1 (*Tfr1*), and stimulator of Fe transport (*Sft*) were unchanged, and *Dmt1*, *Dcytb*, and transferrin receptor 2 (*Tfr2*) were dramatically increased. *Smad4*$^{fl/fl}$ littermates were chosen as controls. The *P* values were calculated by Student *t* test.

accelerate azoxymethane-induced colon carcinogenesis through increased T-cell production of IL-6.[40] Recently, it was reported that T-cell specific deletion of *Smad4* leads to spontaneous epithelial tumors throughout the GI tract. Mice in this study displayed weight loss starting 3 months after birth and eventually developed clinical features of systemic illness. In contrast, mice with epithelial-specific deletion of *Smad4* remained free of polyps, suggesting that polyp formation and tumorigenesis are induced by Smad4-deficient T cells.[41] Our *Mx-Cre;Smad4*$^{\Delta/\Delta}$ mice developed polyps with a much faster kinetics and since they suffered from severe bleeding anemia, we could not examine tumor progression. At age 10 weeks, our *VavCre;Smad4*$^{fl/fl}$ mice did not yet show any signs of weight loss or systemic disease. Furthermore, when *Smad4*$^{\Delta/\Delta}$ mice were used as recipients for the transplantation of normal bone marrow cells, we observed rapid evolution of anemia (Figure 4).

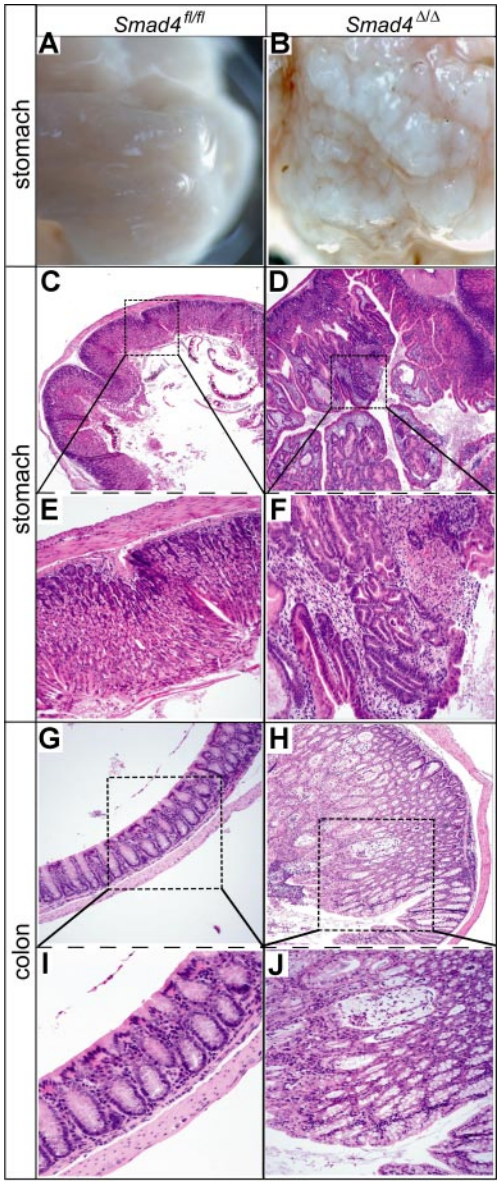

Figure 6. Stomach and colon polyp formation in *Smad4*$^{\Delta/\Delta}$ mice. The left panel shows a *Smad4*$^{fl/fl}$ control mouse, right panel shows a *Smad4*$^{\Delta/\Delta}$ mouse. Gross macroscopy of stomach (A,B), histologic hematoxylin-eosin staining of stomach (C-F), and colon (G-J) with magnified view of the boxed areas. Magnifications for A,B: 2.5×; C,D: 50×; E-H: 100×; I,J: 200×.

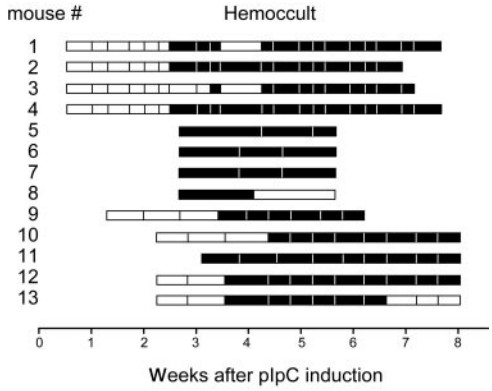

Figure 7. Fecal occult blood test in *Smad4*$^{\Delta/\Delta}$ mice. A total of 13 mice were analyzed (numbered in y-axis). Time in weeks after first pIpC injection is shown on the x-axis. Horizontal bars represent the duration of the stool collection; □, negative hemoccult tests; and ■, positive hemoccult tests.

However, we cannot exclude that some host T cells deficient for *Smad4* remained functional in these mice.

Recently, shRNA-mediated knockdown of bone morphogenic protein type II receptor (*Bmpr2*), another member of the TGF-β receptor family, resulted in severe mucosal hemorrhage, gastrointestinal hyperplasia, and blood loss.[42] This phenotype was related to vascular dysmorphogenesis. Other studies suggested that malformed vessels are present in polyps of patients with juvenile polyposis, found exclusively in patients carrying a mutation up to codon 415 of *SMAD4*.[43] In contrast, no malformations of blood vessels were observed in polyps from our *Smad4*$^{\Delta/\Delta}$ mice.

Our *Smad4*$^{\Delta/\Delta}$ mice showed an almost complete loss of *hepcidin* mRNA expression in liver. This could be due to 2 mechanisms: (1) liver-specific *Smad4* deletion results in markedly decreased hepcidin transcription[44]; and (2) increased erythropoiesis due to anemia suppresses the production of hepcidin.[45] In our mice, both mechanisms are likely to be contributing to the strongly suppressed hepcidin expression.

Our results demonstrate that *Smad4* is dispensable for adult erythropoiesis and suggest that polyp formation in mice with homozygous deletion of Smad4 is not entirely T-cell dependent. The same results were recently reported by Karlsson et al.[46] The *Smad4*$^{\Delta/\Delta}$ mice can be used to further study polyp formation and the interplay between the inflammatory response and alterations in the epithelial cells and the stroma of the GI tract.

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Authorship

Contribution: D.P. performed research, analyzed data, and wrote the paper; T.S. performed research and analyzed data; C.P.K. performed flow cytometric analysis; L.M.T. performed pathology analysis; K.H. and W.K. performed bone marrow transplantations;

H.H.-S. performed genotyping; C.D. analyzed data; and R.C.S. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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