The role of Smad signaling in hematopoiesis and translational hematology

Ulrika Blank[1], Stefan Karlsson[1]

[1]Division of Molecular Medicine and Gene Therapy, Lund Stem Cell Center, Lund University Hospital, Lund, Sweden

Corresponding author:

Dr. Ulrika Blank

Division of Molecular Medicine and Gene Therapy

BMC A12, 221 84 Lund, Sweden

E-mail: ulrika.blank@med.lu.se

Phone: +46-46-2220589

Fax: +46-46-2220568

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Abstract

Hematopoietic stem cells (HSCs) reside in the bone marrow of adult individuals and function to produce and regenerate the entire blood and immune system over the course of an individual's lifetime. Historically, HSCs are among the most thoroughly characterized tissue specific stem cells. Despite this, the regulation of fate options such as self-renewal and differentiation has remained elusive, partly because of the expansive plethora of factors and signaling cues that govern HSC behavior *in vivo*. In the bone marrow, HSCs are housed in specialized niches that dovetail the behavior of HSCs with the need of the organism. The Smad signaling pathway, which operates downstream of the TGF-β superfamily of ligands, regulates a diverse set of biological processes, including proliferation, differentiation and apoptosis, in many different organ systems. Much of the function of Smad signaling in hematopoiesis has remained nebulous due to early embryonic lethality of most knockout mouse models. However, recently new data has been uncovered suggesting that the Smad signaling circuitry is intimately linked to HSC regulation. In this review, we bring the Smad signaling pathway into focus, chronicling key concepts and recent advances with respect to TGF-β-superfamily signaling in normal and leukemic hematopoiesis.

Introduction

Transforming growth factor-β (TGF-β) is the founding member of a large superfamily of secreted polypeptide growth factors, which additionally includes activins, nodal, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and others (1). From early development and continuously throughout adult life, TGF-β members carry out pivotal functions by regulating biological events ranging from gastrulation and organ morphogenesis to homeostatic tissue turnover. Alterations in components of the TGF-β superfamily pathway lead to severe developmental abnormalities and have been shown to underlie a range of human diseases, including autoimmune and cardiovascular disorders as well as cancer (2, 3). A fundamental feature of the TGF-β superfamily is its highly pleiotropic nature, a phenomenon well illustrated within the hematopoietic system; depending on the differentiation stage and environmental context of the target cell, these factors can affect proliferation, differentiation and apoptosis either positively or negatively (4-6). Part of the molecular basis for this is thought to stem from the unique repertoire of transcriptional co-factors expressed by each specific cellular target. The context dependent actions of TGF-β ligands are reflected *in vitro*, often leading to opposing findings between *in vitro* and *in vivo* systems.

In this review, we will discuss the role of TGF-β and Smad signaling in normal hematopoiesis, featuring aspects of translational hematology, particularly the role of Smad signaling in the development of hematologic malignancies and how the Smad circuitry may be exploited for the purpose of stem cell expansion. It should be emphasized that although some of the findings discussed here come from studies of human and patients' cells, the majority of the data derive from studies in well-defined mouse models.

Smad signaling

The Smad signaling circuitry embodies an evolutionary conserved signaling module, which functions to convert biological information from activated TGF-β receptor complexes at the cell surface to concrete transcriptional regulation in the nucleus. TGF-β ligands transmit signals through two types of serine/threonine kinase receptors, known as type I and type II receptors (1). In vertebrates seven different type I receptors (ALK1-7) and five distinct type II receptors have been identified (1). Although some promiscuity occurs each ligand generally signals through a specific combination of receptors (Figure 1). Following ligand binding, the type I receptor becomes activated through phosphorylation by the type II receptor. Activated type I receptors subsequently phosphorylate the Smad proteins at residues in the C-terminus, leading to propagation of the signal intracellularly. The Smad family of intracellular mediators is comprised of eight members in mammals, Smad1-8, which can be further subdivided into three distinct classes based on structural properties and the specific functions they carry out (7). Receptor-regulated Smads (R-Smads), Smad1, 2, 3, 5 and 8, are the only Smads directly phosphorylated and activated by the kinase domain of type I receptors. Phosphorylation of R-Smads results in a conformational change, allowing complex-formation with the common-Smad (Co-Smad), Smad4. Activated complexes subsequently accumulate in the nucleus where they cooperate with other transcriptional co-regulators to modify target gene transcription. The third class of Smads includes the inhibitory Smads (I-Smads), Smad6 and Smad7, which function in a negative feedback loop to inhibit TGF-β superfamily signaling. TGF-β/activin/nodal and BMP/GDF employ different subsets of R-Smads. R-Smad2 and 3 specifically relay signals from TGF-β and activin receptors whereas R-Smad1, 5 and 8 primarily operate downstream of BMP receptors (1, 8).

TGF-β in hematopoiesis

TGF-β is categorized as one of the most potent inhibitors of HSC growth *in vitro* and a large body of work from a variety of culture systems supports this notion (4, 9-11). Owing to the naturally quiescent state of HSCs, TGFβ has been hypothesized to be a cardinal regulator of HSC quiescence, maintaining a slow-cycling state of HSCs *in vivo* (Figure 2). In keeping with this, neutralization of TGF-β *in vitro* was shown to release early hematopoietic progenitor cells from quiescence (12-14). Several molecular mechanisms have been proposed to account for TGF-β-mediated growth inhibition, including alterations in cytokine receptor expression and up-regulation of cyclin-dependent kinase inhibitors (CDKIs), such as p15, p21 and p27 (13, 15-21). However, it has been shown that TGF-β can exert growth inhibitory actions independently of p21 and p27 (22). Additionally, neutralization of TGF-β coupled with antisense knockdown of p27 was shown to result in synergistically increased retroviral gene transfer efficiency in human $CD34^+$ BM cells, implying that TGF-β and p27 work in separate pathways (16). In human $CD34^+$ cells, TGF-β-mediated cell-cycle arrest has been suggested to occur through up-regulation of p57, another member of the CDKI family (23). This finding was further corroborated by the observation that p57 was highly enriched in mouse $CD34^-Kit^+lineage^-Sca1^+$ ($CD34^-$KLS) cells as opposed to the more mature $CD34^+$KLS fraction (24). Interestingly, a high level of p57 was shown to correlate with the activation status of Smad2 and Smad3, which were reported to be uniquely phosphorylated in freshly isolated $CD34^-$KLS cells but not in $CD34^+$KLS progenitors (25). In addition, TGF-β was shown to up-regulate p57 in $CD34^-$KLS cells *in vitro* (25). These findings point to a mechanism where TGF-β functions to induce p57 within the most primitive HSC compartment, thus maintaining their quiescent state *in vivo*.

Most work regarding TGF-β in hematopoiesis has been carried out using TGF-β1. However, TGF-β exists in three isoforms, TGF-β1-3. Although all TGF-βs share significant sequence homology and signal through the same receptor complex (26, 27), differing responses have been reported. Most notably KLS cells exposed to TGF-β2 exhibited a biphasic response, being growth inhibited at high doses and stimulated at low concentrations (28). The findings are intriguing as the stimulatory component of TGF-β2 was shown to be dependent on serum factors, genetic background and age (29). However, this finding showed bearing *in vivo* as *Tgf-β2* heterozygote knockout cells exhibited a defective repopulative capacity upon transplantation. This effect became more pronounced following serial transplantation, suggesting that TGF-β2 functions cell autonomously as a positive regulator of adult HSCs that have undergone replicative stress (28).

Recently evidence has accumulated suggesting that the adult HSC compartment consists of a number of functionally distinct subsets with diverse self-renewal and differentiation potentials (30-32). Challen and colleagues identified the TGF-β pathway as a potential mechanism for differential regulation among discrete HSC subtypes (33). Specifically, TGF-β stimulated proliferation of myeloid-biased HSCs whereas the opposite was true for lymphoid-biased HSCs (33). These bidirectional effects further substantiate the complexity of TGF-β signaling and it remains to be clarified whether or not the Smad pathway is differentially regulated at low and high doses and between diverse HSC subtypes. Additionally, the roles of Smad2 and Smad3 in HSCs have not yet been functionally gauged in conditional knockout mouse models. This will be an important *in vivo* system to characterize, in order to further unwind the intricate nature of TGF-β signaling in HSCs.

TGF-β: Lessons from *in vivo* models

TGF-β can affect most cell types throughout the hematopoietic hierarchy and depending on the context and differentiation stage of the target cell different biological responses are elicited (4-6) *In vivo*, TGF-β plays a principal role as regulator of immune cell homeostasis and function, as unequivocally shown by the development of a lethal inflammatory disorder in both *Tgf-β1*- ligand and receptor knockout mice (34-36). Furthermore, *Tgf-β1* null mice exhibited enhanced myelopoiesis, suggesting that TGF-β acts as a negative regulator of myelopoiesis *in vivo* (35). When *Tgf-β1* knockout mice were analyzed before the onset of multifocal inflammation, a range of HSC properties were shown to be altered (37). Most significantly, BM cells from *Tgf-β1* deficient neonates exhibited impaired reconstitution ability upon transplantation, a finding attributed to defective homing (37). In contrast, mice deficient in the TGF-β type I receptor (*TβRI*), displayed normal HSC self-renewal and regenerative capacity *in vivo*, even under extreme hematopoietic stress (38, 39). Thus, there are both overlapping and non-overlapping phenotypes between ligand and receptor knockout models and it appears to be significantly important at which level TGF-β signaling is disrupted. The apparent discrepancies related to *in vitro* and *in vivo* findings may reflect redundant functions of other type I receptors or alternatively other ligands such as activin, which signals through the same R-Smad pathway. Due to the multifaceted nature of TGF-β coupled with a potentially complex set of redundant mechanisms *in vivo*, its role as a critical regulator of HSC quiescence *in vivo* remains to be fully proven, despite intense research and despite increased knowledge of TGF-β signaling.

TGF-β signaling diversified: a role for TIF1γ in hematopoiesis

Smad4 has traditionally been viewed as the nexus of Smad signaling as it functions as

a core component of both TGF-β/activin and BMP signaling branches. However, Smad2/3 have been shown to partner not only with Smad4 but also with Transcriptional Intermediary Factor-1γ (TIF1γ), suggesting that the Smad pathway is more diversified than previously thought (Figure 3). In a model proposed by He et al. TGF-β was shown to mediate erythroid differentiation concomitantly with balancing growth inhibition in human hematopoietic stem/progenitor cells (40). According to this model, TIF1γ selectively binds Smad2 and Smad3 in competition with Smad4. In response to TGF-β, the TIF1γ/Smad2/3 complex stimulated erythroid differentiation whereas Smad2/3 in association with Smad4 led to growth inhibition of human hematopoietic progenitors. Thus, the relative abundance of Smad4 and TIF1γ appears crucially important for determining the precise outcome of TGF-β stimulation. Interestingly, the zebrafish homolog of TIF1γ, encoded by *moonshine*, has been shown to be essential for blood formation with mutants displaying severe red cell aplasia, indicating that the function of TIF1γ may be preserved across species (41). Furthermore, Bai and colleagues recently uncovered a role for TIF1γ in regulating transcription elongation of erythroid genes (42). The model proposed suggests that TIF1γ functions to release paused Pol II at erythroid genes by recruiting positive elongation factors to the blood-specific transcriptional complex, thus promoting transcription (42). TIF1γ also functions in a broader context as it was recently shown to play a role in erythroid/myeloid lineage bifurcation by modulating GATA1 and PU.1 expression (43). The role of the Smad pathway in these processes has not been investigated and what signaling pathways function to control transcription elongation of blood genes remains to be determined. It is however interesting to note that the BMP pathway has been previously linked to stress erythropoiesis, as will be discussed

below (44, 45). The mechanism by which TIF1γ cooperates with Smads continues to be a controversial issue. Developmental studies from a variety of species have suggested that TIF1γ, also known as Ectodermin, acts as a ubiquitin ligase for Smad4, thus functioning as a direct inhibitor of Smad4 downstream of TGF-β and BMP signaling (46, 47). Mice deficient in TIF1γ die during early somitogenesis, displaying phenotypes which are consistent with excessive TGFβ/nodal signaling, supporting a role for TIF1γ as negative regulator of Smad4 (48). How these seemingly disparate molecular mechanisms can be reconciled will require further investigation, but it is possible that temporal aspects and differences in cellular context play critical roles in determining the precise role of TIF1γ. Regardless of the exact molecular mechanism, accumulated data suggest that TIF1γ functions to restrict Smad signaling downstream of TGF-β/nodal.

BMP signaling in hematopoietic development

BMPs figure early during development as morphogens regulating mesoderm patterning. In mice, targeted deletions of a variety of BMP signaling components, including *Bmp2*, *Bmp4* and *BmpRIa*, resulted in severe mesoderm deficiency and embryonic lethality (49-51). Because blood is derived from mesoderm, BMPs have been implicated as key regulators of blood formation during embryonic development. However, since BMP signaling is required before the onset of hematopoietic development the exact role of BMPs in hematopoietic induction has been challenging to study particularly in the mouse. Therefore, much of the initial knowledge on the role of BMP signaling in hematopoietic development has been derived from studies in lower vertebrates and from culture systems *in vitro* (52). For example, studies performed in *Xenopus*, have revealed an important role for BMP4 in induction of hematopoiesis (53, 54). In zebrafish, BMP2 and BMP7 mutants displayed lack of

ventral mesoderm development and a complete absence of blood cells (55). Additionally, to block BMP signaling after its initial requirement during specification of mesoderm, Schmerer and Evans devised a model where activity of the I-Smad6 could be induced after gastrulation in *Xenopus* explants (56). Using this approach it was shown that Smad signals are required for primitive erythropoiesis and maintenance of Gata1 expression within specified mesoderm. Furthermore, when BMP signaling was disrupted in lateral mesoderm in the zebrafish model, by targeting a dominant negative BMP receptor to Lmo2^{+} cells, it was concluded that BMP signaling continues to function in the regulation of lineage specification after lateral mesoderm commitment (57). However, at this stage BMP signaling functions to restrict hemato-vascular fate in favor of pro-nephric development (57), indicating that the effect of BMP signaling on hematopoiesis is affected by the exact stage of development. Moreover, in both murine and human ES cells exposure to BMP4 has been reported to induce mesoderm formation including hematopoietic commitment (58-61). BMP4 has also been shown to enhance hematopoietic development of rhesus monkey ES cells (62). Data obtained from the murine ES cell system further supports that BMP signaling functions in a two-step manner, its role in mesoderm patterning being separable from its function in blood fate specification (63). Thus, in vertebrates embryonic hematopoiesis depends on BMP-signaling in a mechanism that appears to be independent from its role in mesoderm patterning.

Modeling BMP deficiency *in vivo*

The role of Smad-mediated BMP signaling in hematopoiesis has recently been investigated in both mouse and zebrafish model systems, using mutants and targeted deletions of Smad1 and Smad5 (Table 1). Intriguingly, data accumulated suggest that this pathway is subject to considerable species and temporal variation with a

seemingly more pronounced role for Smad1 and Smad5 in zebrafish as compared to the mouse. Using both loss of function approaches and hypomorphic mutants, the roles of Smad1 and Smad5 were studied in zebrafish (64). Interestingly, distinct phenotypes were generated with respect to primitive erythropoiesis. While *smad1* morphants exhibited enhanced erythropoiesis, knockdown of *smad5* resulted in a failure to maintain the erythroid program and thus erythropoiesis failed (64). Both genes, however, were shown to be required for definitive hematopoiesis as deficiency of either Smad1 or Smad5 caused a failure in the generation of definitive hematopoietic progenitors (64). To what extent this is a cell-autonomous effect is not clear, as Hild et al previously showed that transplantation of *somitabun* (a Smad5 dominant negative mutation) mutant cells survived and formed blood tissue in a wild type environment (65). In the zebrafish model system, the initial requirement for BMP signaling can be overcome by injection of mRNA of the wild-type gene into fertilized eggs, thus allowing analysis of hematopoietic parameters in adult fish. Several allelic mutations of *smad5* were studied, revealing anemia with mutants exhibiting decreased numbers of erythroid progenitors (66). Additionally, *smad5* mutants had an altered response to hemolytic anemia, indicating that Smad5 may be involved in regulating the kinetics of recovery under conditions of acute anemia (66). This is particularly interesting in the context of the *flexed-tail* mouse mutant, which carries a spontaneous mutation in the *Smad5* gene. Neonatal mutant mice are anemic but recover in adulthood, except under conditions of stressed hematopoiesis (44). Mice carrying the *flexed-tail* mutation could not mount an effective response to acute anemia due to a specific defect in splenic erythropoietin-responsive progenitors to respond to BMP4 (44). Subsequently, it was shown that Stem Cell Factor (SCF) and hypoxia synergize with BMP4 to drive effective recovery upon stress anemia (45).

Although acute anemia has never been studied in the context of complete deletion of *Smad5* in the mouse, these studies stand in sharp contrast to studies performed by Singbrant and colleagues. In a series of experiments it was shown that Cre-mediated deletion of *Smad1* and/or *Smad5*, did not impair adult hematopoiesis in the mouse and mutant HSCs displayed normal self-renewal and differentiation capacities upon transplantation (67, 68). Similarly, using a fetal liver-specific *Cre*-driver to induce deletion of *Smad5* or *Smad1*/*Smad5* together, hematopoiesis was shown to occur normally upon transplantation into wild-type hosts (67). These differences are intriguing, but may suggest that Smad-mediated BMP signaling is only required under very specific stressed conditions. Taken together, the canonical BMP signaling pathway does not seem to regulate critical aspects of HSC biology in the adult mouse, in spite of its pivotal function in earlier developmental events. However, BMPs are present in the BM and a role for *BmpRIa*, has been established in the osteoblastic niche (69). *BmpRIa*-deficient mice exhibited an increase in the number of N-cadherin+ osteoblastic cells resulting in an increase in the number of HSCs (69). Thus, BMPs are indirectly involved in regulating HSC-frequency in adult mice.

BMP signaling *ex vivo*

In vitro, BMP4 has been shown to promote maintenance of human HSCs in culture, whereas lower concentrations of BMP4 induced proliferation and differentiation of human hematopoietic progenitors (70). Furthermore, Shh induced proliferation of primitive human hematopoietic progenitors *in vitro*, apparently through a BMP4 dependent mechanism (71). However, while BMP4 has been shown to maintain human NOD/SCID repopulating cells in culture it does not seem to cause an expansion, suggesting that Shh may act through additional mechanisms. The murine fetal liver stromal cell line AFT024 has previously been shown to maintain and

expand HSCs in long-term cultures (72). Interestingly, AFT024 cells were shown to produce BMP4 and this contributed significantly to maintenance of co-cultured human hematopoietic progenitors from cord blood (73). In the murine system, BMP4 does not appear to affect proliferation of purified HSCs *in vitro*, although it is currently unclear whether BMP4 can extend the maintenance of murine HSCs in culture as this study did not assess *in vivo* reconstitution ability following BMP4 exposure (74).

Complete disruption of the Smad pathway

To block the entire Smad signaling network and to sidestep potentially redundant mechanisms within this circuitry, two parallel approaches have been used: overexpression of the inhibitory Smad7 and deletion of *Smad4*. Smad7 was overexpressed in murine HSCs using a retroviral gene transfer approach (75). Forced expression of Smad7 resulted in significantly increased self-renewal capacity of HSCs *in vivo*, indicating that the Smad pathway negatively regulates self-renewal *in vivo*. Importantly, differentiation was unperturbed suggesting that self-renewal is regulated independently of differentiation by Smad signaling. When a similar strategy was used in human SCID repopulating cells (SRCs), overexpression of Smad7 resulted in altered differentiation from lymphoid dominant engraftment toward increased myeloid contribution (76). Thus, in the xenograft model system, forced expression of Smad7 modulates differentiation of primitive multipotent human SRCs. Using a conditional knockout mouse model, disruption of the entire Smad pathway at the level of Smad4 was recently investigated. Intriguingly, *Smad4* deficient HSCs displayed a significantly reduced repopulative capacity of primary and secondary recipients, indicating that Smad4 is critical for HSC self-renewal *in vivo* (77). Since overexpression of Smad7 vs. deletion of *Smad4* would be anticipated to yield similar

hematopoietic phenotypes, it is conceivable that Smad4 functions as a positive regulator of self-renewal independently of its role as a central mediator of the canonical Smad pathway. The precise molecular mechanism for this is currently unknown, but it is possible that Smad4 participates in other signaling cascades such as Wnt or Notch (78-80).

Smad signaling in hematopoietic malignancies

The role of TGF-β in hematologic malignancies has been reviewed in detail, including the role of TGF-β in leukemia, lymphoma/lymphoproliferative disorders, multiple myeloma, myeloproliferative diseases and myelofibrosis (81). Here, we will review briefly the role of TGF-β in hematologic malignancies and myelofibrosis.

Despite the pronounced anti-proliferative effect of TGF-β on HSCs *in vitro* and the fact that alterations in genes encoding components of the TGF-β pathway are frequently found in many epithelial neoplasms, such as pancreatic and colon cancer (82, 83), mutational inactivations involving the TGF-β signaling pathway are uncommon in leukemias and other hematological malignancies (reviewed in (84)). A potential role for TGF-β as a tumor suppressor has been demonstrated *in vivo* where heterozygous knockout mice for *Tgf-β1* developed increased numbers of lung and liver tumors upon exposure to carcinogenic stimuli (85). In addition, *Smad3* homozygous knockout mice live to adulthood, but spontaneously develop metastatic colorectal cancer, clearly supporting a role for TGF-β signaling in tumor suppression (86). The lack of leukemogenesis in our TGF-β signaling-deficient mouse models implies that loss of responsiveness to TGF-β is more important for progression rather than initiation of leukemogenesis (38, 67, 75, 77). These findings suggest that deficient TGF-β signaling alone is not sufficient to induce neoplastic transformation in hematopoietic cells.

Although, loss-of-function mutations that disrupt the TGF-β pathway are rare in hematological malignancies, a number of cases have been reported involving *SMAD4* and *TGFβRII* in patients with acute myelogenous leukemia (AML) (87-90). Using ultra-dense array comparative genomic hybridization on 86 AML genomes, 18 copy number alterations (CNAs) regions were found recurrently modified, one of which represented the deletion of the *SMAD4* gene, demonstrating that *SMAD4* can be an AML-associated gene (Walter, PNAS, 2009). Furthermore, sporadic mutations in both *TβRI* and *TβRII* have been reported in lymphoid neoplasms (91, 92). Another study reported that the SMAD3 protein could not be detected in fresh samples from patients with T-cell acute lymphocytic leukemia (ALL). The mechanism for the SMAD3-deficiency is not known since the *SMAD3* mRNA was present and no mutations could be detected in the *MADH3* gene, which encodes SMAD3 (93). However, T-cell leukemogenesis was promoted in mice with haploinsufficiency of *Smad3* and a complete deficiency of *p27* (93). These findings are interesting because the *p27* gene is frequently mutated in pediatric ALL, due to translocations and deletions or germline mutations (94, 95). Impaired TGF-β signaling in hematologic malignancies can also be caused by suppression of Smad-dependent transcriptional responses by oncoproteins like TAX, EVI-1 and AML1-ETO (96-98). Similarly, downregulation of the transcription factor ZEB1 and overexpression of Smad7 contribute to resistance to TGF-β1-mediated growth suppression in adult T-cell leukemia/lymphoma without known mutations in TGF-β pathway genes (99). In addition, there have been a number of reports on oncoproteins, which generate leukemias and simultaneously neutralize the growth inhibitory signal of the Smad pathway by binding or interacting with Smads. Fusion oncoproteins, like TEL-AML1 and AML1-EVI1 have been shown to bind to Smad3, impairing both TGF-β signaling

and apoptosis of transduced HSCs *in vitro* (97, 100-102). Furthermore, Smad4 has been shown to physically associate with HoxA9, thus reducing its ability to regulate transcriptional targets in hematopoietic cells *in vitro* (103). Recently, *in vivo* studies have shown that in wild type mice overexpressing HoxA9 or Nup98-HoxA9, Smad4 binds to the oncoproteins and sequestrates them to the cytoplasm, suggesting a protective role of Smad4 against further promotion and growth of leukemic cells (104). Therefore, Smad signaling is often reduced or neutralized in hematopoietic malignancies, but in a majority of cases this is not due to primary mutations in genes encoding proteins of the Smad circuitry. Rather, malignant cells may exploit other mechanisms to reduce Smad signaling indirectly, through altered expression or function of co-factors and oncoproteins, or alternatively via loss of or disruption of TGF-β target genes.

Although TGF-β plays a major role as tumor suppressor, TGF-β can also paradoxically facilitate tumor growth, particularly in the later stages of disease. This is due to effects on the tumor microenvironment and the immunosuppressive function of TGF-β, rendering the patient/animal with reduced possibilities to reject tumor cells by immunological means.

Myelofibrosis with myeloid metaplasia is a chronic myeloproliferative disease characterized by clonal myeloproliferation and reactive bone marrow fibrosis (81). Myelofibrosis appears in the later stages of chronic myeloid leukemia and polycytemia vera while it occurs early in myelofibrosis with myeloid metaplasia. Several cytokines have been reported to contribute towards accumulation of reticulin fibers in the bone marrow of patients with myelofibrosis. These include TGF-β, mainly TGF-β1, basic fibroblast growth factor and platelet-derived growth factor (105-108). Some of the strongest evidence for a prominent role of TGF-β in the

generation of myelofibrosis involved the use of bone marrow cells from *Tgf-β1* null mice. To induce myelofibrosis, irradiated mice were transplanted with bone marrow cells transduced with vectors containing the TPO gene. Importantly, prominent myelofibrosis only developed in mice receiving transduced wild type cells but not *Tgf-β1* null cells (107). These data indicate that TGF-β1 produced by hematopoietic cells is a vital component in the development of myelofibrosis.

Stem cell expansion towards advanced cell therapy

One of the most important therapeutic modalities in hematology is blood and marrow transplantation to cure leukemia and genetic disorders. The process of finding donors that have compatible histocompatibility antigens for patients that need blood and marrow transplantation (BMT) is often a challenge and a limiting factor in current cell therapy is the shortage of available donors. As of today, umbilical cord blood (CB) is being used increasingly as a source of HSCs due to the common availability of CB cells and the diversity of histocompatibility gene haplotypes that are available in banked CB samples (109). However, the number of HSCs in each CB sample is limited and it would, therefore, greatly increase the applicability of CB-derived HSCs if efficient expansion could be safely achieved *ex vivo* prior to transplantation. In order to achieve stem cell expansion, a detailed understanding of cell signaling is required, including Smad signaling but also other major signaling pathways. Therefore, we will discuss the possible role of Smad signaling and that of other pathways in future efforts to expand stem cells *in vitro*.

Successful stem cell expansion involves symmetric self-renewal divisions of HSCs, where both daughter cells retain HSC properties (110). More commonly, HSCs grown *in vitro* undergo asymmetric divisions characterized by the production of one HSC and a more differentiated progenitor, or alternatively, a symmetric division where

both progeny cells have lost their HSC potential. Although BMPs have been reported to contribute to HSC expansion *ex vivo*, manipulations of the Smad signaling pathway alone are not likely to result in effective stem cell expansion (70, 71). Rather, stimulation of several signaling circuitries and suppression of other pathways will be essential to generate stem cell expansion *in vitro* prior to transplantation in clinical settings. Positive and negative regulators ultimately balance the transition from quiescence to proliferation of HSCs. Thus, the strategies for stem cell expansion should involve activation of regulators that encourage HSC self-renewal and/or inhibition of pathways that mediate quiescence, differentiation or apoptosis of HSCs. Some approaches, such as over-expression of HoxB4, require viral vector-mediated gene transfer to HSCs for efficient expansion (111). However, this strategy is not likely to be accepted for clinical therapy due to the risk of insertional mutagenesis (112, 113). The safest approaches would involve soluble factors, for example cytokines, developmental cues or components such as Angiopoietin-like (Angptl) proteins.

Developmental cues that activate Notch and Wnt signaling in HSCs have been shown to affect self-renewal positively *ex vivo*. Most notably, Wnt3A was shown to expand murine repopulating HSCs and injection of Wnt5A into NOD/SCID mice repopulated with human hematopoietic cells increased the reconstitution and number of primitive hematopoietic cells (114, 115). Similarly, a soluble form of the Notch ligand, Jagged 1, was reported to stimulate growth of human HSCs and may therefore be used to expand stem cells *ex vivo* (116). However, the Angptl proteins Angptl 2 and 3 are by far the most promising soluble factors identified to date for expansion of murine HSCs (117). Using cultures containing Angptl 5 together with SCF, Thrombopoietin (TPO), Fibroblast Growth Factor-1 (FGF-1) and insulin growth factor binding protein

2 (IGFBP2), the number of Scid Repopulating Cells (SRCs) could be expanded by a factor of 20 (118). Clinical benefits can also be achieved by expanding hematopoietic progenitors *ex vivo* to prevent delayed myeloid engraftment following HSC transplantation. Engineered Notch ligand attached to tissue culture plates was used to significantly expand SRCs and was also used in a clinical trial to improve engraftment and prevent delayed myeloid reconstitution following transplantation of CB $CD34^+$ cells (119). As more detailed knowledge is unearthed concerning the regulatory pathways that govern HSC self-renewal, it may be possible to modulate these pathways with small molecule drugs. Recently, it was demonstrated that chemicals that increase prostaglandin E2 synthesis could expand HSC numbers in both zebrafish and mice (120). Similarly, a chemical screen identified a purine derivative, StemRegenin 1, which was shown to promote *ex vivo* expansion of human $CD34^+$ cells and SRCs (121). In the future, more detailed information on single signaling pathways that determine cell fate options will be required, in order to increase our understanding of how integration of major signaling modules may be exploited *in vitro*. Such increased knowledge will open up new avenues for maintaining and expanding HSCs *in vitro*.

Concluding remarks

The Smad pathway constitutes a fundamental signaling component downstream of the TGF-β superfamily of ligands. Manipulations of this pathway at various levels and in a range of species have revealed important functions of this signaling circuitry in HSC self-renewal and differentiation.

Apart from Smad-mediated signals, TGF-β and related ligands have been shown to activate TGF-β activated kinase 1 (TAK1), a component of the mitogen-activated protein kinase (MAPK) pathway, in a range of cell types (122-124) (Figure 3).

Interestingly, TAK1 has been studied in the context of hematopoiesis resulting in severe hematopoietic failure upon *Mx1-Cre* mediated deletion (125). Thus, TAK1 appears to be crucially important for hematopoietic maintenance, but the link to TGF-β has not been established. To what degree non-canonical pathways function downstream of TGF-β and BMPs in HSCs remains unclear and constitutes an exciting avenue for future research. Additionally, the complexity of the Smad pathway continues to be exposed as new layers of regulatory mechanisms are established. For example, the linker region of Smads has been shown to be subject to negative regulation by GSK3 and FGF or EGF (126-129). The importance of this type of signal integration in hematopoietic cells has not been studied, but it will be an interesting area to explore as both WNTs and FGFs have been shown to be involved in HSC regulation (114, 130). In the future, more detailed mechanistic studies are required to precisely define how the Smad signaling pathway may be manipulated in relation to other signaling circuitries, ultimately controlling self-renewal decisions in a manner compatible with expansion of HSCs for advanced cell therapy.

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Figure legends

Figure 1. The TGF-β superfamily. Schematic representation of the various components of the TGF-β superfamily pathway, including ligands, receptors and Smads. Following ligand binding to type I and type II receptors, R-Smads become phosphorylated by activated type I receptors. Phosphorylation of R-Smads results in a conformational change allowing for complex-formation with Smad4. The R-Smad/Smad4 complex subsequently translocates to the nucleus where target gene transcription is modified in cooperation with other DNA-binding factors. P indicates phosphorylation. R-Smad denotes receptor-activated Smad; Co-Smad common Smad; and I-Smad indicates inhibitory Smad.

Figure 2. The bone marrow niche. Quiescent HSCs reside in the bone marrow endosteal region in close proximity to osteoblastic cells and other cellular and structural components with supportive and regulatory functions. BMPs signal via ALK3 on osteoblastic cells, regulating the size of the osteoblastic niche and consequently the size of the HSC pool. Autocrine and/or paracrine TGF-β is thought to induce quiescence of HSCs, contributing to maintenance of HSCs. Loss of Smad4 in HSCs results in impaired self-renewal capacity whereas overexpression of Smad7 leads to increased self-renewal of HSCs.

Figure 3. Summary of TGF-β signaling. TGF-β signaling results in growth inhibition of HSCs when Smad2/3 partners with Smad4. In human cells binding of TIF1γ to Smad2/3 stimulates erythroid differentiation in response to TGF-β. Activation of non-canonical MAPK signaling downstream of TGF-β has been shown in other cell types, but is largely unexplored in HSCs. However, TGF-β-activated kinase1 (TAK1) is expressed in the LSK compartment and is essential for HSC survival *in vivo*.

Table 1. Smad knockout/overexpression or mutant models and associated hematopoietic phenotypes.

Smad signaling component	Organism	Model system	Hematopoietic phenotype	Reference
Smad1	Mouse	Conditional knockout , *Mx-Cre*	Normal HSC properties.	(67)
Smad1	Zebrafish	Morpholino knockdown	Enhanced primitive erythropoiesis, failure to generate definitive hematopoietic progenitors.	(64)
Smad5	Zebrafish	*piggytail* mutant, morpholino knockdown	Defective primitive erythropoiesis, failure to generate definitive hematopoietic progenitors.	(64)
Smad5	Mouse	Conditional knockout, *Mx-Cre*, *Vav-Cre*	Normal adult HSC parameters. Unperturbed fetal liver hematopoiesis.	(67, 68)
Smad5	Mouse	*flexed-tail* mutant	Defective erythroid response during acute anemia.	(44)
Smad1/5	Mouse	Conditional knockout , *Mx-Cre*, *Vav-Cre*	Reduced myeloid component in PB upon transplantation of fetal liver cells, but otherwise normal adult and fetal liver hematopoietic parameters.	(67)
Smad4	Mouse	Conditional deletion, *Mx-Cre*	Impaired self-renewal of HSCs.	(77)
Smad7	Mouse	Retroviral over-expression	Enhanced self-renewal of HSCs *in vivo.*	(75)
Smad7	Human/xenograft	Retroviral over-expression in cord blood scid-repopulating cells	Altered cell fate, favoring myeloid commitment over lymphoid.	(76)

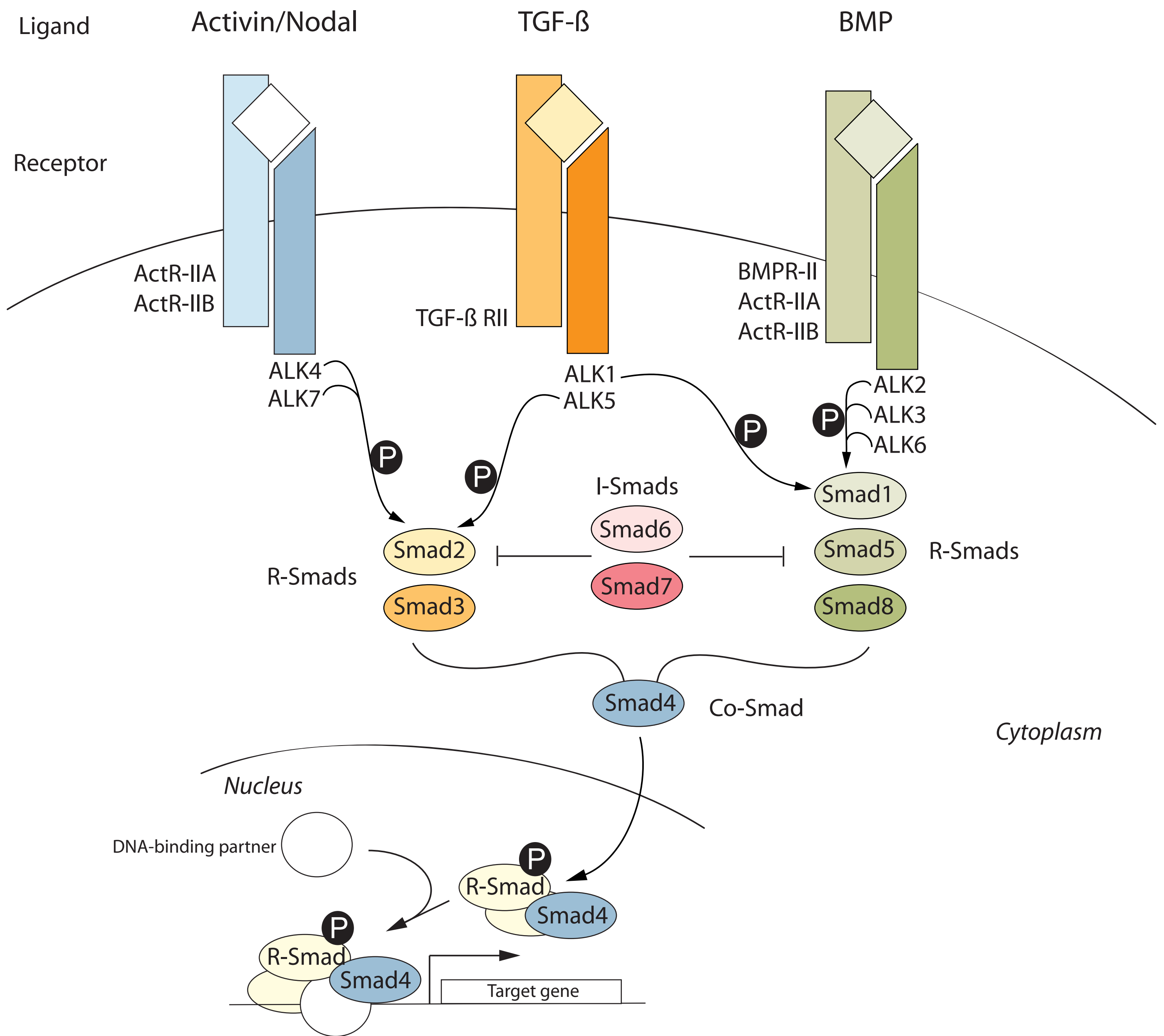

Figure 1
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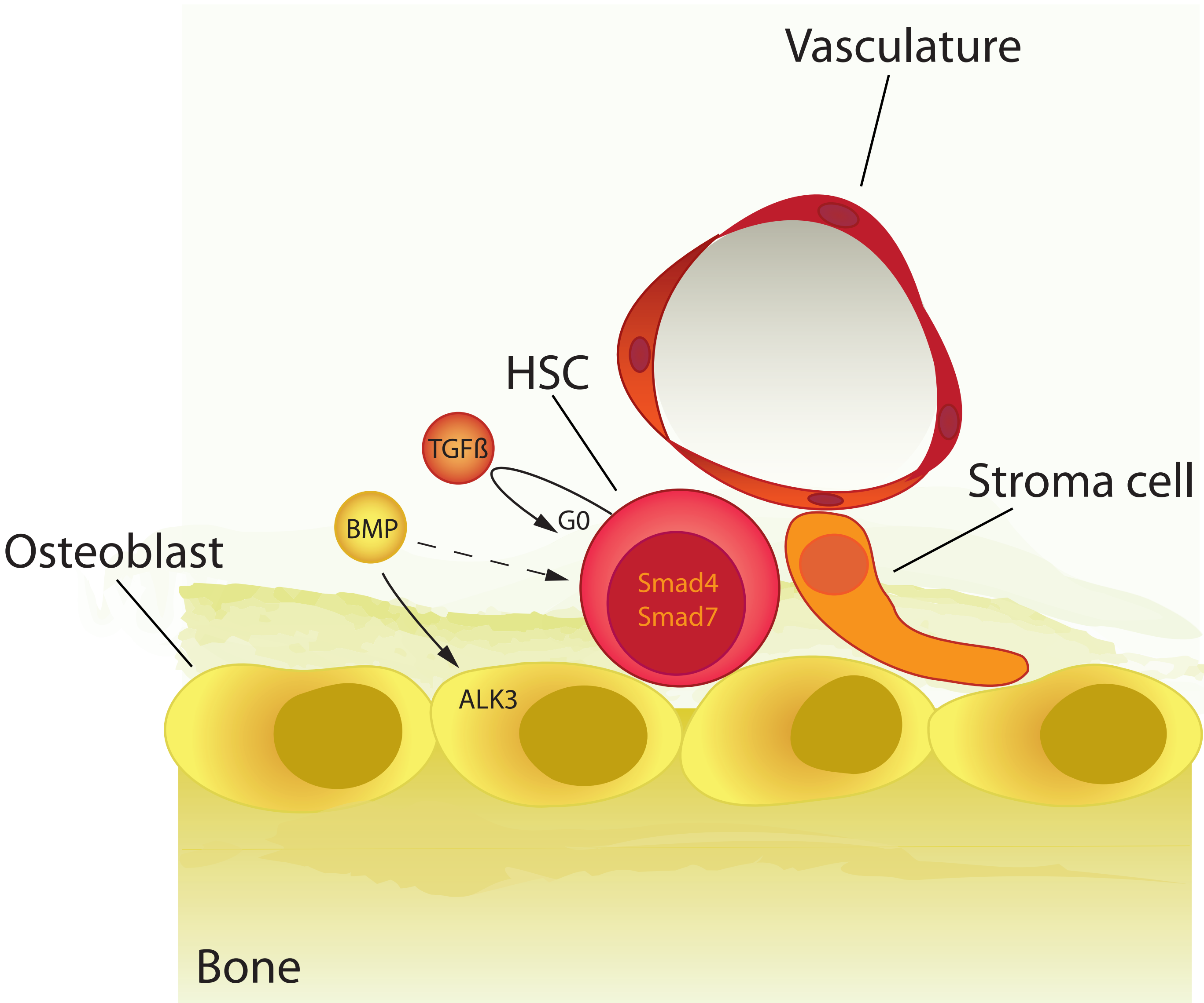

Figure 2
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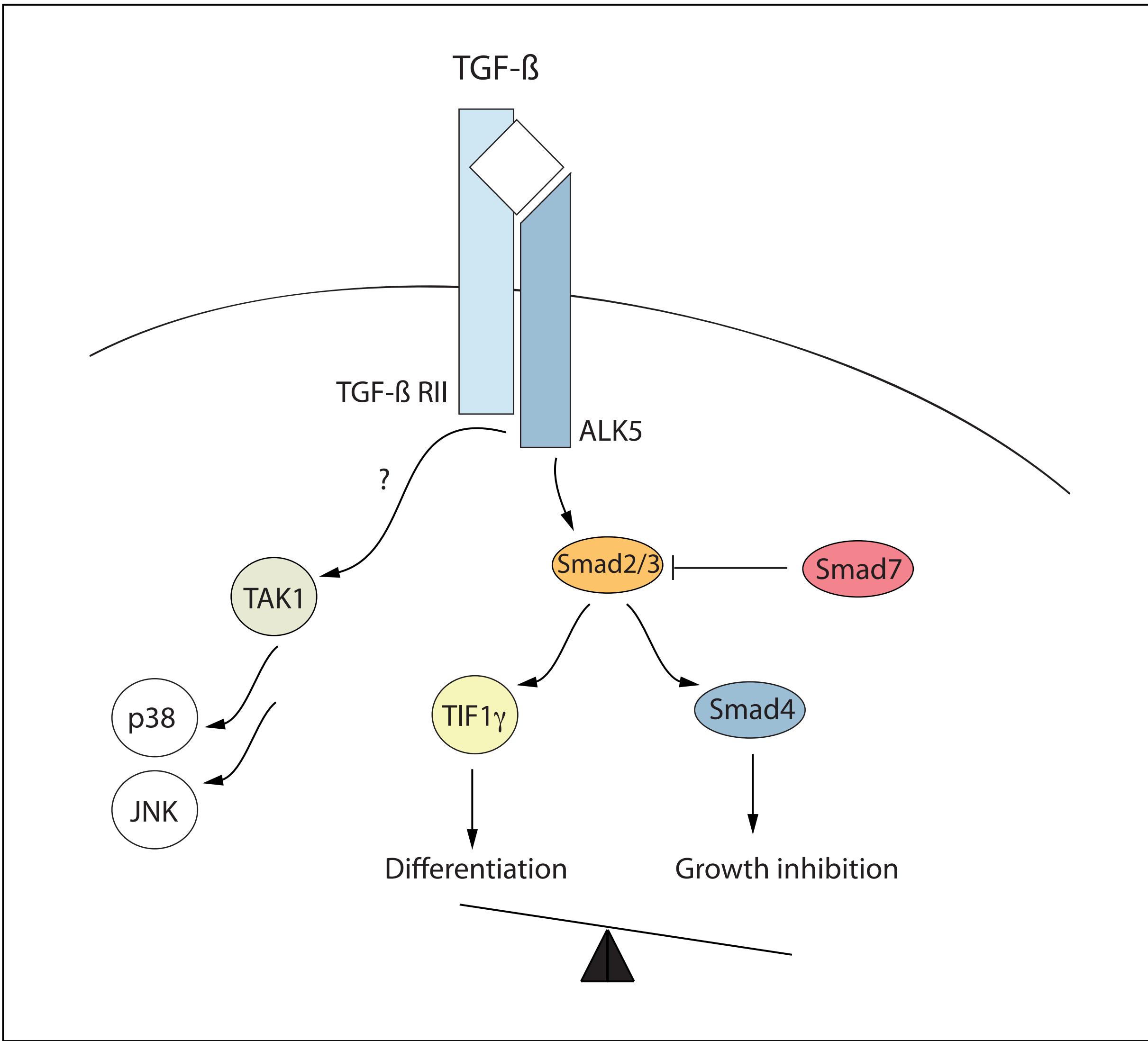

Figure 3
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