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# **Role of Transforming Growth Factor- $\beta$ Superfamily Signaling Pathways in Human Disease**

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**Abstract:** Transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily signaling pathways are ubiquitous and essential regulators of cellular processes including proliferation, differentiation, migration, and survival, as well as physiological processes, including embryonic development, angiogenesis, and wound healing. Alterations in these pathways, including either germline or somatic mutations or alterations in expression of members of these signaling pathways often results in human disease. Appropriate regulation of these pathways is required at all levels, particularly at the ligand level, with either a deficiency or an excess of specific TGF- $\beta$  superfamily ligands resulting in human disease. TGF- $\beta$  superfamily ligands and members of these TGF- $\beta$  superfamily signaling pathways also have emerging roles as diagnostic, prognostic or predictive markers for human disease. Ongoing studies will enable targeting of TGF- $\beta$  superfamily signaling pathways for the chemoprevention and treatment of human disease.

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## Introduction

During embryonic development human cells are organized into a complex network of tissues, organs, and systems that carry out the normal physiological processes of life. Homeostasis, both during development and in the adult, is achieved through a careful balance of signaling pathways which transmit signals through ligands circulating through the body and through cell surface receptors and their downstream signaling pathways. Most human diseases arise from either inappropriate activation or inhibition of these signaling pathways. One of the most ubiquitous regulators of embryonic development, physiological, and cellular processes is the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily of cytokines.

The TGF- $\beta$  superfamily of cytokines contains more than 30 structurally related polypeptide growth factors including TGF- $\beta$ s (1-3), activins (A, B), inhibins (A, B), bone morphogenetic proteins (BMPs 1-20), growth differentiation factors including myostatin, nodal, leftys (1,2), and Müllerian-inhibiting substance (MIS) [1-3]. The TGF- $\beta$  superfamily members share a conserved cysteine knot structure, and are ubiquitously expressed, with nearly every cell in the human body expressing and responding to at least one member of this superfamily [4]. The TGF- $\beta$  superfamily normally functions to regulate embryonic development and cellular homeostasis, including regulation of proliferation, differentiation, apoptosis, and extracellular matrix remodeling in a cell and context specific manner [3-7].

Alterations in TGF- $\beta$  superfamily pathways, including either germline or somatic mutations or alterations in the expression of members of these signaling pathways often results in human disease, including developmental disorders, vascular diseases, and

cancer [8, 9]. Here we review the role of TGF- $\beta$  superfamily signaling pathways in human diseases including the role in hereditary diseases, and conclude with assessing the potential for these pathways to serve either as diagnostic, prognostic or predictive biomarkers, or as targets for the prevention or treatment of human disease.

### *TGF- $\beta$ Superfamily Signaling*

Mechanisms of TGF- $\beta$  superfamily signaling have been the focus of several excellent recent reviews [2, 3, 5, 10-12]. Briefly, TGF- $\beta$  superfamily ligands initiate their cellular effects by binding to high affinity cell surface receptors, including type I (activin like kinase (ALK) 1-7) and type II TGF- $\beta$  superfamily receptors, which together comprise the only known family of serine/threonine kinase receptors (Table I, Figure 1). In addition, there are a growing number of TGF- $\beta$  superfamily co-receptors, receptors that bind ligand and regulate ligand binding to and signaling through their corresponding signaling receptors, but have been traditionally thought not to signal directly (Table I, [13]). The type II receptors are constitutively active serine/threonine kinases, with ligand binding resulting in conformational changes that induces recruitment and complex formation with an appropriate type I receptor (Table I, ; [9, 12-14], Figure 1). The type II receptor then phosphorylates the type I receptor in the GS domain (glycine serine rich) immediately amino-terminal to the kinase domain, activating its serine/threonine kinase activity [1, 3, 6, 12]. The activated type I receptors mediate their cellular effects through interaction and phosphorylation of Smad proteins, a family of conserved transcription factors. The receptor Smads (R-Smads) are directly phosphorylated by the type I receptors (Table 1, Figure 1). Upon phosphorylation, two activated R-Smads form a

complex with the common Smad, Smad4, and this complex accumulates in the nucleus, where it can interact with various transcription factors, co-activators or co-repressors to modulate the expression of a multitude of genes (Figure 1 [1, 10]).

TGF- $\beta$  superfamily co-receptors, like other signaling co-receptors, regulate and contribute to TGF- $\beta$  superfamily signaling through a variety of mechanisms. Although originally defined for their role in either conferring or enhancing ligand binding to the signaling receptors, recent studies support additional roles for these co-receptors in: (1) establishing morphogen gradients during embryonic development; (2) antagonizing ligand function by shedding their extracellular domains into the extracellular space; (3) regulating receptor localization and internalization; (4) regulating cellular adhesion; and (5) orchestrating signaling [13]. For example, the TGF- $\beta$  superfamily co-receptor, T $\beta$ RIII (or betaglycan), regulates the function of inhibin [15-17], BMP [18], and TGF- $\beta$  ligands [19-21] through binding to these ligands, mediating the interaction of these ligands with other TGF- $\beta$  superfamily receptors (through the action of both cell surface and soluble T $\beta$ RIII) [19, 20], and regulating the trafficking and localization of these receptors [21]. Established ligands and binding partners for specific TGF- $\beta$  superfamily co-receptors are listed in Table I.

TGF- $\beta$  superfamily signaling is not limited to Smad-mediated pathways, as they can also mediate signaling responses through non-Smad pathways, in particular 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 NF- $\kappa$ B pathway (Figure 1 [2, 10, 22]). The precise mechanism by which TGF- $\beta$  superfamily ligands activate these pathways has not been

fully established [10]; however, progress is being made. For example, recent studies have defined a novel interaction of  $\beta_3$  integrins with the type II TGF- $\beta$  receptor (T $\beta$ RII), resulting in Src-mediated phosphorylation of Y284 on T $\beta$ RII, recruitment of Grb2, and subsequent downstream activation of p38 [23-25]. TGF- $\beta$  superfamily signaling pathways also crosstalk extensively with many signaling pathways including the MAPK, PI(3)K/Akt and tyrosine kinase receptor-mediated pathways (Figure 1 [2, 26]).

TGF- $\beta$  superfamily signaling is regulated at all levels, beginning at the ligand level. TGF- $\beta$ 1 is synthesized and secreted into the extracellular matrix as an inactive precursor protein consisting of a signal peptide, the latency associated peptide (LAP) domain, and mature TGF- $\beta$ 1. Activation of TGF- $\beta$ 1 is thought to involve proteolytic cleavage of LAP [27]. This cleavage is induced by acidic environmental conditions, or executed by extracellular proteases including thrombospondin-1 [28], plasmin, cathepsin D [29], matrix-metalloproteinases 2 and 9 [30], and furin convertase [31]. There is also evidence that integrins are involved in activating TGF- $\beta$  in the absence of proteolytic cleavage. The LAP domain of TGF- $\beta$  has a RGD sequence that mediates binding to integrins, and  $\alpha_v\beta_6$  integrin was demonstrated to activate TGF- $\beta$ 1 [32], and  $\alpha_v\beta_8$  was shown to activate TGF- $\beta$ 1 via an MT1-MMP dependent mechanism [33]. Unlike TGF- $\beta$ , BMPs are secreted in their active form. Their activity is regulated by BMP antagonists, proteins that bind directly to BMPs and prevent them from interacting with their respective type I and type II receptors (Figure 1 [34]). There are three subfamilies of BMP antagonists based on the size of their cysteine knot; (1) CAN, (2) twisted gastrulation, and (3) the chordin/noggin families [35]. These BMP antagonists

have differential affinities for BMPs. For example, Sclerostin binds to BMP-6 and BMP-7 with high affinity and to BMP-2 and BMP-4 with low affinity [36]; whereas, noggin binds to BMP-2 and BMP-4 with high affinity and to BMP-7 with low affinity [34]. These BMP antagonists also have differential expression patterns, localizing their effects to specific tissues [34]. The importance of these antagonists in regulating BMP signaling during development is evident from the phenotypes of the knock-out mice. *noggin(NOG)/chordin* double knock-out mice display severe craniofacial malformations [37] and in single *NOG* knock-outs, growth plates are enlarged and joint initiation is disrupted [38].

TGF- $\beta$  superfamily signaling is also regulated at the levels of the receptors, with FK506-binding protein 12 (FKBP12) binding the GS domain of the type I superfamily receptors, preventing phosphorylation by their respective type II receptors, and maintaining the type I receptor in an inactive conformation [39, 40]. Localization and trafficking of the signaling receptors are also important for regulating TGF- $\beta$  signaling. Smad anchor for receptor activation (SARA), which links receptors and Smads and is localized on the plasma membrane and early endosomes, has been linked to an increase in TGF- $\beta$  signaling following clathrin-mediated endocytosis of the receptors [41]. In contrast, lipid raft or caveolin-mediated endocytosis results in receptor ubiquitination and degradation [41]. In addition, we have demonstrated that the TGF- $\beta$  superfamily co-receptors, T $\beta$ RIII (or betaglycan) and endoglin regulate the cell surface localization, internalization, and signaling of their respective signaling receptors through interactions with the scaffolding proteins, GAIP-interacting protein C-terminus (GIPC) [42] and  $\beta$ -arrestin2 [21, 43].

At the Smad level, Smad2/3 binding to SARA retains them in the cytoplasm awaiting activation by C-terminal phosphorylation by their respective type I receptor [44]. The ability of phosphorylated Smads to interact with Smad4 is also regulated, as the ErbB2/Her2-interacting protein (Erbin) functions to sequester Smad2/3 in the cytoplasm away from Smad4, thus preventing TGF- $\beta$  signaling [45]. In addition, dephosphorylation of nuclear accumulated Smads results in export back to the cytoplasm to terminate signaling [46].

Once the pathway is activated, a number of feedback mechanisms are turned on to regulate the duration of signaling. For example, the inhibitory Smads (I-Smads), Smad6 and Smad7, which lack the MH1 domain and whose production is directly increased by both TGF- $\beta$  and BMP signaling, inhibit TGF- $\beta$  superfamily signaling by competing with R-Smads for association with their respective type I receptors [47-50]. They also recruit the E3 ubiquitin ligases Smad ubiquitin-related factor-1 (Smurf1) and Smurf2, which ubiquitinate the Smads and type I receptors resulting in protein degradation and termination of signaling [51, 52]. In addition, TGF- $\beta$  superfamily signaling pathways also stimulate the production of secreted proteins, which function to both mediate and regulate signaling. For example, TGF- $\beta$  increases the production of secreted protein acidic rich in cysteine (SPARC), which enhances TGF- $\beta$  signaling [53]; cystatin C, which inhibits TGF- $\beta$  signaling [54, 55], and fibulin-5 [56], which mediates TGF- $\beta$ 's effects on inhibiting VEGF signaling and angiogenesis [57, 58]. These data indicate that modification to components of the TGF- $\beta$  signaling cascade regulates their availability for signaling and ensures proper cellular responses. A summary of the TGF- $\beta$  superfamily signaling pathways is presented in Figure 1.

Taken together, the complexity of TGF- $\beta$  superfamily signaling and regulation of these signaling pathways permits this superfamily to elicit diverse and context-dependent cellular functions to regulate normal homeostasis. Not surprisingly, misregulation of TGF- $\beta$  superfamily signaling pathways often results in human disease [8].

### Cardiovascular Disease

TGF- $\beta$  superfamily members critically regulate many different processes within the cardiovascular system (CVS), including cardiac development and angiogenesis. The predominant TGF- $\beta$  superfamily ligands expressed in the CVS are TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, BMP-2, BMP-4, BMP-6, and BMP-7 [59]. The importance of the TGF- $\beta$  superfamily in cardiovascular development is evident from the significant phenotypes of knock-out mice: (1) *TGFBI*<sup>-/-</sup> mice are embryonic lethal due to an excessive systemic inflammatory response with massive infiltration of macrophages and lymphocytes into the heart and lungs [60]; (2) *TGFB2*<sup>-/-</sup> mice are embryonic lethal due in part to ventricular septum defects (VSD), myocardial thinning, and a double outlet right ventricle (DORV) [61]; (3) *TGFBR3*<sup>-/-</sup> mice are embryonic lethal, exhibiting cardiac defects similar to the *TGFB2*<sup>-/-</sup> mice, with a failure of coronary vessel development and epicardial development [62, 63]; (4) specific *BMPRIA* loss in the myocardium of atrioventricular cushions results in valve malformations [64], while deletion in the endothelium reduces the formation of mesenchyme through reduced epithelial to mesenchymal transition (EMT) and mesenchymal cell survival, resulting in defective cardiac valve and septa formation [65]; and (5) specific knock-out of *SMAD4* in the heart results in hypocellular myocardial wall defects contributing to heart failure [66]. While a recent review focused

on the extracellular regulation of TGF- $\beta$  signaling and its effects on vascular development and disease [67], here we will discuss the contribution of TGF- $\beta$  superfamily members to specific cardiovascular diseases.

*Hereditary Hemorrhagic Telangiectasia (Rendu-Osler-Weber Syndrome)*

Hereditary Hemorrhagic Telangiectasia (HHT) is an autosomal dominant disease with variable penetrance in which vascular dysplasia results in telangiectasia and arteriovenous malformations [68]. Clinically, these patients present as young adults and onward with telangiectases of the skin, spontaneous recurrent epistaxis, gastrointestinal bleeding, high cardiac output heart failure, or neurological sequelae due to arteriovenous malformations [68, 69]. There are two forms of HHT, HHT1 and HHT2, which are often difficult to differentiate clinically; however, HHT1 is associated with a higher incidence of pulmonary, hepatic, and cerebral arteriovenous malformations and HHT2 has a later onset, lower penetrance, and is associated with more severe liver involvement [68-70].

Genetic linkage studies of families with HHT identified disease causing mutations in two TGF- $\beta$  superfamily family receptors, the co-receptor endoglin for HHT1 [71], and the type I receptor ALK1 for HHT2 [72], that together account for approximately 80% of HHT patients. Most of the mutations identified in *ENG* and *ACVRL1* in HHT patients are thought to inactivate the receptors. In HHT1, 155 unique mutations in *ENG*, all in the extracellular domain, have been identified [69]. Most of these mutations result in a truncated or mutated protein that does not reach the cell surface [69]. In HHT1, 123 unique mutations in *ACVRL1* have been identified, and more than 50% of these are missense mutations [69]. In contrast to endoglin, these mutations span the extracellular,

transmembrane, and cytoplasmic domains of ALK1, with the majority affecting the cytoplasmic domain. Protein modeling studies predict that the majority of these mutations would affect protein folding and stability. In both cases, HHT patients are heterozygous for these disease causing alleles, suggesting a haploinsufficiency model of disease pathogenesis [73].

Genetic analysis of *ACVRL1* in a large HHT2 family established that 58% of the offspring from two heterozygous individuals died in utero or shortly after death, while all of the surviving offspring were heterozygotes [74]. This analysis suggests that ALK1 is essential for embryonic development and viability, as has been confirmed in murine models [75, 76].

Other genes that contribute to HHT have been identified, including mutations in the carboxy terminus of *Smad4*, with resulting patients exhibiting a combined syndrome of juvenile polyposis ([73], see section on juvenile polyposis) and HHT; and an unidentified locus on chromosome 5 has been linked to HHT patients without mutations in *ENG*, *ACVRL1*, or *SMAD4* [77]. Whether mutations in *SMAD4* and/or an unidentified gene on chromosome 5 accounts for the 20% of remaining cases of HHT remains to be elucidated [69]. In addition, a subset of HHT2 patients also have primary pulmonary hypertension ([78], see section on primary pulmonary hypertension), suggesting that disruption of ALK1 signaling may also contribute to this human disease.

The vascular dysplasias present in patients with HHT are thought to form from dilation of post-capillary venules and consist of a dilated vessel with a single layer of endothelium attached to a continuous basement membrane. HHT is thought to arise through defective function of endoglin and ALK1 in endothelial cells. The importance of

endoglin and ALK1 in endothelial cell function is evident from the phenotypes of the *ENG* and *ACVRL1* knock-out mice. *ENG*<sup>-/-</sup> mice die at embryonic day 10.5 due to defects in angiogenesis, including failure to develop blood vessels in the yolk sac, and severe cardiac defects [71]. *ACVRL1*<sup>-/-</sup> mice also die at embryonic day 10.5-11.5 due to severe vascular abnormalities, including hyperfusion and hyperdilation of blood vessels, and impaired placental development [76, 79]. Supporting a haploinsufficiency model for HHT; *ENG* heterozygous mice exhibit symptoms of HHT1 [80], and mice heterozygous for *ACVRL1* exhibit HHT2 symptoms including vascular lesions, gastrointestinal bleeding, and hyperfusion and hyperdilation of arterial and venous capillaries [81].

While the mutations of *ENG* and *ACVRL1* in HHT and the phenotypes of the knock-out mice support an important role for these TGF- $\beta$  superfamily receptors in endothelial cell biology and angiogenesis, the mechanisms by which these receptors normally function and how disruption of this signaling contributes to the disease pathology is still being explored. Moreover, insight into these processes has been hampered by several layers of complexity in TGF- $\beta$  superfamily signaling in endothelial cells. First, in endothelial cells, TGF- $\beta$  signals not only through the ALK5/Smad2/3 pathway (as in most other cell types), but also through ALK1/Smad1/5/8 [82]. These pathways have opposing effects on endothelial cell proliferation and migration; although, it remains controversial which pathway inhibits and which pathway stimulates these processes [82, 83]. ALK1 and ALK5 signaling is also not mutually exclusive, as ALK5 has been reported to be required for ALK1 signaling [59, 82]. The presence of these two signaling pathways with divergent effects might provide a mechanism for the dose dependent, dichotomous function of TGF- $\beta$  on endothelial cells, promoting proliferation

and migration at low concentrations while inhibiting these responses at higher ligand concentrations [82]. In any case, the effects of endoglin and ALK1 on TGF- $\beta$  signaling appears to be important as primary endothelial cells isolated from HHT1 and HHT2 patients exhibit reduced TGF- $\beta$ -mediated transcription of Smad1 and Smad2/3 responsive promoters [84]. The precise mechanism and role of ALK1 and ALK5-mediated TGF- $\beta$  signaling in endothelial cells is still being defined. Second, the role of endoglin in regulating TGF- $\beta$  superfamily signaling in endothelial cells appears to be multifaceted. Unlike many other co-receptors, endoglin does not bind ligand independently; thus, it is unlikely to present ligand [85]. However, endoglin does bind several TGF- $\beta$  superfamily ligands, including TGF- $\beta$ 1, TGF- $\beta$ 3, activin, BMP-2 and BMP-7, in conjunction with their respective binding receptors [85]. As mutation in *SMAD4*, which is downstream of all TGF- $\beta$  superfamily ligands, also results in an HHT phenotype, and BMP-9 has been identified as a bona fide ligand for ALK1 [86], which or whether all TGF- $\beta$  superfamily signaling pathways are responsible for the disease pathology in HHT patients remains unknown. In terms of effects on signaling, endoglin has been reported to both increase and decrease, or have no effect on ALK1 and ALK5 signaling, depending on the methods and model systems used. Endoglin also has defined functions in inhibiting migration through direct interactions of its cytoplasmic domain with zyxin [87] and zyxin related protein-1 [88], which affects focal adhesion complex assembly. Endoglin can also regulate cyclooxygenase-2 production and activity to control synthesis of prostaglandins (vasodilators), and can stimulate production of the potent vasodilator nitric oxide [89]. Recently, endoglin was demonstrated to antagonize TGF- $\beta$ -mediated ERK signaling in endothelial cells to inhibit endothelial cell migration

[43]. Endoglin is also phosphorylated on its cytoplasmic domain by ALK1, ALK5, and T $\beta$ R2 [90, 91]. The functional consequences of endoglin phosphorylation are still being defined. Third, endoglin and ALK1 expression appears to be broader than initially thought, with expression in vascular smooth muscle cells and fibroblasts, suggesting that these compartments could mediate an effect on HHT disease pathology as well [82, 92, 93]. Finally, ALK1 might signal through different pathways. For example, decreasing ALK1 levels in microvascular endothelial cells resulted in decreased migration mediated by JNK and ERK [94], and interactions of ALK1 with the nuclear receptor, LXR- $\beta$ , to inhibit ALK1 signaling have been reported [95]. Clearly, further investigation is required to fully define the role of this superfamily signaling pathway in the disease pathology of HHT.

*Diseases of the Aorta: Loeys-Dietz Syndrome, Familial Thoracic Aortic Aneurysm Syndrome, and Arterial Tortuosity Syndrome*

Loeys-Dietz syndrome is an autosomal dominant aortic aneurysm syndrome with affected patients having widely spaced eyes, cleft palate, and widespread vascular dilation placing them at very high risk for aortic dissection or rupture [96]. Based on an emerging role for the TGF- $\beta$  signaling pathway in aortic dissections associated with Marfan syndrome (see section on Marfan syndrome), sequencing of *TGFBR1* and *TGFBR2* in 52 families with a history of Loeys-Dietz syndrome identified disease causing somatic mutations in *TGFBR2* and *TGFBR1*; 29 mutations were identified in *TGFBR2*, and 13 were identified in *TGFBR1*. The majority of mutations identified were either adjacent to or within the serine/threonine kinase domains of T $\beta$ R2 and T $\beta$ R1.

There were no phenotypic differences between those patients that had *TGFBR1* versus *TGFBR2* mutations. These receptor mutations identified were specific to this syndrome, as unrelated patients with aortic aneurysms did not exhibit these genetic alterations [96]. While these mutations would be predicted to result in loss of function, experimentally, patients with these mutations paradoxically have increased TGF- $\beta$  signaling in their aortic walls, suggesting that these mutations are either gain of function, or that compensatory mechanisms overcome these mutations [96].

Familial Thoracic Aortic Aneurysm Syndrome (TADD) is an autosomal dominant disease that predisposes patients to progressive enlargement of aorta aneurysms that can result in type A aortic dissection, tearing of the arterial wall, and leakage of blood into the layers of the vessel wall [97]. This is a life threatening condition as aortic dissection often results in massive blood loss. TADD is the major cardiovascular complication of Marfan Syndrome, a hereditary connective tissue disorder (see Marfan Syndrome section). 19% of TADD patients that do not have Marfan Syndrome have affected first degree relatives, suggesting that there is a common genetic contributor. Germline mutations in *TGFBR2* have been identified in patients with TADD, including mutation of a conserved arginine to a cysteine within the kinase domain of the receptor [97], missense mutations of highly conserved nucleotide bases within the kinase domain, and a missense mutation downstream of the kinase domain [98]. In addition, a missense mutation in the cytoplasmic domain of *TGFBR1* has been identified in patients with TADD [98]. Protein structural modeling predicts that all of these TADD associated receptor mutations would affect the ability of receptors to transduce signals through their kinase domain [98].

Arterial tortuosity syndrome (ATS) is characterized by vascular and skeletal

manifestations that overlap with Loeys-Dietz syndrome, including generalized tortuosity and elongation of all major arteries, including the aorta, and hyperextensibility and laxity of the skin and joints [99]. Patients with ATS have loss of function mutations in the glucose transporter, *GLUT10*, which result in decreased glucose-dependent expression of decorin, a potent extracellular inhibitor of TGF- $\beta$ . As expected, patients with ATS exhibit elevated TGF- $\beta$  signaling in their vessel walls [99].

#### *Primary Pulmonary Hypertension and Familial Pulmonary Hypertension*

Primary pulmonary arterial hypertension (PAH) is characterized by intense remodeling of small pulmonary arteries by myofibroblast and smooth muscle proliferation, resulting in sustained elevation of pulmonary vascular resistance, arterial pressure, and ultimately right ventricular heart failure leading to cardiac arrest [100, 101]. There are two types of PAH; a sporadic or idiopathic PAH (IPAH), and hereditary or familial PAH (FPAH). Genetic analysis has identified a heterozygous germ line mutation in *BMPR2* associated with both IPAH and FPAH. *BMPR2* mutations have been identified in 70% of families with FPAH and in up to 40% of patients diagnosed with IPAH [102, 103]. 71% of the mutations in *BMPR2* are nonsense, frameshift, splice-site defects, or gene re-arrangements resulting in a truncated mRNA transcript that is prone to nonsense-mediated decay. Additional mutations have been identified that affect the ligand binding, transmembrane, and cytoplasmic domains [103]. As disease onset varies between family members and unrelated carriers have the same mutation, there are likely additional genetic and/or environmental modifiers that contribute to the pathogenesis of this disease and to full disease penetrance [103]. There is a subset of HHT patients that

also present with clinical symptoms of PAH. These patients have mutations in both *ACVRL1* and *BMPR2* [104], suggesting that ALK1 signaling may also contribute to PAH.

The mechanisms by which *BMPR2* mutations result in pulmonary hypertension remain unclear. A disease causing role for BMPRII is supported by transgenic mouse models. BMPRII is critical for early embryonic development as homozygous mutant mice die shortly after conception, due to the inability to derive mesoderm [105]. To define the function of BMPRII during development, a knock-out mouse was developed that expresses a BMPRII mutant lacking half of the ligand-binding domain. These mice die mid-gestation with severe liver defects and outflow tract defects in the heart, including lack of septation of the conotruncus below the valve level and an interrupted aortic arch, a phenotype known in humans as persistent truncus arteriosus [106]. In addition, when BMPRII expression was reduced in mice using shRNA, the pulmonary vessel walls had regions lacking smooth muscle cells, were highly disorganized, and were more prone to rupture [107]. These mouse phenotypes exemplify the importance of BMPRII in development of the pulmonary vasculature and suggest why mutations in *BMPR2* result in PAH.

Heterozygous *BMPR2* mice exhibit mild pulmonary hypertension and have an impaired vascular remodeling response to prolonged low oxygen conditions [108], suggesting that haploinsufficiency of BMPRII contributes to this disease. These heterozygous mice also develop elevated right ventricular systolic pressure and vascular remodeling when exposed to inflammatory agents [109]. In addition, expression of a dominant-negative BMPRII in smooth muscle cells in mice results in elevated pulmonary arterial hypertension, and an increase in pulmonary arterial muscularization [110]. The

benefits of increasing BMPRII levels on reducing the clinical manifestations of PAH have been assessed using a rat model; delivery of an adenoviral vector expressing BMPRII to the pulmonary vascular endothelium significantly reduced the pulmonary hypertensive response to chronic hypoxia [111].

In terms of signaling mediated by BMPRII, BMPRII is unique in the context of TGF- $\beta$  superfamily type II receptors in that it possesses a long cytoplasmic domain after the kinase domain. Mutations in this region have been identified in PAH, and some of these mutations disrupt interaction of BMPRII with receptor activated C-kinase 1 (RACK1). As RACK1 is required for BMP-mediated Smad1 phosphorylation and inhibition of proliferation in pulmonary arterial smooth muscle cells, this defines one potential mechanism of action for *BMPR2* mutations in this disease [112]. Recently, a link was established between the inflammatory cytokine interleukin 6, which has been implicated in PAH, and BMP signaling in a PAH mouse model. *BMPR2* knock-out mice have high levels of IL-6 and overexpressing IL-6 induced the transcription of the BMP responsive gene *Id1* [113]. Finally, the effects of serotonin, which induces PAH in rats, are more pronounced in *BMPR2* knock-out mice, suggesting that loss of BMP signaling synergizes with several signaling pathways to contribute to the pathogenesis of PAH [114]. This mechanistic data suggests that PAH is a complex disease that clinically manifests as a result of a variety of genetic and environmental modifiers, explaining the clinical heterogeneity of PAH.

#### *Pre-eclampsia*

Pre-eclampsia, or pregnancy-associated hypertension, is a major cause of maternal, fetal, and neonatal mortality world-wide. A diagnosis of pre-eclampsia is made

when patients develop new hypertension and increased proteinuria during their pregnancy. Early diagnosis and treatment is important as this condition, if untreated, can progress to a severe form resulting in maternal central nervous system, liver, and renal dysfunction, bleeding related to thrombocytopenia, preterm delivery, fetal growth restriction, abruption placenta, and perinatal death, as well as predisposing the mother to a greater long term risk of developing coronary heart disease and stroke [115].

Several recent studies identified high levels of soluble endoglin, formed by ectodomain shedding of cell surface endoglin, in patients exhibiting pre-eclampsia, with levels rising prior to clinical pre-eclampsia, and correlating with disease severity [116, 117]. Soluble endoglin may function by binding to TGF- $\beta$ 1 and preventing it from binding to its receptors on endothelial cells, resulting in decreased activation of eNOS and decreased production of the potent vasodilator, nitric oxide. This results in vasoconstriction, which induces hypertension in patients [116]. The precise mechanisms of how soluble endoglin contributes to pre-eclampsia are still being defined. However, the use of endoglin as a predictive or prognostic marker for pre-eclampsia is currently being evaluated using serum from expectant mothers, with current data supporting a strong relationship between disease severity and endoglin levels [118].

### *Atherosclerosis*

Atherosclerosis is a disorder involving endothelial dysfunction, vascular inflammation, and accumulation of lipids, cholesterol, and cellular debris within the intima of medium and large sized arteries resulting in plaque formation and acute and chronic luminal obstruction. Atherosclerosis is the primary cause of cardiovascular

disease and stroke. TGF- $\beta$  ligands and their receptors are expressed by endothelial cells, smooth muscle cells, macrophages, and T cells in human atherosclerotic lesions [119]. Although the role of the TGF- $\beta$  signaling pathway in atherosclerosis is controversial, with some studies suggesting a pro-atherosclerotic effect [119, 120], most studies support a role for TGF- $\beta$  in inhibiting atherosclerosis and atherosclerotic progression. First, TGF- $\beta$  mediates cellular effects that would be expected to inhibit atherosclerosis including inhibiting the proliferation and migration, and stimulating apoptosis of vascular smooth muscle cells and endothelial cells, and potently inhibiting the immune system. Second, in murine models of atherosclerosis; blocking TGF- $\beta$  activation through expression of apolipoprotein [121], blocking systemic TGF- $\beta$  signaling through expression of soluble T $\beta$ RII [122] or neutralizing TGF- $\beta$  antibodies [123], blocking TGF- $\beta$  signaling in T-cells through expression of dominant-negative type II receptors [124], or decreasing circulating levels of TGF- $\beta$ 1 by targeted deletion of one allele is sufficient to increase atherosclerosis [125], while increasing TGF- $\beta$  levels indirectly with Tamoxifen is sufficient to decrease atherosclerosis [126]. Third, smooth muscle cells isolated from human atherosclerotic lesions are resistant to the antiproliferative and apoptotic effects of TGF- $\beta$ , and contain mutations and/or decreased T $\beta$ RII expression [127, 128]. Fourth, TGF- $\beta$  appears to stabilize atherosclerotic plaques by stimulating collagen secretion and promoting the formation of a collagen-rich fibrous cap [120]. Finally, in an atherosclerotic mouse model (*LDLR*<sup>-/-</sup>), delivery of an adeno-associated virus type 2 vector expressing active TGF- $\beta$ 1 suppressed the formation of atherosclerotic lesions [129].

TGF- $\beta$  signaling has been linked to risk of myocardial infarction (MI), which

occurs when the heart is deprived of oxygen. Several *TGFBI* polymorphisms have been examined in MI patients [130]. The -509C/T polymorphism is associated with an increased incidence of myocardial infarction in men, independent of other risk factors including smoking, hypertension, diabetes, and high cholesterol levels. Interestingly, this polymorphism was only associated with increased risk of MI in males and not in females [131], which was also the case for another polymorphism, T29C, which was only associated with an increased risk of MI in middle-aged Japanese men [132]. However, a recent study performed on a larger cohort did not confirm an association of the -509C/T polymorphism with MI; however, it was associated with an increased risk of stroke, as was the case for the codon 10 Leu/Pro *TGFBI* polymorphism [133].

While the role of TGF- $\beta$  in atherosclerosis has been well defined, the role of other superfamily members, including the BMPs and activins is only beginning to be established. Similar to TGF- $\beta$ 1, activin appears to suppress atherosclerosis, as overexpression of activin decreases vascular smooth muscle cell migration into the intima in an atherosclerotic mouse model [134]. BMP-2, BMP-4, and BMP-6 levels are increased in advanced atherosclerotic lesions and these elevations are associated with increased levels of calcification, suggesting a role for BMPs in promoting advanced atherosclerotic lesions [135, 136].

### *Restenosis*

Restenosis refers to the exuberant fibrotic reaction, intimal proliferation, and remodeling in response to revascularization therapies including angioplasty, stenting, and atherectomy. Restenosis remains a major barrier to the effectiveness of these

interventions. While TGF- $\beta$  is thought to protect from atherosclerosis, several lines of evidence support increased TGF- $\beta$  signaling as a major contributor to restenosis. First, expression of all TGF- $\beta$  isoforms, T $\beta$ RI, and T $\beta$ RII is increased after angioplasty-induced vascular injury [137]. Second, over-expression of TGF- $\beta$ 1 is able to stimulate neo-intimal hyperplasia and fibrosis [138], a major contributor to restenosis. Indeed, TGF- $\beta$  is one of the most important ECM regulators in the cell; increasing extracellular matrix (ECM) protein expression and inhibiting the degradation of ECM components, resulting in a net accumulation of ECM [139]. The major ECM proteins produced by fibroblasts in the heart are Type I and Type III collagen, and these are responsible for most fibrotic processes occurring after vascular injury, including angioplasty [140]. Third, decreasing TGF- $\beta$  signaling by decreasing TGF- $\beta$ 1 levels with ribozymes [141], or using pharmacological inhibitors of TGF- $\beta$  signaling [142] is able to decrease neo-intimal hyperplasia and remodeling. Finally, overexpression of Smad7 in rat carotid arteries was able to abrogate restenosis after angioplasty, suggesting that blocking TGF- $\beta$  signaling is capable of preventing restenosis [143].

### *Hypertension*

Hypertension is a global medical problem that places individuals at risk for long term renal, cerebral, and coronary heart disease. While environmental factors including obesity and salt intake contribute to the onset of hypertension, approximately 50% of hypertension is thought to have a genetic basis [101], with several documented links between increased TGF- $\beta$  signaling and hypertension [140]. Emilin1, a secreted glycoprotein associated with the extracellular matrix of blood vessels, functions to

antagonize TGF- $\beta$ 1 signaling by binding to the precursor form of TGF- $\beta$  and decreasing its proteolytic processing into the active form. In both *EMILIN1* homozygous null mice and heterozygous mice, the systolic blood pressure is significantly increased in all animals, regardless of age, with decreased Emilin1 expression resulting in increased TGF- $\beta$ 1 signaling, reduced vessel size, and increased vascular resistance. A direct role for TGF- $\beta$ 1 in the *EMILIN1* null phenotype is supported, as genetic suppression of TGF- $\beta$ 1 in *EMILIN1* knock-out mice results in a return to a normotensive state [144]. In addition, in a rat hypertension model, TGF- $\beta$  neutralizing antibodies are sufficient to decrease blood pressure and subsequent renal failure [145]. Importantly, in humans, polymorphisms in *TGFBI* resulting in increased TGF- $\beta$ 1 expression have been linked to the development of hypertension, with a direct correlation between TGF- $\beta$ 1 levels and mean arterial pressure [146].

TGF- $\beta$  signaling is also upregulated by the renin-angiotensin-aldosterone axis [147], a critical regulator of blood pressure. Angiotensin II (Ang II) directly stimulated TGF- $\beta$ 1 expression at the mRNA level, and enhances its conversion to the active form at the protein level by increasing Thrombospondin-1 levels [148, 149], see Figure 2. In addition, Ang II increases T $\beta$ R2 mRNA expression [150], angiotensin III stimulates TGF- $\beta$ 1 expression at the mRNA level [151], and aldosterone induces TGF- $\beta$ 1 expression [152], see Figure 2. An important role for the renin-angiotensin-aldosterone axis in increasing TGF- $\beta$  signaling is supported by the ability of angiotensin converting enzyme (ACE) inhibitors and angiotensin I receptor antagonists, both used in the treatment of hypertension, to reduce renal TGF- $\beta$ 1 production. Renin-angiotensin system inhibitors including ACE inhibitors are effective in reducing renal disease progression

associated with diabetic nephropathy in rat models. When a TGF- $\beta$  neutralizing antibody was administered in combination with an ACE inhibitor, there was a synergistic effect on inhibiting renal injury and proteinuria [153]. The exact mechanisms of how TGF- $\beta$  superfamily members contribute to renal injury warrants further investigation.

#### *Hypertrophic Cardiomyopathy/Congestive Heart Failure*

Cardiomyopathy refers to diseased heart muscle and is classified into three different types, dilated, hypertrophic and restrictive, based on anatomical and physiological parameters. Eventually, if untreated, cardiomyopathy will progress, resulting in congestive heart failure due to contractile dysfunction, arrhythmias, ultimately leading to death [147]. In the pathogenesis of cardiomyopathy, elevated levels of angiotensin II increase TGF- $\beta$  levels and TGF- $\beta$  signaling, as discussed above. These elevated levels of TGF- $\beta$  are important for the pathogenesis of cardiac hypertrophy as angiotensin II is unable to induce hypertrophy in mice lacking *TGFBI* [147, 154]. Importantly, elevated circulating levels of TGF- $\beta$ 1 are found in patients with dilated, hypertrophic, and restrictive cardiomyopathy, and as with hypertension, ACE inhibitors and angiotensin I receptor antagonists, which are used therapeutically for cardiomyopathy patients, may function by reducing TGF- $\beta$ 1 production and subsequent collagen production in the heart. In addition, in humans, polymorphisms in *TGFBI* resulting in increased TGF- $\beta$ 1 expression have been linked to dilated cardiomyopathy [155].

Other TGF- $\beta$  superfamily members have also been implicated in the pathogenesis of cardiomyopathy. Specifically, BMP-10 expression is increased in the ventricles of

hypertensive rats with cardiac hypertrophy, and in humans, a polymorphism in *BMP10*, which leads to increased BMP-10 secretion/bioactivity, was identified in patients with dilated cardiomyopathy [156]. In mice, expression of BMP-10 increases in the ventricular trabecular myocardium when cardiac development shifts from patterning to growth and chamber maturation, and BMP-10 is required for both of these processes [157]. When BMP-10 is absent, there are elevated levels of p57<sup>Kip2</sup>, which is associated with reduced proliferation of cardiomyocytes, and reductions in the cardiogenic transcription factors NKX2.5 and MEF2C in the developing myocardium. This results in severe cardiac malformations including hypoplastic ventricular walls and absence of ventricular trabeculae, both of which contribute to the early embryonic lethal phenotype [157]. In addition, myostatin (GDF-8) has been implicated in contributing to cardiac hypertrophy, as myostatin expression is elevated in hypertrophic cardiac tissue and myostatin increases the growth of cultured cardiomyocytes [158].

## **Connective Tissue Diseases**

### *Marfan Syndrome and Marfan-Like Disorders*

Marfan syndrome (MS) is a hereditary autosomal dominant multisystem connective tissue disorder, which is characterized clinically by cardiac abnormalities including aortic aneurysms, aortic dissections, and mitral valve abnormalities; skeletal manifestations, and vision problems [159, 160]. MS has been linked to a mutation in the extracellular matrix protein fibrillin-1 [98]. Fibrillin polymerizes to form microfibrils, which normally stabilize latent TGF- $\beta$ -binding proteins and maintain TGF- $\beta$  in its inactive state, while the mutant fibrillin proteins in MS patients fail to do so, resulting in

elevated levels of active TGF- $\beta$ . Fibrillin mutations have been identified in the majority of patients with MS. However, up to 34% of patients have no identified mutation in *FBNI*, suggesting that others genes contribute to this disease. Recently, mutations were identified in *TGFBR1* and *TGFBR2* in patients with MS [96, 98, 160-162]. Those patients with *TGFBR1* and *TGFBR2* mutations are referred to as having MS type II, while those with *FBNI* mutations are referred to as having MS type I. These *TGFBR1* and *TGFBR2* mutations affect the kinase domain of the receptors and are predicted to result in loss of function [161]. While similar mutations in Loeys-Dietz syndrome paradoxically result in increased TGF- $\beta$  signaling [96], whether this is the case in MS remains to be established. *TGFBR2* mutations resulting in MS are associated with more extensive and progressive aortic and peripheral vascular disease, earlier disease onset, and more diffuse arterial disease than those patients with the *FBNI* mutation, suggesting that patients with TGF- $\beta$  receptor mutations may have a distinct disease pathogenesis than those with *FBNI* mutations [159]. Clearly, context dependent alterations in TGF- $\beta$  signaling are involved in the pathogenesis of MS.

Mutations in TGF- $\beta$  receptors have also been identified in Marfan-like disorders. Specifically, a mutation affecting the kinase domain of T $\beta$ RI has been identified in patients with Furlong Syndrome, which is associated with premature closure of skull sutures and aortic dissection [163]. In addition, a *TGFBR2* mutation resulting in defective splicing of *TGFBR2* was identified in patients with Sphrintzen-Goldberg syndrome, which is associated with premature closure of skull sutures and mental retardation [162, 164]. Since these phenotypes overlap for these Marfan-like disorders,

more clinical analysis of patients is required to understand the role of TGF- $\beta$  superfamily signaling in progression of these diseases.

In mouse models in which *FBNI* mutants identified in MS patients are expressed, there is increased TGF- $\beta$  signaling in multiple organs including in the lungs, heart, and aorta, confirming that fibrillin normally functions to downregulate TGF- $\beta$  signaling [161]. Importantly, administering a TGF- $\beta$  neutralizing antibody to these mice diminishes the lung and mitral valve manifestations of MS [161, 165], demonstrating the importance of increased TGF- $\beta$  signaling in the pathogenesis of this disease.

#### *Fibrotic Diseases*

Fibrosis is a complex tissue disease that results from excessive and abnormal deposition of extracellular matrix (ECM), which is composed of fibronectin, laminin, vitronectin, and collagen, by long term activation of myofibroblasts [166]. Myofibroblasts are a specialized type of fibroblast that are normally activated during wound healing [167]. If the build-up of ECM occurs in organs such as the lungs, liver, kidneys, and skin, it can interfere with organ function, and unabated, results in organ failure. Examples of fibrotic diseases include skin fibrosis, vascular fibrosis, systemic sclerosis, renal fibrosis, and liver fibrosis. As mentioned briefly during the discussion of restenosis, TGF- $\beta$  is a critical regulator of extracellular matrix assembly and remodeling [166], which stems from its first defined function as a potent stimulator of wound healing [168]. As fibrosis has been described as a “wound-healing response that has gone out of control” [169], excessive TGF- $\beta$  signaling has long been implicated in the pathogenesis of fibrotic diseases, and has been the subject of several excellent recent reviews [166,

169]. TGF- $\beta$  signaling through Smad3 directly promotes expression of type I collagen, a major component of the ECM, during fibrosis [170]. Smad3 has a central role in TGF- $\beta$  induced fibrosis in several organ systems as: (1) skin from *SMAD3*<sup>-/-</sup> mice is resistant to injury from ionizing radiation with reduced recruitment of myofibroblasts [171]; (2) hepatic stellate cells mediate fibrosis in the liver, and they require Smad3 to induce collagen expression [172]; (3) Smad3, and not Smad2, is required for angiotensin-II induced vascular fibrosis [173]; (4) increased levels of phosphorylated Smad3 are observed in keloid (scar) fibroblasts [174]; and (5) *SMAD3*<sup>-/-</sup> mice are resistant to bleomycin and TGF- $\beta$ -mediated pulmonary fibrosis [175, 176].

Another important effector of TGF- $\beta$  induced fibrosis is connective tissue growth factor (CTGF). TGF- $\beta$  promotes CTGF expression in fibroblasts, which promotes collagen synthesis and myofibroblast differentiation [177, 178]. CTGF binds directly to TGF- $\beta$ , and enhances its activity resulting in increased binding to T $\beta$ RI and T $\beta$ RII [179]. CTGF can stimulate fibrosis directly by promoting mesangial cell survival, via stabilization of the anti-apoptotic protein Bcl-2, which is thought to promote renal fibrosis under chronic pathological conditions such as diabetic nephropathy [180]. It is thought that CTGF synergizes with other pathways, including TGF- $\beta$ , to induce fibrosis, as overexpressing CTGF alone induced only mild pulmonary fibrosis in mice [181], but potentiated the effects of bleomycin-induced fibrosis [178, 182].

An additional contributor to fibrosis is epithelial to mesenchymal transition, a phenotypic conversion of epithelial cells into mesenchymal cells, which is associated with increased expression of fibroblast-associated proteins, such as alpha smooth muscle actin (SMA). The contribution of EMT to fibrosis has been extensively studied in the

kidney, as it converts renal tubular epithelial cells into fibroblasts, which produce large amounts of ECM that accumulates in the kidneys, and interferes with their function [183]. Progressive renal fibrosis is often associated with diabetes [184]. The ERK and JNK MAPK pathways, and Smad2 mediate TGF- $\beta$ -induced EMT in renal tubular epithelial cells [185]. Integrin linked kinase (ILK) has also been implicated in TGF- $\beta$ -induced renal fibrosis, as expression of a dominant negative ILK inhibited TGF- $\beta$ -induced EMT of renal tubular epithelial cells and inhibiting ILK in vivo inhibited EMT and fibrosis in a renal fibrosis mouse model [186]. The importance of increased TGF- $\beta$  signaling in fibrosis has been validated therapeutically. A T $\beta$ RI kinase inhibitor significantly reduced renal fibrosis in the db/db diabetic nephropathy model, which was associated with reduced expression of collagen and fibronectin in the kidneys [184]. Alternatively, delivering a T $\beta$ RI inhibitor to proximal tubular cells by conjugating it to the renal carrier protein lysozyme suppressed the activation of tubular cells and fibroblasts, reduced inflammation, and decreased levels of the fibrotic markers vimentin and  $\alpha$ SMA in the unilateral ureteral obstruction rat model of renal fibrosis [187].

In addition, TGF- $\beta$  regulates the expression of other proteins involved in executing the fibrotic cascade including: (1) regulation of the expression and activity of extracellular remodeling proteases including matrix-metalloproteinases, and their inhibitors, the tissue inhibitors of metalloproteinases [188]; (2) increasing the expression of the vasoconstrictive peptide endothelin-1, which has been shown to contribute to persistent fibrosis by inducing fibroblasts to produce and secrete ECM [189]; and (3) regulating the activity of the Rho family of small GTPases, which have been implicated in mediating TGF- $\beta$ -induced fibrosis [139]. Overall TGF- $\beta$  is a critical regulator of

extracellular matrix production; and therefore, increased TGF- $\beta$  signaling is a critical contributor to the progression of fibrotic diseases.

### **Skeletal and Muscular Disorders**

Bone is a mineralized tissue that provides mechanical support, protects organ systems, regulates calcium levels in the blood, and provides an environment for hematopoiesis. Bone forms through the deposition and mineralization activity of osteoblasts, and is constantly remodeled through the coordinated action of osteoblasts and osteoclasts, which serve to resorb bone [190]. TGF- $\beta$  superfamily members are abundantly expressed in the bone environment and regulate a number of important bone processes. TGF- $\beta$ 1 signaling has been extensively studied in bone since it is the predominant TGF- $\beta$  isoform expressed in bone. TGF- $\beta$ 1 has been implicated in every stage of bone formation including recruitment of osteoblast progenitors, stimulation of their proliferation and differentiation, and inhibition of osteoblast apoptosis [191]. The TGF- $\beta$ 1 knock-out mice have a skeletal phenotype including reduced bone growth and mineralization, as well as reduced elasticity [192], and reduced alkaline phosphatase activity [193].

BMPs critically regulate bone homeostasis; they were initially given their name by Urist based on their ability to induce ectopic bone formation [194]. When *BMP2* and *BMP4* are both knocked out in the limb bud mesenchyme of mice, there is severe impairment of bone formation [195]. Although knocking out *BMP2* alone results in no obvious bone defects in mice; these mice are more susceptible to bone fractures [196]. When the BMP type I receptors *BMPRIA* and *BMPR1B* are knocked out in chondrocytes,

there are defects in chondrocyte proliferation and differentiation, resulting in chondrodysplasia and disrupted bone formation [197].

#### *Camurati-Engelmann Disease*

Camurati-Engelmann Disease (CED) is an autosomal dominant disorder that affects the diaphysis of long bones and results in increased bone density, bone marrow dysfunction, muscular weakness, and severe bone pain [190]. This disease has been linked to mutations in *TGFBI*, with 9 distinct mutations identified thus far [198]. The majority of CED linked *TGFBI* mutations are in the latency associated domain of TGF- $\beta$ 1 [190], while one mutation has been identified that affects the signal peptide of TGF- $\beta$ 1 [198]. The location of these mutations suggests that increased TGF- $\beta$  signaling is responsible for the pathogenesis of CED. Indeed, when known CED *TGFBI* mutants are expressed in human epithelial kidney cells, these mutants result in increased autocrine TGF- $\beta$ 1 production and TGF- $\beta$ -mediated transcription [198].

#### *Fibrodysplasia Ossificans Progressiva*

Fibrodysplasia Ossificans Progressiva (FOP) is an autosomal dominant disease characterized by the progressive ossification of skeletal tissue either spontaneously or in response to trauma. A heterozygous mutation in *ACVRI* (encodes ALK2) has been tightly linked to the disease, with a single mutation conserved in all familial and sporadic cases of FOH [199]. The disease causing mutation in *ACVRI* results in an arginine to histidine substitution in the GS domain [190, 199]. While this mutation does not affect the expression of ALK2, structural protein modeling predicts that this mutation would

create a pH-sensitive switch within the activation domain of the receptor resulting in ligand-independent activation of ALK2 [200]. Indeed, increased ALK2 receptor activity as a contributor to the pathogenesis of FOP is supported by data from signaling studies, as lymphocytes from FOP patients exhibit elevated BMP-mediated p38 activation and increased basal expression of BMP responsive genes [201]. Lymphocytes from FOP patients also have increased expression of BMP-4 and increased expression and activity of ALK3 [201]. As ALK2, BMP-4, and ALK3 are all altered in FOP, this suggests that aberrant signaling through the BMP signaling pathway contributes to the pathogenesis of FOP. In support of this, mice overexpressing BMP-4 under control of the neuron-specific enolase promoter develop severe postnatal heterotopic ossification, which closely resembles human FOP. Crossing these mice with mice overexpressing noggin inhibits disease progression supporting increased BMP signaling as a contributor to the pathogenesis of FOP [202].

#### *Hunter-Thompson and Grebe-type Chondrodysplasias*

Hunter-Thompson (CHTT) and Grebe-type chondrodysplasias (CGT) patients both have pronounced shortening of limb bones and are predisposed to severe knee dislocations; however, CGT patients have a more severe phenotype than CHTT patients. Both CHTT and CGT are caused by mutations in *GDF5*, with CGT patients having a mutation in a conserved cysteine in the functional domain of GDF-5 [203], while CHTT patients have missense mutations in *GDF5* that result in loss of GDF-5 expression [204]. *GDF5* deficient mice have similar phenotypes to humans with these syndromes, including structurally weaker Achilles tendons with reduced collagen composition [205].

Analysis of GDF-5 function in chick skeletal development demonstrates that GDF-5 is required for cartilage condensation and chondrocyte proliferation [203].

### *Osteoporosis*

Osteoporosis is a common disease that occurs when there is an imbalance between bone resorption and formation. Patients present with decreased bone mass and increased susceptibility to fractures. Women are at a two-fold greater risk than men for developing this disease. The genetic contributors to osteoporosis are still being defined as there are likely many genetic modifiers that contribute to this disease [206]. Several TGF- $\beta$  superfamily members have been implicated as candidate genes in osteoporosis, including *TGFB1*, *BMP2*, *BMP4*, and *SOST* (sclerostin) [206]. Several polymorphisms in *TGFB1* have been identified in patients with osteoporosis; G(-1639)-A, C(-1348)-T, C(-765)insC, T(29)-C, G(74)-C, 713-8delC, C(788)-T, and T(816-20)-C [207]. Many of these *TGFB1* polymorphisms are associated with higher serum TGF- $\beta$ 1 levels and significantly lower bone mass [208], and patients with one of these polymorphisms exhibit a reduced response to vitamin D treatment [209]. Another study identified single-nucleotide polymorphisms in several TGF- $\beta$  pathway components in patients with osteoporosis including *TGFB1*, *TGFB2*, *SMAD2*, *SMAD3*, *SMAD4*, and *SMAD7* [210]. The relevance of these polymorphisms remains to be established.

Bone morphogenetic proteins have also been implicated in contributing to osteoporosis since they are critical regulators of bone homeostasis. Polymorphisms in *BMP2* have been associated with low bone mineral density and increased fracture incidence [211], although a subsequent study on a larger population did not confirm this

result [212]. In addition, a polymorphism in *BMP4* has been identified that affects hip bone density in post-menopausal women [213]. Finally, Resveratrol, a natural compound with estrogenic activity, has been explored as a potential osteoporosis therapy due to its ability to potently stimulate osteoclast differentiation and increase bone mass in a rat model. Resveratrol may function in part by inducing BMP-2 mRNA expression and stimulating BMP-2 mediated osteoblast differentiation [214].

#### *Sclerosteosis and Van Buchem Disease*

Sclerosteosis and Van Buchem Disease (VBD) are closely related rare autosomal recessive diseases that affect the skeletal system [215]. Both diseases are associated with overgrowth of the skull and jaw, facial palsy, hearing loss, and loss of smell. Sclerosteosis is more severe than VBD as patients have increased intracranial pressure and have been reported to die suddenly from brain stem impaction. Sclerosteosis has more clinical features including syndactyly, gigantism, dental malocclusion, and nail hypoplasia. These diseases have been linked to the same gene with Sclerosteosis resulting from mutations within *SOST* (encodes sclerostin) and VBD resulting from a 52kB deletion 32kB downstream of *SOST*, which deletes an enhancer element that regulates *SOST* expression [215]. Four mutations in *SOST* have been identified in patients with Sclerosteosis, including two mutations that result in a premature stop codon in exon 2, one mutation that results in a premature stop codon in exon 1, and a base substitution, that results in an incorrectly spliced message [215-217].

Sclerostin is a BMP antagonist abundantly expressed in long bones and cartilage [34, 215, 218]. This restricted expression is consistent with patients exhibiting only

skeletal clinical manifestations. Sclerostin is expressed exclusively by osteocytes in human bone, and it inhibits the differentiation and mineralization activity of osteoblasts in vitro. Therefore, sclerostin is thought to negatively regulate bone mass. The current model for the pathogenesis of these diseases is that sclerostin is normally produced by osteocytes and transported to the bone surface, where it inhibits the later stages of bone formation by binding and sequestering BMPs. In Sclerosteosis and VBD patients, as there is no inhibitory signal, osteoblasts continue to deposit bone leading to increased bone mass [218]. Patient's clinical manifestations support this model with bone biopsies indicating increased activity of osteoblasts and higher serum alkaline phosphatase levels, which contribute to patients being resistant to acquiring bone fractures [215]. The importance of sclerostin in inhibiting bone mass is evident from the sclerostin overexpressing mouse, which has reduced osteoblast activity and bone mass, as well as disorganized bone architecture [219]. Taken together these studies demonstrate that careful regulation of sclerostin is critical for normal bone development and growth.

#### *Brachydactyly and Symphalangism*

Brachydactyly type C (BDC) is associated with shortening and occasionally the complete loss of digits. Brachydactyly type A2 (BDA2) is an autosomal dominant disorder resulting in shortening and lateral deviation of the index fingers and, to a variable degree, shortening and deviation of the first and second toes. These diseases are both associated with defects in signaling through GDF-5 and ALK6, with mutations in *GDF5* that reduce the binding affinity of GDF-5 to ALK6 in BDC, and heterozygous missense mutations in the GS and kinase domains of ALK6 in BDA2 [220]. The

importance of GDF-5 and ALK6 in skeletal development is evident from the phenotype of the knock-out mice, with *GDF5* knock-out mice having severe limb reduction and joint malformations [221], and *BMPR1B* knock-out mice failing to generate digit cartilage, similarly to the human phenotype [220]

Symphalangism (SYM1) is a disease that is characterized by fusion of the joints in the hands and feet. SYM1 is also associated with defects in signaling through GDF-5 and ALK6, with mutations in *GDF5* increasing the binding affinity of GDF-5 for ALK6. SYM1 has also been linked to mutations in the BMP antagonist *NOG* (encodes noggin), which are predicted to decrease noggin's ability to bind to BMPs and GDFs [222]. *NOG* knock-out mice exhibit cartilage overproduction and joint malformations, while *NOG* heterozygotes have a similar phenotype to human SYM1 with loss of joint cartilage and fusion [221], supporting a role for dysregulated noggin function in this disease.

#### *Duchenne Muscular Dystrophy*

Duchenne muscular dystrophy (DMD) is the most common inherited lethal muscle wasting disease. Patients present at a young age, with muscle weakness that interferes with their mobility. Eventually patients die from cardiovascular or respiratory failure. This disease is caused by mutations in muscle dystrophin, resulting in insufficient protein levels. There is some phenotypic variability in patients, suggesting that there are other genetic or environmental modifiers that contribute to this disease [223]. Recently, several missense mutations in *GDF8* (encodes myostatin) were identified in DMD patients [223]. Myostatin is a muscle-specific TGF- $\beta$  superfamily ligand that functions to limit muscle growth; as evident from myostatin deficient mice

and cattle, which show a marked increase in body weight, muscle mass, and strength [224, 225]. In addition, crossing a DMD mouse model with a *GDF8* knock-out mouse produced mice with increased muscle mass and stronger muscle [226], while administration of myostatin blocking antibodies to a DMD mouse model increased body weight, muscle mass, size, and strength with no long term obvious side effects [225, 226]. Finally, a homozygous mutation in *GDF8* was identified in an infant that exhibited gross muscle hypertrophy [227]. Taken together, these observations suggest mutations in myostatin may contribute to the pathogenesis of DMD, and that targeting myostatin function may be a useful therapeutic strategy for treating the muscle wasting symptoms in DMD patients.

### **Reproductive Disorders**

TGF- $\beta$  superfamily members exert critical functions in the female reproductive system. They regulate all aspects of ovarian follicle development; primordial follicle recruitment, granulosa and theca cell proliferation, gonadotropin receptor expression, oocyte maturation, ovulation, luteinization, and corpus luteum formation [228]. The most well studied TGF- $\beta$  superfamily members in the reproductive system are activin and inhibin, which were originally identified as components of gonadal fluid that stimulated or inhibited follicle stimulating hormone (FSH) release, respectively [229]. Inhibin was subsequently found to antagonize activin signaling by binding to T $\beta$ RIII and sequestering activin type II receptors into a complex so they cannot transduce activin mediated signals [15]. Follistatin is another activin antagonist that binds to activin and prevents activin from binding to cell surface receptors. Follistatin and inhibin also antagonize specific

BMP-mediated signals [229]. The importance of activin and inhibin in normal reproductive processes is evident from the phenotypes in murine models: (1) inhibin A overexpressing male mice have decreased testis size and female mice do not have mature follicles due to inadequate FSH secretion from the pituitary gland [230]; (2) *ACVR2* knock-out females are infertile and males have small testis, phenotypes that are similar to the inhibin overexpressing mice [231]; (3) *activin-beta* subunit knock-out mice have delayed child-birth and nursing defects [232]; and (4) knocking out *FST* (encodes follistatin) in granulosa cells of the postnatal ovary results in reduced reproductive capacity and often infertility [233]. We will now discuss the contribution of these TGF- $\beta$  superfamily members to specific reproductive diseases.

#### *Premature Ovarian Failure*

Premature ovarian failure (POF) occurs in up to 1% of women and is a common cause of infertility. POF is diagnosed when there is absent menstruation or premature depletion of ovarian follicles before the age of 40 years. POF is associated with high levels of gonadotropins and estrogen [234]. A contributor to POF is ovarian dysgenesis (OD), which is defective gonadal development where the ovaries can be replaced by a gonadal streak rather than true ovaries. Most cases of OD are associated with major X-chromosome abnormalities; however, OD can affect patients with normal X chromosomes. BMP-15 has been implicated in contributing to OD, with several mutations identified in *BMP15* in patients with OD, but not in control subjects, including a heterozygous substitution mutation in the pro region of *BMP15* (Y235C) [235, 236]. Animal models have supported a role for BMP-15 in ovarian development, as

*GDF9/BMP15* double knock-out mice have fewer fertilized oocytes and those that do become fertilized take longer to develop to the blastocyst stage [237]. Mutations in *BMP15* in sheep are also associated with an increased ovulation rate and infertility in a dosage-sensitive manner [238].

A mutation in *INHA* (encodes inhibin alpha subunit) has been identified in many patients with POF (A257T) [239, 240]. The effects of this mutation on inhibin-mediated suppression of activin activity were assessed by transfecting an activin responsive luciferase promoter into multiple activin responsive cell lines. Adding wild-type inhibin alpha suppressed the activity of this promoter 5 fold, whereas the POF-associated *INHA* mutant form exhibited no inhibitory activity. This inability to suppress activin signaling was not a result of inappropriate dimerization with the inhibin beta subunit [239]. An inhibin binding receptor, *TGFBR3*, has also been implicated in POF as there is an increased occurrence of a polymorphism in exon 12 of *TGFBR3* in patients with POF. The consequences of this mutation in patients with POF requires further investigation [241].

#### *Persistent Müllerian Duct Syndrome*

The Sertoli cells of the testes produce two hormones seven weeks post gestation that are required for the male embryo to develop a normal internal reproductive tract; (1) testosterone induces the Wolffian ducts to differentiate into epididymidis, vas deferens, and seminal vesicles; and (2) Müllerian inhibiting substance (MIS), which causes regression of the Müllerian ducts. If MIS is absent, then these ducts develop into the Fallopian tubes, uterus, and upper vagina as observed in female embryos [242, 243]. In a

rare autosomal recessive disorder, there is persistence of Müllerian duct derivatives including the uterus and fallopian tubes in genetic males otherwise normally virilized. Persistent Müllerian duct syndrome (PMDS) results from a mutation in *MIS*, that leads to very low levels of circulating MIS even before puberty, when MIS levels would normally be high [244]. Patients with PMDS can be homozygous or heterozygous. Although most patients are homozygous, the clinical manifestations in heterozygous patients indicate that PMDS can arise from MIS haploinsufficiency [244]. There have been 38 mutations in *MIS* identified to date, mostly missense that affect the bioactive carboxy terminus. *MIS* mutants, when expressed in epithelial cells, are retained in the endoplasmic reticulum and not secreted. Structural modeling predicts that many of these mutations would affect the folding and stability of MIS [243].

In PMDS patients with normal levels of serum MIS, mutations in *AMHR2* [encodes MIS Type II receptor (MISR2)] have been identified [244]. Patients with *MIS* or *AMHR2* mutations are clinically indistinguishable. Mutations in *AMHR2* affect the entire gene and result in inappropriate targeting of the receptor to the cell surface [245], truncation before the transmembrane domain leading to a soluble unstable receptor [243, 245], truncation immediately after the transmembrane domain that results in a dominant negative receptor, or disruption of the substrate-binding site of the kinase domain [245, 246]. There is a subset of PMDS patients that do not carry mutations in *MIS* or *AMHR2*. These patients have been screened for mutations in other TGF- $\beta$  superfamily members including *ACVR1*, *BMPRIA*, and *BMPR1B*; however, no mutations in these genes have been identified to date [243].

## Hereditary Cancer Syndromes

### *Juvenile Polyposis Syndrome*

Juvenile Polyposis Syndrome (JPS) is an autosomal dominant disorder that causes multiple hamartomatous polyps in the gastrointestinal tract, predominantly in the colon, at a young age, and predisposes individuals to gastrointestinal tract cancers, including colorectal, gastric, small intestinal, and pancreatic cancer, with a lifetime risk for colorectal cancer as high as 50%. Germline mutations in three TGF- $\beta$  superfamily members have been identified in JPS patients, with 20% having *SMAD4* mutations, 20% having *BMPRIA* mutations, and *ENG* mutations have been established in a very small subset of patients [73, 247, 248]. Similarly to other human cancers, the majority of the *SMAD4* mutations are in the MH2 domain; however, five mutations have also been identified in the linker region and one in the MH1 domain. The majority of mutations in *BMPRIA* are within the kinase domain. As about a third of JPS patients do not have mutations in *SMAD4* or *BMPRIA*, other TGF- $\beta$  superfamily members might be mutated in this disease. Thus far, *BMPR1B*, *BMPR2*, and *ACVRL1* have been excluded as potential genes that contribute to JPS [73].

### *Hereditary Nonpolyposis Colorectal Cancer*

The most common hereditary condition that predisposes patients to developing colorectal cancer is the autosomal dominant disorder Hereditary Nonpolyposis Colorectal Cancer (HNPCC). HNPCC results from germ-line mutation of genes within the DNA mismatch repair system, which contribute to microsatellite instability. Microsatellites are repetitive DNA sequences that are spread across the genome and are prone to mutation

during DNA replication, particularly when there are defects in the DNA mismatch repair system [249]. The *TGFBR2* gene is a hot spot for inactivation in patients with HNPCC as it contains a 10 base pair polyadenine repeat microsatellite sequence [250]. Indeed, up to 80% of colon cancer patients with HNPCC have a *TGFBR2* mutation. Recently, a polymorphism in *TGFBRI* (*TGFBRI\*6A*) was identified in a subset of patients with HNPCC [251]. The significance of this finding remains to be established.

#### *Bannayan-Riley-Ruvalcaba and Cowden Syndromes*

Cowden-syndrome (CS) is an autosomal dominant cancer syndrome associated with abnormal growths in various organs that predisposes patients to breast, thyroid, and endometrial cancers. Bannayan-Riley-Ruvalcaba (BRR) is a disorder that manifests at birth and is associated with gastrointestinal polyposis. These diseases are usually associated with a *PTEN* mutation; however, one patient with CS and BRR symptoms with no *PTEN* mutation was identified as having a mutation in *BMPRIA* [252]. The contribution of this mutation to these syndromes remains to be defined.

#### **Sporadic Cancer**

The contribution of TGF- $\beta$  signaling to human cancer has been extensively studied and we and others have previously reviewed this topic [253-255]. The TGF- $\beta$  signaling pathway has complex and contradictory roles in the pathogenesis of human cancers. In general, TGF- $\beta$  acts as a tumor suppressor early in carcinogenesis, inhibiting growth and promoting differentiation and apoptosis when appropriate, but then switches to a tumor promoter later in carcinogenesis, promoting the growth, survival, motility,

invasion, and metastasis of tumors that have formed. TGF- $\beta$  affects both the cancer cells, regulating their growth and survival, and the tumor microenvironment, regulating the composition of the extracellular matrix, angiogenesis, and immune surveillance. Indeed, TGF- $\beta$  contributes to all aspects of tumorigenesis including: (1) growth stimulation; (2) evasion of apoptosis; (3) promotion of angiogenesis; (4) immune evasion; and (5) promotion of invasion and metastasis [253, 256]. The importance of the TGF- $\beta$  signaling pathway in human cancers is evident from the frequent alteration of TGF- $\beta$  signaling components in hereditary human cancers (Table 2) and sporadic cancers (Table 3).

There is increasing evidence that the BMP signaling pathway, in addition to being mutated in several hereditary cancer predisposition syndromes, also contributes to sporadic cancer progression. Similarly to TGF- $\beta$ , BMPs contribute to numerous aspects of tumorigenesis including: (1) increasing the motility and invasiveness of prostate [257] and colon [258] cancer cells, (2) stimulating the growth of pancreatic [259], and prostate and bladder cancer cells [260] and (3) inhibiting the apoptosis of colon cancer cells [258].

### *Breast Cancer*

TGF- $\beta$  critically regulates normal mammary growth and development and numerous alterations in the TGF- $\beta$  signaling pathway have been described in human breast cancer. At the ligand level, a polymorphism in the *TGFB1* gene, specifically a thymine to cytosine transition in the 29th nucleotide in the coding sequence that results in a leucine to proline substitution at the 10th amino acid has been identified and linked to increased serum levels of TGF- $\beta$ 1 and an increased risk of breast cancer [261, 262]. However, some studies have failed to confirm an increased risk for carriers of this

polymorphism in their cohort [263]. In addition, this polymorphism has been examined as a prognostic marker, with the C allele of the T29C polymorphism predicting decreased survival for breast cancer patients [264]. A polymorphism in the promoter of *TGFB2* has also been identified that enhances gene transcription and increases TGF- $\beta$ 2 protein levels. This polymorphism has been associated with a 5 fold increase in lymph node metastasis [265].

In terms of the TGF- $\beta$  signaling receptors, two polymorphisms in *TGFBRI* (*TGFBRI*\*6A, Int7G24A) have been identified in patients with breast cancer [266, 267]. These polymorphisms may serve as low penetrance tumor susceptibility alleles and have also been described in colon, cervix, and ovarian cancer patients. Recently the effect of these allelic variations on breast cancer risk was assessed, with one study supporting an association of the *TGFBRI*\*6A allele with an increased risk of familial breast cancer and as a potential marker for poorly differentiated breast cancer [268], and another supporting an association of the Int7G24A allele with invasion and metastasis [269]. Mutations or alterations in *TGFBRI* and *TGFB2* are infrequent in human breast cancers. While a specific mutation of *TGFBRI*, a C to A transversion at nucleotide 1160 of the gene, has been reported to occur at high frequency (42%) in breast cancer metastases [270], a subsequent study failed to confirm this observation [271]. We recently demonstrated that there is frequent loss of T $\beta$ RIII expression in human breast cancers, due to loss of heterozygosity of the *TGFB3* gene locus, transcriptional downregulation by elevated TGF- $\beta$  levels, and perhaps epigenetic regulation. Loss of T $\beta$ RIII expression contributes to the pathogenesis of breast cancer, with a specific role in regulating breast cancer progression and metastasis [272]. Loss of T $\beta$ RIII expression correlated with the

progression from pre-invasive disease to invasive disease and from primary to metastatic disease. Importantly, restoring T $\beta$ RIII expression in breast cancer cells inhibited tumor invasiveness in vitro and tumor invasion, angiogenesis, and metastasis in vivo. Finally, low T $\beta$ RIII levels predicted decreased recurrence-free survival in breast cancer patients [272].

Mutations in *SMAD2*, *SMAD3*, and *SMAD4* are infrequent in human breast cancers [273]. Tissue array studies have supported this observation with 92% of human breast cancers expressing Smad2 and Smad4, and are TGF- $\beta$  responsive as demonstrated by phospho-Smad2 staining, while only 2% lacked Smad4 expression [274].

Other TGF- $\beta$  superfamily ligands are also likely to have a role in breast carcinogenesis. Breast cancers metastasize to the bone frequently, suggesting that breast cancer cells express genes that enable them to thrive within the bone environment. As BMPs are critical regulators of bone homeostasis [275], and breast cancer cells express all the components of the BMP pathway and are BMP responsive, BMPs are poised to regulate breast cancer pathogenesis [276]. Recently decreased BMP-7 expression in primary breast cancer specimens was associated with bone metastases, while increasing BMP-7 levels in a bone metastasis mouse model inhibited breast cancer metastasis to the bone [277]. In addition, BMP-2 promotes the proliferation of breast cancer cells in vivo [278], high levels of ALK6 are associated with increased tumor grade, proliferation, and a poorer prognosis for patients [279], while BMP-4 increases breast cancer cell invasiveness [280]. These studies suggest that the BMP axis promotes breast cancer progression but inhibits metastasis of breast cancer to the bone.

*Colorectal Cancer*

TGF- $\beta$  is a critical regulator of normal colonic epithelial homeostasis. Loss of TGF- $\beta$  responsiveness occurs frequently during colon cancer progression, usually through mutational inactivation of *TGFBR2* or *SMAD4* [253]. Colorectal cancers can be divided based on their genetic stability, with about 12% of colorectal cancer exhibiting microsatellite instability (MIS) due to errors in mismatch repair. While some MIS(+) colorectal cancers are due to HNPCC, most are sporadic. In either case, MIS(+) tumors are more likely to have mutations in *TGFBR2*, due to the polyadenine repeat sequence in the coding sequence of the *TGFBR2* gene [281]. Overall approximately 30% of colorectal cancers exhibit mutations in *TGFBR2*. Mouse models support a role for T $\beta$ RII in colon cancer progression as crossing *APC* null mice with mice lacking T $\beta$ RII in their colonic epithelium significantly increases the number of intestinal adenocarcinomas as compared to *APC* null mice mated to wild-type T $\beta$ RII expressing mice [282].

While the *SMAD4* gene is mutated or deleted in about 16-25% of colorectal cancers [281], *SMAD2* is mutated in only about 6% of colorectal cancers [283], and mutations in *SMAD3* have not been identified. However, Smad3 may also contribute to colon carcinogenesis, as one murine model lacking Smad3 developed metastatic colon adenocarcinomas by six months of age [283]. TGF- $\beta$  signaling in the tumor microenvironment is also critical for tumor progression. When *SMAD4* is knocked out in T-cells, there was a marked increase in the occurrence of gastrointestinal tumors [284]. An important tumor suppressive function for TGF- $\beta$  in the colon is inhibition of cyclooxygenase 2, an enzyme involved in prostaglandin synthesis, which is significantly

increased early in colon tumorigenesis, and has been shown in mice to be required for tumor progression [285].

Recently a study characterized the risk of advanced colorectal adenoma with *TGFB1* polymorphisms. The genetic alterations that resulted in increased serum levels of *TGFB1* (*TGFB1*-509TT, 10Pro/Pro) were associated with a greater risk of advanced colorectal cancer, especially in patients with multiple adenomas and rectal adenoma [286].

There is much less known about the contribution of other TGF- $\beta$  superfamily members to colon cancer; however, there is evidence for a role of BMPs in colon cancer progression. As mentioned previously, loss of *BMPRIA* contributes to hereditary colon cancer progression, BMPs are upregulated in colon cancer [287], and BMPs can increase the invasiveness of colon cancer cells in vitro [287, 288]. Recently, *BMPR2* was knocked out in stromal cells of the colorectal mucosa in mice and this resulted in hyperplasia throughout the colonic epithelium due to increased proliferation and decreased apoptosis. These mice developed polyps throughout the colon and experienced intestinal bleeding [289]. This data supports a potential tumor suppressor role of *BMPRII* in the tumor microenvironment.

### *Pancreatic Cancer*

TGF- $\beta$  is a critical regulator of pancreatic cancer homeostasis [290], and components of the TGF- $\beta$  pathway are often the target of disruption during pancreatic carcinogenesis. In a majority of human pancreatic cancers and cell lines; mutations in *TGFBR1* and *TGFBR2* have been identified that alter protein and/or mRNA levels [291-

293]. In addition, *SMAD4* is mutated in 50% of all human pancreatic cancers, supporting an important tumor suppressor role for the TGF- $\beta$  pathway [294, 295]. Mouse models support the importance of altered TGF- $\beta$  signaling in pancreatic cancer progression. *KRAS* is mutated in 90% of human pancreatic cancers and is thought to initiate the events that promote tumor progression. When one copy of *SMAD4* is inactivated in mice harboring this mutation, they develop mucinous cystic neoplasms that progress to invasive ductal adenocarcinomas [296]. When *TGFBR2* is knocked out in mice with an active *KRAS* mutation, 100% of the mice develop aggressive pancreatic ductal adenocarcinoma [297]. In human patients, TGF- $\beta$ 1 and TGF- $\beta$ 2 levels are high and this correlates with tumor growth and metastasis [298, 299]. Recently we determined that T $\beta$ RIII mRNA and protein expression is reduced in pancreatic adenocarcinoma, and this loss in expression correlates with increased tumor grade [300]. This loss of T $\beta$ RIII expression occurs during epithelial to mesenchymal (EMT), a process in which non-motile polarized epithelial cells acquire motility and invasiveness which contributes to metastasis. The loss of T $\beta$ RIII is required for the induction of motility and invasiveness of pancreatic cancer cells, but not the other aspects of EMT, such as decreases in E-cadherin or induction of Snail expression [300].

### *Lung Cancer*

Lung cancer is categorized into small cell lung cancer and nonsmall cell lung cancer (NSCLC), which includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and giant cell carcinoma [301]. TGF- $\beta$  signaling critically regulates lung development and homeostasis. In NSCLC, the progression of premalignant

bronchoepithelial lesions towards invasive carcinoma is associated with a decrease in T $\beta$ RII expression at the mRNA and protein level [302-304]. A 16bp microdeletion in exon 4 of *TGFBR2* has been identified in patients with giant and large cell variants of nonsmall cell lung carcinoma. This mutation results in a premature stop codon that produces a truncated receptor that is expressed at the cell surface, which has a mutated transmembrane domain and no kinase domain. Lung cancer cells harboring this mutation are unresponsive to TGF- $\beta$ -mediated growth inhibition and fibronectin synthesis [301]. Loss of T $\beta$ RII expression increases the invasiveness of lung cancer cells [304], and this invasion is dependent on the activity of RANTES, a chemokine that is secreted by inflammatory cells and tumor cells [305]. T $\beta$ RIII mRNA and protein expression are also frequently reduced in NSCLC patients, with loss correlating with increased tumor grade and disease progression [306]. This loss of T $\beta$ RIII expression results in increased migration, invasion, and anchorage-independent growth *in vitro* and tumor growth *in vivo*, supporting T $\beta$ RIII as a tumor suppressor gene in NSCLC [306]. NSCLC patients also occasionally harbor mutations in *SMAD4* and *SMAD2*, in 7% and 4% of cases, respectively. In contrast to NSCLC, most small cell lung cancer patients become resistant to TGF- $\beta$  through loss of T $\beta$ RII expression, through mechanisms that remain to be determined [307].

The lungs are often the target of metastasis, especially by breast cancer cells. There is also evidence for TGF- $\beta$  in promoting metastasis to the lungs; (1) administration of a T $\beta$ RI kinase inhibitor inhibited lung metastasis in an orthotopic xenograft model of lung metastasis and in an intracardiac injection model of experimental lung metastasis by the breast cancer cell line, MDA-MB-435-F-L [308], and (2) TGF- $\beta$  promotes expression

of inflammatory chemoattractants and myeloid cell recruitment in the lungs, which attract primary tumor cells to infiltrate the lungs [309].

BMPs also have an emerging role in lung cancer. There is aberrant expression of BMP-2 in 98% of human lung cancers, and there are increased levels of phosphorylated Smad1/5, and the BMP-responsive gene, Id-1 in human lung tumors, supporting increased BMP signaling in human lung cancer [310]. BMP-2 increases the growth of human lung cancer epithelial cells both in vitro and in vivo in nude mice, [310], and it was recently shown that BMP-2 induces the transformation of lung cancer cells via activation of the PI(3)K/mTOR pathway [311]. Overall these data indicate that BMP signaling critically contributes to lung cancer progression.

#### *Prostate Cancer*

TGF- $\beta$  is thought to critically regulate androgen-dependence in the prostate and to contribute to the progression of prostate cancer by promoting the resistance of prostate cancer cells to inhibitory hormonal growth signals [312]. A polymorphism has been identified in *TGFBI* (C-509T) that increases its expression and is associated with a two-fold greater risk for developing late stage prostate cancer [313]. Numerous animal model studies suggest that aberrant TGF- $\beta$  signaling contributes to prostate cancer progression including: (1) blocking TGF- $\beta$  signaling by expressing a dominant negative T $\beta$ RII suppressed androgen-independent growth of prostate tumor cells in nude mice [314]; (2) expression of a dominant negative T $\beta$ RII in the prostate of a prostate cancer mouse model increased metastasis [315]; (3) expression of wild-type T $\beta$ RII in prostate cancer cells reduced their growth by inducing apoptosis [316]; and (4) expression of a dominant

negative Smad3 abrogated the growth and metastasis of prostate cancer cells in nude mice [317].

In human patients, there is evidence for increased TGF- $\beta$  signaling in advanced prostate cancer including: (1) elevated serum levels of TGF- $\beta$ 1 [318]; (2) a positive correlation between TGF- $\beta$ 1 serum levels and Prostate Specific Antigen (PSA) levels, a bio-marker associated with prostate cancer [319]; (3) increased expression of Smad3 in prostate tumors [317]; and (4) nuclear overexpression of Smad4 in prostatic tumor tissue [320]. We have recently demonstrated that loss of T $\beta$ RIII expression is an important contributor to human prostate cancer progression. T $\beta$ RIII expression is reduced in prostatic tumor tissue and re-expression of T $\beta$ RIII in prostate cancer cells reduced their invasiveness and growth in nude mice [321].

Several other TGF- $\beta$  superfamily members have been implicated in suppressing prostate cancer progression. GDF-15 has been associated with early stages of prostate cancer; in a prostate cancer mouse model, there is a significant increase in the levels of GDF-15 during hyperplasia and prostatic intraepithelial neoplasia development [322]. In addition, BMP-7 expression is reduced in advanced prostate adenocarcinoma, and administration of BMP-7 to mice potently inhibits prostate cancer bone metastasis [323].

### **Developmental Disorders**

Appropriate TGF- $\beta$  superfamily signaling is critical during embryogenesis from the initial stages of blastula formation, during gastrulation, and throughout the multiple stages of organ development. Misregulation of TGF- $\beta$  superfamily signaling is often

lethal, as demonstrated by the embryonic lethal phenotypes of knock-out mouse models of numerous TGF- $\beta$  superfamily signaling components [67].

### *Cleft Palate*

Cleft Palate (CP) is one of the most common congenital malformations, which can be associated with other diseases (see Marfan Syndrome and Loeys-Dietz sections). CP arises from failure of the adherence of the two palatal shelves or from failure of the medial edge epithelial cells of the palate to undergo epithelial to mesenchymal transition (EMT), in which adherent and non-motile medial edge epithelial cells acquire motility and invasiveness [324]. EMT is associated with the loss of epithelial markers including E-cadherin and cytokeratins, which are important in the maintenance of epithelial cell junctions, and gain of mesenchymal markers including vimentin and N-cadherin. The TGF- $\beta$  pathway is an important regulator of EMT, as TGF- $\beta$  initiates and maintains EMT in many different systems in vitro and in vivo [325]. CP is thought to arise from genetic and environmental modifiers. Several polymorphisms have been identified in *TGFB2* and *TGFB3* that are associated with CP [326, 327].

Although, there have not been many polymorphisms in *TGFB1* identified in CP patients and the TGF- $\beta$  signaling mechanisms are still being defined, mouse models indicate an essential role for TGF- $\beta$  in proper palate development. When *TGFB1* is knocked out in embryonic ectoderm and neural crest lineages in mice, they develop CP [328]. *TGFB3* knock-out mice also exhibit CP with complete penetrance [329], and this phenotype can be rescued by crossing these mice with mice overexpressing Smad2 [330].

*Situs Inversus and Situs Ambiguus*

Humans have conserved left-right visceral asymmetries, for example the left-sided heart and spleen and right-sided liver and gall bladder. When there are errors in establishing this asymmetry, serious human diseases can arise such as Situs Inversus (SI), which is a mirror-image reversal of normal symmetry or Situs Ambiguus (SA), abnormal placement of certain organs. SA is usually more severe as it is associated with fatal cardiac malformations that often lead to death, and affects the digestive system including the liver, biliary tract, and bowel. Chromosome abnormalities, maternal diabetes, and retinoic acid exposure during pregnancy have all been implicated in contributing to SI and SA. However, recently several TGF- $\beta$  superfamily members have been shown to directly regulate left-right axis determination, including nodal [331]. Nodal is normally expressed on the left side of embryos during development, and in a mouse model that exhibits mirror-image reversal of normal symmetry, nodal is expressed on the right-side [332]. Mice deficient in nodal exhibit left-right malformations including abnormal placement of the heart, lungs, and digestive organs [333]. These potent effects of Nodal on left-right axis determination in mice prompted sequencing analysis of the *NODAL* gene in human patients with SA. A mutation in the prodomain of *NODAL* (R183Q) was identified in a patient with SA, as well as in her mother who did not exhibit SA [334].

Similarly to Nodal, Lefty-1 and Lefty-2 are also expressed asymmetrically during development [335, 336]; they are expressed only in the left half of gastrulating mouse embryos, and in mice that exhibit SI, there is an inversion of Lefty-1 expression [335, 336]. Lefty-1 knock-out mice exhibit left-sided morphology of both lungs, cardiac malformations, abnormalities of the inferior vena cava, and there are alterations in the

asymmetric expression of Lefty-2 and Nodal, suggesting that Lefty-1 regulates the expression of these TGF- $\beta$  superfamily members [337]. Mutations in *LEFTY1* have been identified in human patients with left-right malformations. In 112 sporadic cases of left-right axis malformations, 2 mutations were found in *LEFTY1*. Both mutations affect the cysteine knot, which mediates receptor binding. One mutation introduces a premature stop codon, producing a truncated protein, and the other is a single base change that affects a conserved serine. The patients with these two mutations had similar clinical manifestations including left ventricle hyperplasia, atrioventricular canal defect, left-sided superior vena cavae, and left-sided lungs, which is similar to the phenotype of the *LEFTY1*<sup>-/-</sup> mouse. These mutations were not identified in the affected individual's parents, supporting the somatic nature of these mutations [338].

GDF-1 has also been implicated in left-right patterning in mice. Unlike Nodal and Lefty-1, GDF-1 is expressed throughout the embryo at first, and then is localized to the primitive node, ventral neural tube, and lateral plate mesoderm. Mice deficient in *GDF1* exhibit SA, and have misexpression of Lefty-1, Lefty-2, and Nodal, suggesting that GDF-1 may be upstream of these other TGF- $\beta$  superfamily members [339]. No mutations in *GDF1* have been identified thus far in humans with SA or SI.

### **Emerging role for TGF- $\beta$ superfamily members in other Human Diseases**

This review has focused on diseases in which TGF- $\beta$  superfamily signaling has been extensively studied and importantly (Figure 3, Tables II and III), where alterations in TGF- $\beta$  superfamily signaling components have been directly linked to human disease (Table II). However, TGF- $\beta$  superfamily members are continually being linked to other

diseases. For example, alterations in TGF- $\beta$ 1 expression have been observed in several neurological disorders including: autism [340], schizophrenia [341], Parkinson's disease [342], multiple sclerosis [343], and Alzheimer's disease [344], while activin expression is altered in Huntington's and Parkinson's disease [345]. Mouse models have been developed for many of these diseases, so it will be beneficial to determine how targeting TGF- $\beta$  superfamily members affects the progression of these diseases. Recently, *TGFBI* polymorphisms were identified in patients with otosclerosis [346], a progressive loss of hearing that results from increased remodeling of the otic capsule, as well as in psoriasis [347], biliary cirrhosis [348], and childhood asthma [349]. Thus the range of diseases in which TGF- $\beta$  superfamily members have a role will likely continue to grow.

Other TGF- $\beta$  superfamily members, particularly the BMPs are continually being implicated in human diseases. Recently, BMPs were implicated in iron metabolic disorders, including anemia and hemochromatosis (iron overload). Hemojuvelin is a BMP co-receptor that participates in the positive regulation of levels of the liver hormone hepcidin, which regulates iron balance by decreasing the cell surface expression of the iron exporter ferroportin and thus decreases iron absorption in the stomach [350]. Mutations in BMP family members have been identified in patients with hemochromatosis including *BMP2*, *BMP4*, and *HJV* [351]. Modulating the BMP pathway in patients with these disorders has recently been proposed, as administering BMP-2 to mice increases hepcidin expression and decreases serum iron levels, while administering soluble hemojuvelin has a reciprocal effect [352].

### **TGF- $\beta$ Superfamily Members as Diagnostic, Prognostic or Predictive Markers**

Since specific alterations in TGF- $\beta$  superfamily members contribute to numerous human diseases (Table II, Table III and Figure 3), these alterations could serve as valuable diagnostic, prognostic, or predictive markers. In particular, assessing ligand levels or levels of soluble TGF- $\beta$  superfamily receptors in serum is useful due to the ease and accessibility of these specimens, and ability to monitor over time.

Several TGF- $\beta$  superfamily signaling components have been assessed as prognostic markers for several cardiovascular diseases. Recently high serum levels of soluble endoglin during the second trimester of pregnancy were determined to be predictive of developing pre-eclampsia [118]. This information is useful clinically as it enables physicians to determine which patients need closer supervision and how aggressively to treat patients at risk for pre-eclampsia. In addition, in patients with coronary artery disease, high levels of TGF- $\beta$ 1 in the blood are significantly associated with increased patient survival, with fewer coronary events and need for interventions [353]. In contrast, after angioplasty, there is a greater risk of developing restenosis in those patients that have higher levels of TGF- $\beta$ 1 in their blood 15min, 24hr, and 2 weeks after the procedure was performed [354], suggesting that these patients would benefit from additional interventions to prevent restenosis.

Since TGF- $\beta$  exerts potent tumor promoting activity later in tumorigenesis, levels of TGF- $\beta$  signaling components have frequently been assessed as potential prognostic markers in several cancer types. Increased serum levels of TGF- $\beta$ 1 has been implicated as a marker of advanced disease and poor prognosis in multiple cancers including esophageal adenocarcinoma [355], gastric carcinoma [356], colorectal cancer [357],

pancreatic cancer [358], hepatocellular cancer [359], and breast cancer [360]. Decreased levels of TGF- $\beta$  receptors within the tumor cells have also been associated with poor outcome including: (1) reduced T $\beta$ RII expression is associated with increased lymph node metastasis and reduced survival of colon cancer patients [361]; (2) reduced expression of T $\beta$ RI and T $\beta$ RII is associated with the pathologic stage, metastasis, and decreased survival of esophageal cancer patients [361]; (3) reduced expression of T $\beta$ RII is associated with the progression of premalignant lung lesions to carcinoma in situ [302]; and (4) reduced expression of T $\beta$ RIII is associated with disease progression and a poorer prognosis for breast and prostate cancer patients [272, 321]. Measuring levels of TGF- $\beta$  superfamily downstream signaling effectors has also been proposed as a prognostic marker as loss of phosphorylated Smad2/3 expression is associated with a shorter disease-free survival in breast cancer patients [362], patients with gastric tumors with high Smad4 expression and low Smad7 expression have higher survival rates [363], and loss of Smad2 and Smad4 expression in colon tumors is associated with advanced disease including lymph node metastasis and a shorter survival rate [364]. These studies suggest that profiling the TGF- $\beta$  signaling pathway may aid in defining an appropriate treatment approach for individual patients.

Other TGF- $\beta$  superfamily members have been assessed as prognostic markers in various tumor types including: (1) the promoter of *BMP6* is hypermethylated, resulting in decreased BMP-6 expression, in aggressive diffuse large B cell lymphoma and this is associated with reduced survival [365]; (2) BMP-7 expression is increased in malignant melanomas and their metastases, and this correlates with shorter tumor recurrence [366];

and (3) loss of BMPRII in prostate cancer cells correlates with the Gleason score in prostate cancer patients and is associated with reduced disease-free survival [367].

TGF- $\beta$  superfamily receptors have also been used to facilitate imaging of solid tumors. Endoglin is overexpressed on endothelial cells associated with tumor induced angiogenesis [368]. Accordingly, a radioactively labeled-antibody directed to endoglin has been used to visualize solid tumors that were not detected by more conventional means in a canine model of breast cancer [369], suggesting that this might serve as a sensitive diagnostic marker of solid tumors.

### **Targeting TGF- $\beta$ Superfamily Members Therapeutically**

As with any signal transduction pathway involved in human disease, there are a myriad of methods for targeting the pathway. In addition, as either increased or decreased activity of these pathways has been implicated in the pathogenesis of different human diseases (Tables II and III), methods for both increasing and decreasing signaling through these pathways are required.

In terms of diseases with either decreased TGF- $\beta$  superfamily signaling including HHT and hereditary cancer syndromes, or where elevating TGF- $\beta$  superfamily signaling would be beneficial, including atherosclerotic heart disease; several mechanisms for elevating TGF- $\beta$  signaling are available including: (1) administering the ligand directly; (2) using agonists; (3) increasing expression of the appropriate receptors; or (4) decreasing the expression of signaling antagonists. Recently, the efficacy of the anti-fibrinolytic drug Tranexamic Acid (TA) was assessed in preventing recurrent nosebleeds in HHT patients. TA effectively reduced the frequency and severity of nosebleeds in

patients. Mechanistically, TA increased ALK1 and endoglin mRNA and protein expression in primary endothelial cells and increased ALK1 and endoglin mediated signaling as measured using transcriptional reporter assays, migration, and tube formation assays, suggesting that TA functions to restore TGF- $\beta$  superfamily signaling by increasing receptor-mediated signaling in this disease [370]. Another example of a potential stimulator of TGF- $\beta$  signaling is the statin class of drugs, used to suppress cholesterol levels and decrease risk of cardiovascular disease. Recently, it was demonstrated that cholesterol decreases the binding of TGF- $\beta$  to ALK5 and T $\beta$ RII, and effectively suppresses TGF- $\beta$  signaling; whereas, statins, drugs that inhibit cholesterol synthesis, increase TGF- $\beta$  responsiveness. Therefore, patients with atherosclerosis that take statins will have decreased serum cholesterol levels as well as the added benefit of increased TGF- $\beta$  signaling [371]. These examples highlight the importance of using drugs that stimulate TGF- $\beta$  signaling to treat diseases that manifest from insufficient TGF- $\beta$  signaling.

Alternatively, TGF- $\beta$  superfamily ligands could be administered directly to patients. In an atherosclerosis mouse model, overexpression of TGF- $\beta$ 1 by viral delivery suppressed atherosclerosis [129]. In addition, administering myostatin directly to patients with Duchenne muscular dystrophy may be a useful therapeutic strategy to rebuild muscle mass. Due to BMP's potent stimulation of bone formation, most therapeutic interventions thus far have targeted this role; BMP-2 has been administered locally to the upper or lower jaw of patients receiving dental implants to assess whether it can stimulate bone formation [372], and at fracture sites to determine if it can accelerate healing [373].

In terms of diseases with increased TGF- $\beta$  superfamily signaling including Camurati-Engelmann Disease, Fibrodysplasia Ossificans Progressiva, Marfan syndrome, and most advanced cancers, decreasing TGF- $\beta$  superfamily signaling would be beneficial. There are many ways to inhibit TGF- $\beta$  superfamily signaling including; (1) neutralizing antibodies; (2) soluble receptors; (3) natural or synthetic antagonists; (4) antisense nucleotides that reduce expression; and (5) small molecule inhibitors of the receptor serine/threonine kinases. Neutralizing antibodies are frequently the therapeutic method of choice for inhibiting TGF- $\beta$  superfamily signaling since they are particularly effective; they bind directly to the ligand and prevent it from binding to its receptor, effectively shutting down superfamily signaling. Numerous clinical trials have been performed with neutralizing antibodies, especially in fibrotic diseases including: (1) a TGF- $\beta$ 2 neutralizing antibody (lerdelimumab) effectively decreased the amount of scarring after glaucoma surgery [374]; (2) a TGF- $\beta$ 1 neutralizing antibody (metelimumab) is being administered to patients with systemic sclerosis, which causes scarring in skin and internal organs [375]; and (3) a pan-TGF- $\beta$  (GC-1008) neutralizing antibody is currently being administered to patients with idiopathic pulmonary fibrosis [376]. TGF- $\beta$  neutralizing antibodies have frequently been assessed as anti-cancer therapeutics. The efficacy of the GC-1008 neutralizing antibody is also being assessed in patients with advanced renal carcinoma and malignant melanoma [377]. In mice, a TGF- $\beta$  neutralizing antibody suppressed radiation-induced acceleration of metastatic breast cancer progression [378].

Soluble TGF- $\beta$  superfamily receptors have also been utilized as therapeutic agents. Similar to neutralizing antibodies, they abrogate signaling at the ligand level by

binding ligand and preventing it from binding to cell surface receptors. For example, soluble T $\beta$ RIII has demonstrated efficacy against renal damage progression associated with diabetes in mice [379], and inhibits the growth and angiogenesis of human colon and breast cancer cells in vivo [380]. Soluble T $\beta$ RII has similar anti-cancer effects in mice, it suppresses the growth and metastasis of pancreatic cancer cells [381] and inhibits breast cancer cell growth, migration, invasion, and metastasis [382]. Whether this strategy will work in humans warrants further investigation.

An alternative strategy is to identify and/or develop antagonists of TGF- $\beta$  superfamily signaling. In Marfan syndrome (MS), there is increased TGF- $\beta$  activity due to insufficient binding by fibrillin [see MS section], suggesting use of inhibitors of TGF- $\beta$  signaling in this disease. In fibrillin-1 deficient mice, a murine model of MS, the angiotensin receptor blocker losartan, which should antagonize TGF- $\beta$ 1 signaling through inhibition of the renin-angiotensin system (see Figure 2), prevented the development and progression of aortic aneurysms by reducing aortic wall thickness and aortic root growth, and improved aortic wall architecture [383]. This effect was attributed to suppression of TGF- $\beta$  signaling as losartan reduced levels of phosphorylated Smad2 levels in the aortic walls and administration of a TGF- $\beta$  neutralizing antibody was able to mimic the effects of Losartan in this model system [383]. Losartan also rescued the alveolar septation defect phenotype, which commonly occurs in human MS patients [383]. Another clinical manifestation of MS is myopathy, which is also thought to be a result of increased TGF- $\beta$  signaling in muscle fibers. Similarly, losartan treatment of fibrillin-1 deficient mice also resulted in regeneration and restoration of normal muscle architecture, while reducing levels of phosphorylated Smad2 and 3, and these effects

could also be achieved by administration of a TGF- $\beta$  neutralizing antibody [384]. Interestingly, these effects may not be limited to MS-associated myopathy as losartan has similar effects on muscle architecture and regeneration in the *MDX* Duchenne muscular dystrophy (DMD) mouse model (arises from mutations in dystrophin, see DMD section) [384]. Losartan may prove to be a valuable tool for treating MS and DMD, with a current study evaluating whether losartan treatment reduces aortic root enlargement in MS patients [385].

Recently, Burmester et. al. screened a chemical library and identified 5 small molecule antagonists that specifically abrogate the ability of TGF- $\beta$  to bind to its receptor and attenuate TGF- $\beta$  mediated growth inhibition [386]. It will be interesting to see if these compounds are also effective in mouse models of TGF- $\beta$  superfamily diseases, especially in MS and DMD where increased TGF- $\beta$  signaling contributes to disease pathology.

Using antisense oligonucleotides to reduce expression of TGF- $\beta$  superfamily members is a relatively new therapeutic tool that has been successful in treating cancer. A TGF- $\beta$ 2-specific antisense oligodeoxynucleotide (AP 12009) prolonged the survival of high grade glioma patients with two patients experiencing complete remission [387], and it is now being considered for use in other TGF- $\beta$ 2 overexpressing tumors. A TGF- $\beta$ 2 antisense transgene also prolonged the survival of patients with NSCLC [388].

Small molecule inhibitors have been developed to block TGF- $\beta$  superfamily signaling at the receptor level. A small molecule inhibitor (Ly573636) of T $\beta$ RI/ALK5 kinase activity is currently being assessed in patients with malignant melanoma [389], soft tissue sarcoma [390], NSCLC [391], and ovarian cancer [392]. ALK5 inhibitors

have demonstrated efficacy in mouse models including reduction of the growth of mesothelioma, and they reduce the rate of recurrence after surgical resection [393]. Development of ALK2 inhibitors would be useful for treatment of Fibrodysplasia Ossificans Progressiva, for which there are currently no cures or effective treatments. The therapeutic benefit of specifically inhibiting TGF- $\beta$  superfamily receptors in diseases other than cancer is just beginning to be realized.

### **Concluding Remarks**

As we have described, the TGF- $\beta$  superfamily regulates a plethora of vital functions throughout embryonic development and adulthood. There is tight regulation at all levels of TGF- $\beta$  superfamily signaling, and when components are altered by germline or sporadic alterations, human diseases frequently arise (Figure 3). Understanding the molecular basis of these TGF- $\beta$  superfamily alterations is critical to make a correct diagnosis, and to design effective therapies based on the genetic alterations in each individual patient. For many of the diseases with altered TGF- $\beta$  superfamily signaling, there are no effective treatments available and patients will eventually die from progressive complications. Therefore, further work is necessary to elucidate precisely how TGF- $\beta$  superfamily signaling is altered in each disease, and to define effective strategies for targeting TGF- $\beta$  superfamily signaling pathways.

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## Figure Legends

**Figure 1: TGF- $\beta$  Superfamily Signaling Pathways.** TGF- $\beta$  superfamily ligands in the extracellular space bind to their respective type II and type I receptors. This binding is facilitated by the presence of co-receptors, including T $\beta$ RIII. Within the extracellular space, there are negative regulators, including BMP antagonists, which bind to TGF- $\beta$  superfamily ligands and prevent them from binding to their respective cell surface receptors. When a TGF- $\beta$  superfamily ligand binds its specific receptor complex, a signal transduction cascade is initiated. The type I receptor's kinase domain becomes activated via phosphorylation by an appropriate type II receptor, enabling the type I receptor to interact with and phosphorylate the Smad transcription factors. There are two classes of receptor Smads; the BMP responsive Smads, which include Smad1, 5, and 8, and the TGF- $\beta$  responsive Smads, which include Smad2 and 3. When these receptor Smads are phosphorylated, they form a trimeric complex with the common Smad4 and this complex accumulates in the nucleus. Within the nucleus, the Smads interact with other transcription factors to affect the transcriptome, resulting in regulation of cellular responses including growth, motility, and apoptosis.

**Figure 2: The Renin-Angiotensin-Aldosterone Axis Increases TGF- $\beta$ 1 expression and activity.** Renin mediates the conversion of Angiotensinogen to Angiotensin I, which is then converted to Angiotensin II by Angiotensin Converting Enzyme (ACE). Angiotensin II binds to its receptor (AT1R) to activate a NADPH oxidase/p38 MAPK/AP-1 signaling cascade to increase TGF- $\beta$ 1 mRNA levels. Angiotensin II also directly increases p38 and JNK MAPKs signaling to increase thrombospondin-1 (TSP-1)

expression, which cleaves the LAP domain of TGF- $\beta$ 1 and converts TGF- $\beta$ 1 into its active form. Furthermore, Angiotensin II promotes aldosterone secretion, which also increases TGF- $\beta$ 1 mRNA levels. Aminopeptidase A converts Angiotensin II into Angiotensin III, which also increases TGF- $\beta$ 1 expression. The renin-angiotensin-aldosterone axis effects on TGF- $\beta$ 1 expression and activity can be attenuated by ACE inhibitors or angiotensin receptor blockers, such as losartan.

**Figure 3: Contribution of TGF- $\beta$  Superfamily Signaling to Human Disease.** The TGF- $\beta$  superfamily regulates many critical physiological processes throughout development into adulthood. TGF- $\beta$  superfamily signaling is tightly regulated at all levels, from the availability of ligand, to the amount of receptors on the cell surface, and the levels of the downstream transcription factors that will ultimately dictate the outcome of TGF- $\beta$  superfamily signaling. When any level of the pathway is altered, either through germ-line or somatic mutation, the pathway no longer effectively regulates physiological processes, and human diseases arise. Indeed, human diseases are associated with alterations in each level of the TGF- $\beta$  superfamily signaling pathway, as demonstrated here.

Table I: TGF- $\beta$  Superfamily Signaling Components

	Receptor Gene/Protein	Ligands, Binding Partners	Receptor Smads
<b>Type I Receptors</b>	<i>ACVRL1</i> /ALK1	Activin A, BMP-9, TGF- $\beta$ 1, TGF- $\beta$ 3	1,5,8
	<i>ACVRI</i> /ALK2	Activin A, BMP-6, BMP-7, MIS, TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3	1, 5, 8
	<i>BMPRIA</i> /ALK3(BMPRIa)	BMP-2, BMP-4, BMP-6, BMP-7	1, 5, 8
	<i>ACVR1B</i> /ALK4	Activin A, GDF-1, GDF-11, Nodal	2, 3
	<i>TGFBR1</i> /ALK5(T $\beta$ RI)	TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3	2,3
	<i>BMPRI1B</i> /ALK6(BMPRIb)	BMP-2, BMP-4, BMP-6, BMP-7, GDF-5, GDF-6, GDF-9b, MIS	1,5,8
	<i>ACVR1C</i> /ALK7	Nodal	2,3
<b>Type II Receptors</b>	<i>ACVR2</i> /ActRII	Activin A, BMP-2, BMP-6, BMP-7, GDF-1, GDF-5, GDF-8, GDF-9b, GDF-11, Inhibin A, Inhibin B	
	<i>ACVR2B</i> /ActRIIb	Activin A, BMP-2, BMP-6, BMP-7, GDF-5, GDF-8, GDF-11, Inhibin A, Inhibin B, Nodal	
	<i>BMPR2</i> /BMPRII	BMP-2, BMP-4, BMP-6, BMP-7, GDF-5, GDF-6, GDF-9b	
	<i>AMHR2</i> /MISRII	MIS	
	<i>TGFBR2</i> /T $\beta$ RII	TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3	
	<i>TGFBR3</i> /T $\beta$ RIII	Inhibin, TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, BMP-2, BMP-4, BMP-7, GDF-5	
<b>Co-receptors</b>	<i>ENG</i> /Endoglin	In conjunction with appropriate type II receptor; Activin A, BMP-2, BMP-7, TGF- $\beta$ 1, TGF- $\beta$ 3	
	<i>CRIPTO</i> /Cripto	GDF-1, GDF-3, Nodal, TGF- $\beta$ 1	
	<i>RGMA</i> /RGMa	BMP-2, BMP-4	
	<i>RGMB</i> /RGMb(DRAGON)	BMP-2, BMP-4	
	<i>HJV</i> /Hemojuvelin(RGMc)	BMP-2, BMP-4	
	<b>Pseudo-receptor</b>	<i>BAMBI</i> /BAMBI	Associates with Type I receptors
<b>Ligand Antagonists</b>	<i>CHRD</i> /Chordin	BMP-2, BMP-4	
	<i>FST</i> /Follistatin	Activin A, Activin B, Inhibin	
	<i>LEFTY1</i> /Lefty1	Binds to Cripto, competes with Nodal	
	<i>NOG</i> /Noggin	BMP-2, BMP-4, BMP-7	
	<i>SOST</i> /Sclerostin	BMP-2, BMP-4, BMP-6, BMP-7	

Table II: Germ-line Mutations in TGF- $\beta$  Superfamily Members in Human Disease

	DISEASE	SUPERFAMILY SIGNALING COMPONENT		
		Antagonists	Ligands	Receptors Smads
Cardiovascular System	Hereditary Hemorrhagic Telangiectasia			<i>ENG, ACVRL1</i> <i>SMAD 4</i>
	Loeys-Dietz Syndrome			<i>TGFBR1, TGFBR2</i>
	Familial Thoracic Aortic Aneurysm Syndrome			<i>TGFBR1, TGFBR2</i>
	Primary Pulmonary Hypertension			<i>BMPR2 ACVRL1</i>
Connective Tissue	Marfan Syndrome	<b>FBN1</b>		<i>TGFBR1, TGFBR2</i>
	Sphrintzen-Goldberg syndrome			<i>TGFBR2</i>
	Furlong Syndrome			<i>TGFBR1</i>
Skeletal and Muscular System	Camurati-Englemann Disease		<i>TGFB1</i>	
	Fibrodysplasia Ossificans Progressiva			<i>ACVRI</i>
	Hunter-Thompson and Grebe type Chondrodysplasia		<i>GDF5</i>	
	Sclerosteosis	<i>SOST</i>		
	Van Buchem Disease	Enhancer for <i>SOST</i>		
	Brachydactyly type C		<i>GDF5</i>	
	Brachydactyly type A2			<i>BMPR1B</i>
	Symphalangism	<i>NOGGIN</i>	<i>GDF5</i>	
Reproductive System	Premature Ovarian Failure		<i>BMP15, INHA</i>	
	Persistent Mullerian Duct Syndrome		<i>MIS</i>	<i>AMHR2</i>
Developmental Disorders	Situs Ambiguus	<i>LEFTY1</i>	<i>NODAL</i>	
	Cleft palate		<i>TGFB2, TGFB3</i>	
Metabolic Disorders	Hereditary Hemochromatosis Juvenile Hemochromatosis			<i>HJV</i>
Hereditary Cancer	Juvenile Polyposis			<i>BMPRIA, ENG</i> <i>SMAD 4</i>
	Hereditary Non-polyposis Colorectal Cancer			<i>TGFBR1</i>
	Bannayan-Riley-Ruvalcaba Syndrome			<i>BMPRIA</i>
	Cowden Syndrome			<i>BMPRIA</i>

Table III: Alterations in TGF- $\beta$  Superfamily Members in Sporadic Human Disease

	DISEASE	COMPONENT	SUPERFAMILY		SIGNALING
			Ligands	Receptors	Smads
Cardiovascular System	Atherosclerosis	Decreased TGF- $\beta$ 1		<i>TGFBR2</i> mutations	
	Hypertension	<i>TGFB1</i> polymorphisms (Increased TGF- $\beta$ 1)			
	Dilated Cardiomyopathy	<i>TGFB1</i> polymorphisms (Increased TGF- $\beta$ 1) <b><i>BMP10</i> polymorphisms (Increased <i>BMP10</i>)</b>			
	Pre-eclampsia			Increased sEnd	
	Restenosis	Increased TGF- $\beta$ 1 levels			
Skeletal and Muscular System	Osteoporosis	<i>TGFB1</i> polymorphisms (Increased TGF- $\beta$ 1)			
	Breast Cancer	<i>TGFB1</i> polymorphisms (Increased TGF- $\beta$ 1) <i>TGFB2</i> polymorphisms (Increased TGF- $\beta$ 2) Increased BMP-4 Decreased BMP-7		<i>TGFBRI</i> polymorphisms; decreased T $\beta$ RIII, T $\beta$ RII, T $\beta$ RI Increased ALK6	
Cancer	Colon Cancer	<i>TGFB1</i> polymorphisms (Increased TGF- $\beta$ 1) Increased BMP		<i>TGFBR2</i> mutations	<i>SMAD2</i> , <i>SMAD4</i> mutations
	Lung Cancer			<i>TGFBR2</i> mutations, decreased T $\beta$ RII	<i>SMAD2</i> , <i>SMAD3</i> mutations
	Pancreatic Cancer	Increased TGF- $\beta$ 1, TGF- $\beta$ 2		<i>TGFBR2</i> mutations, Decreased T $\beta$ RIII, T $\beta$ RII, T $\beta$ RI	<i>SMAD4</i> mutation
	Prostate Cancer	<i>TGFB1</i> polymorphisms (Increased TGF- $\beta$ 1) Increased GDF-15		Decreased T $\beta$ RIII	Increased Smad3, Smad4 nuclear localization

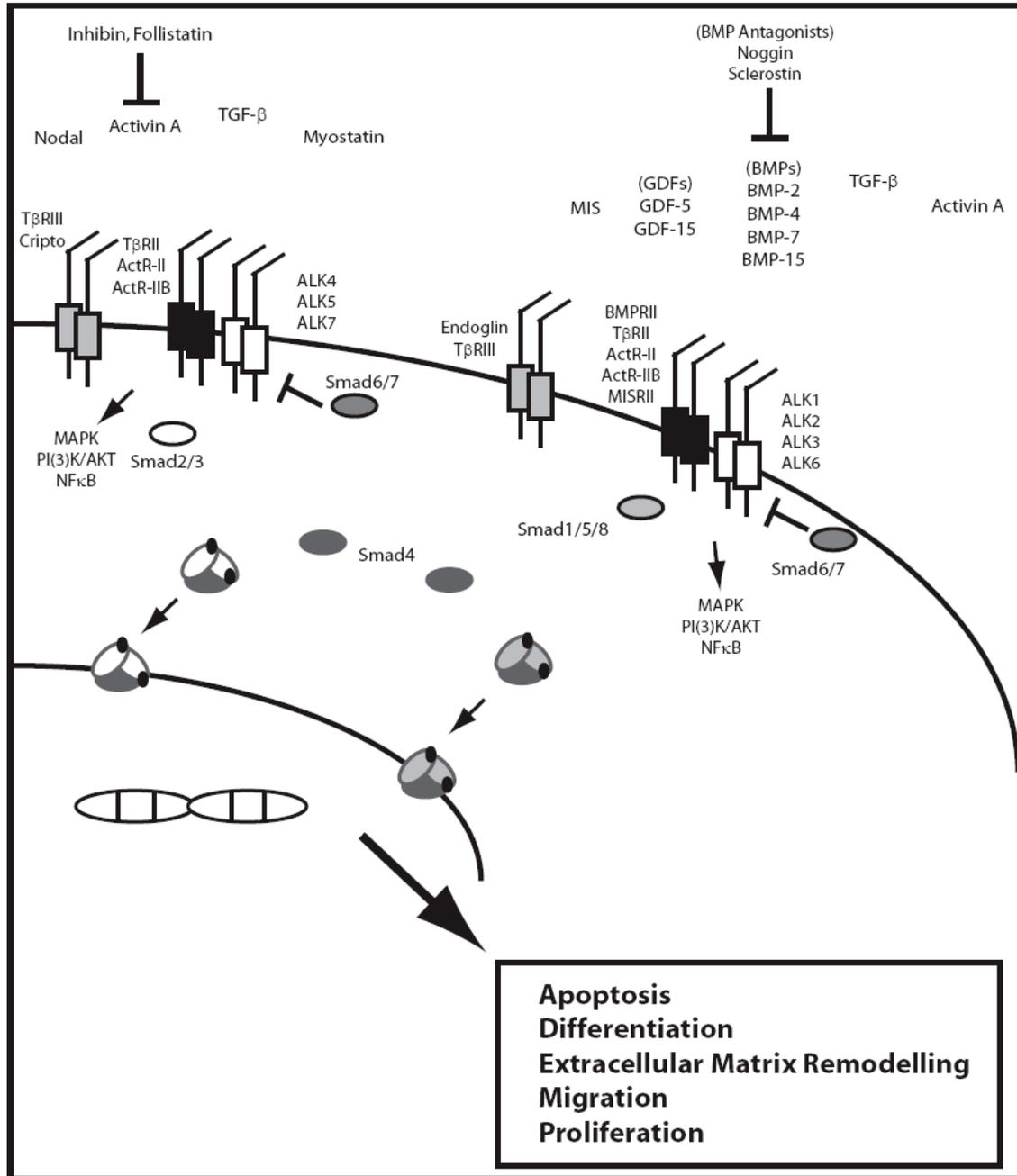
**Figure 1: TGF- $\beta$  superfamily Signaling**

Figure 2

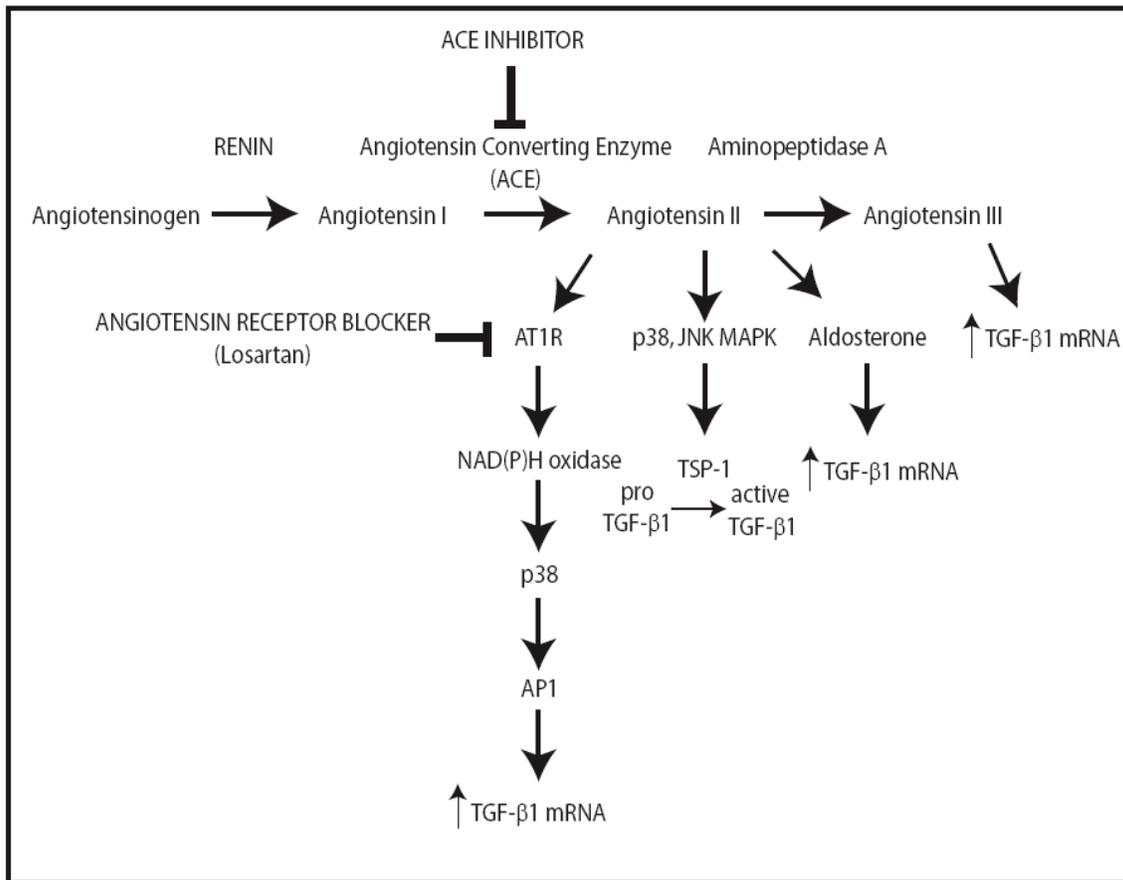


Figure 3

