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Genetic Analysis of the Mammalian Transforming Growth Factor-β Superfamily

HUA CHANG, CHESTER W. BROWN, AND MARTIN M. MATZUK

Departments of Pathology (H.C., M.M.M.), Molecular and Human Genetics (C.W.B., M.M.M.), Molecular and Cellular Biology (M.M.M.), and Pediatrics (C.W.B.), and Program in Developmental Biology (H.C., M.M.M.), Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030

Members of the TGF-β superfamily, which includes TGF-βs, growth differentiation factors, bone morphogenetic proteins, activins, inhibins, and glial cell line-derived neurotrophic factor, are synthesized as prepropeptide precursors and then processed and secreted as homodimers or heterodimers. Most ligands of the family signal through transmembrane serine/threonine kinase receptors and SMAD proteins to regulate cellular functions. Many studies have reported the characterization of knockout and knock-in transgenic mice as well as humans or other mammals with naturally occurring genetic mutations in superfamily members or their regulatory proteins. These investigations have revealed that TGF-β superfamily ligands, receptors, SMADs, and upstream and downstream regulators function in diverse developmental and physiological pathways. This review attempts to collate and integrate the extensive body of *in vivo* mammalian studies produced over the last decade. (*Endocrine Reviews* 23: 787–823, 2002)

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Abbreviations: ActRIB, Activin receptor type IB; ACVR2, activin receptor type IIA; ALK, activin receptor-like kinase; AV, atrial-ventricular; BMP, bone morphogenetic protein; Bmpr, BMP receptor; *bp*, brachypodism; CBP, CREB binding protein; CDMP, cartilage-derived morphogenetic protein; DPC4, deleted in pancreatic carcinoma locus 4; dpp, decapentaplegic; E, embryonic day; ES, embryonic stem; *FecB*, Booroola fecundity gene; FGF, fibroblast growth factor; FoxH1, forkhead or winged helix DNA-binding protein 1; GDF, growth differentiation factor; GDNF, glial cell line-derived neurotrophic factor; HTC, Hunter-Thompson acromesomelic chondrodysplasia; *Inhba*, activin/inhibin βA; *Inhbb*, activin/inhibin βB; InhBP, inhibin-binding protein; LAP, latency associated peptide; LPM, lateral plate mesoderm; MAD, mothers against dpp; MEE, medial edge epithelium; MH1, MAD homologous region 1; MIS, Müllerian inhibiting substance; PFP, putative ventral floor plate; PGC, primordial germ cells; PTX3, pentraxin; R-SMAD, receptor-regulated SMAD; SARA, SMAD anchor for receptor activation; SF-1, steroidogenic factor-1; SHH, sonic hedgehog; SMA DIP1, SMAD-interacting protein 1; SNIP1, SMAD nuclear interacting protein 1; TAB1, TAK1-binding protein 1; TAK1, TGF-β-activating kinase-1; TβRI, TGF-β type I receptor; TGIF, transforming growth interacting factor; TRIP-1, TGF-β type II receptor-interacting protein; TSP-1, thrombospondin 1.

I. Introduction

THE TGF-β SUPERFAMILY is a large group of extracellular growth factors controlling many aspects of development (1–5). Homo- or hetero-dimers of the TGF-β family ligands bind to and activate two types of transmembrane serine/threonine kinase receptors, which then stimulate downstream regulatory SMAD proteins to localize from the cytoplasm to the nucleus where they can function as transcriptional regulators (3, 6–8). TGF-β superfamily signaling is regulated at multiple levels, including extracellular binding and processing of TGF-β superfamily ligands and intracellular interactions of the receptors. Gene inactivation studies in mice during the past decade have greatly expanded our understanding of TGF-β superfamily signaling in animal development, and many of these studies have been summarized in the abovementioned reviews (1, 2). In this review, we will focus on the latest progress in this field, with emphasis on the roles of TGF-β superfamily signaling in embryonic development, reproduction, and tumor formation.

II. Components of the TGF-β Superfamily Signal Transduction Pathway

A. Ligands

The TGF-β superfamily consists of more than 35 members in vertebrates, including TGF-βs, BMPs (bone morphogenetic proteins), GDFs (growth differentiation factors), activins, inhibins, MIS (Müllerian inhibiting substance), Nodal, and leftys (2, 9, 10; Fig. 1). The TGF-β family ligands are translated as prepropeptide precursors with an N-terminal signal peptide followed by the prodomain and the mature domain. Six to nine conserved cysteine residues in the mature domain form intra- and intermolecular disulfide bonds characteristic of this family of proteins (3, 10, 11). Several members of the family (*i.e.*, GDF-9, BMP-15, GDF-3, lefty-1, and lefty-2) have a substitution of a serine for the cysteine normally involved in intermolecular disulfide bond formation. Therefore, although dimers of most family members are held together covalently, the proteins with this substitution would be expected to be noncovalently associated and possibly more labile.

TGF-βs are secreted as biologically latent forms (12, 13). The activity of the mature domain of the TGF-β ligand is masked by the propeptide, LAP (latency associated peptide), which is cleaved from the mature domain by a furin-like endoproteinase during secretion but remains associated with the mature domain via noncovalent interactions (14). Dissociation from LAP activates the TGF-β subfamily of ligands and possibly other members of the superfamily. The extracellular matrix protein thrombospondin 1 (TSP-1; Refs. 15 and 16), as well as integrin $\alpha_v\beta_6$ (17), also mediates TGF-β activation under physiological conditions. Other mechanisms, such as proteolysis (16, 17), may also be involved in the activation of TGF-β ligands *in vivo* (18).

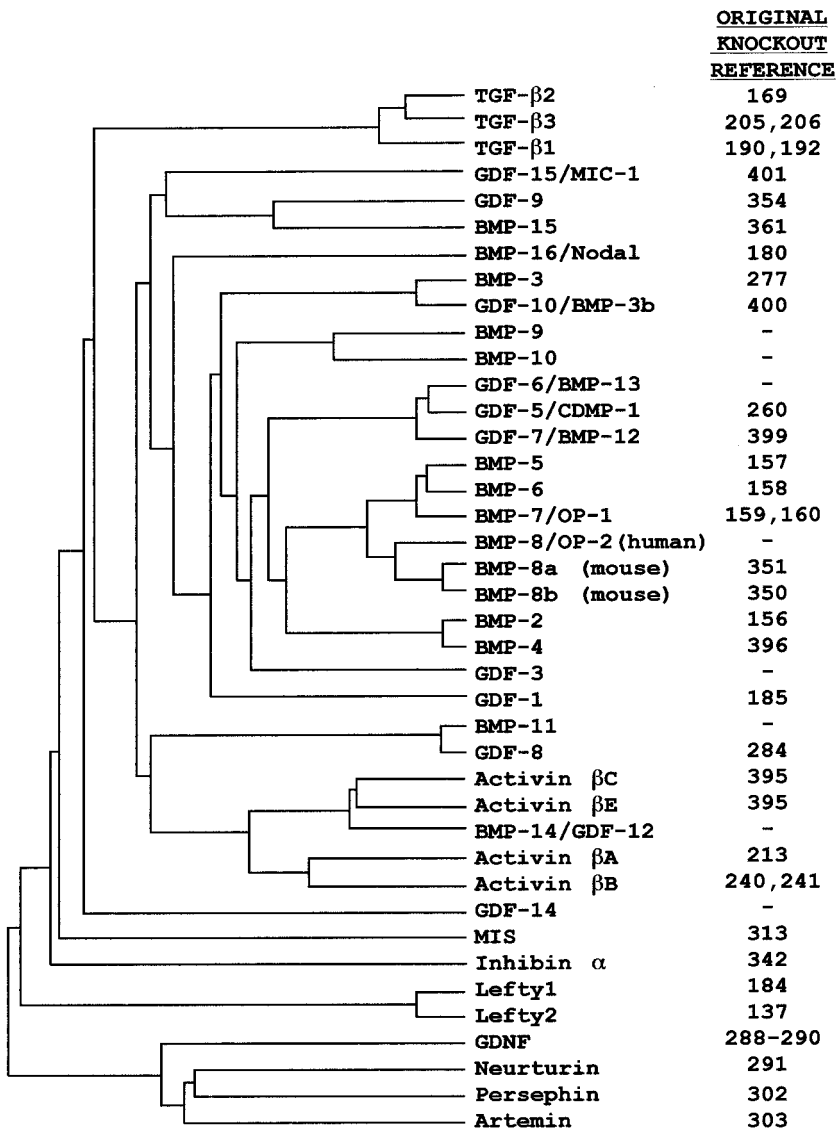

FIG. 1. TGF-β superfamily. Amino acid sequences of the carboxyl-terminal polypeptides of the mouse TGF-β superfamily members (and human BMP-8) were aligned using the PILEUP program (Genetics Computer Group, Madison, WI). Mouse and human sequences are available for all sequences except BMP-8. In the mouse, there are two BMP-8 sequences (BMP-8a and BMP-8b), unlike one in humans because of a duplication of the ancestral gene.

B. Receptors

TGF-β superfamily ligands signal through a family of transmembrane serine/threonine kinases known as the receptors for the TGF-β superfamily. On the basis of their structural and functional properties, the TGF-β receptors are divided into two subfamilies: type I and type II receptors. Type I and type II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, which interact upon ligand binding. The extracellular regions of these receptors contain about 150 amino acids with 10 or more cysteines that determine the folding of this region. A unique feature of the type I receptors is a highly conserved 30 amino acid intracellular region immediately preceding the kinase domain (Fig. 2); this 30-amino acid stretch is called the GS domain because of the SGSGSG sequence it contains (19). Ligand-induced phosphorylation of the GS domain in the type I receptor by the type II receptor is required for the activation of signaling (19–21).

Type I receptors received different names as a result of being cloned by independent groups simultaneously. For example, ALK (activin receptor-like kinase) 4 is commonly known as ActRIB (activin receptor type IB) because it can bind activin and mediate certain activin responses in cultured cells (22–25); BMPR1A and BMPR1B are BMP receptors that are also known as ALK3 and ALK6, respectively. ALK8 is a novel type I receptor for BMP-2b and BMP-7, which is

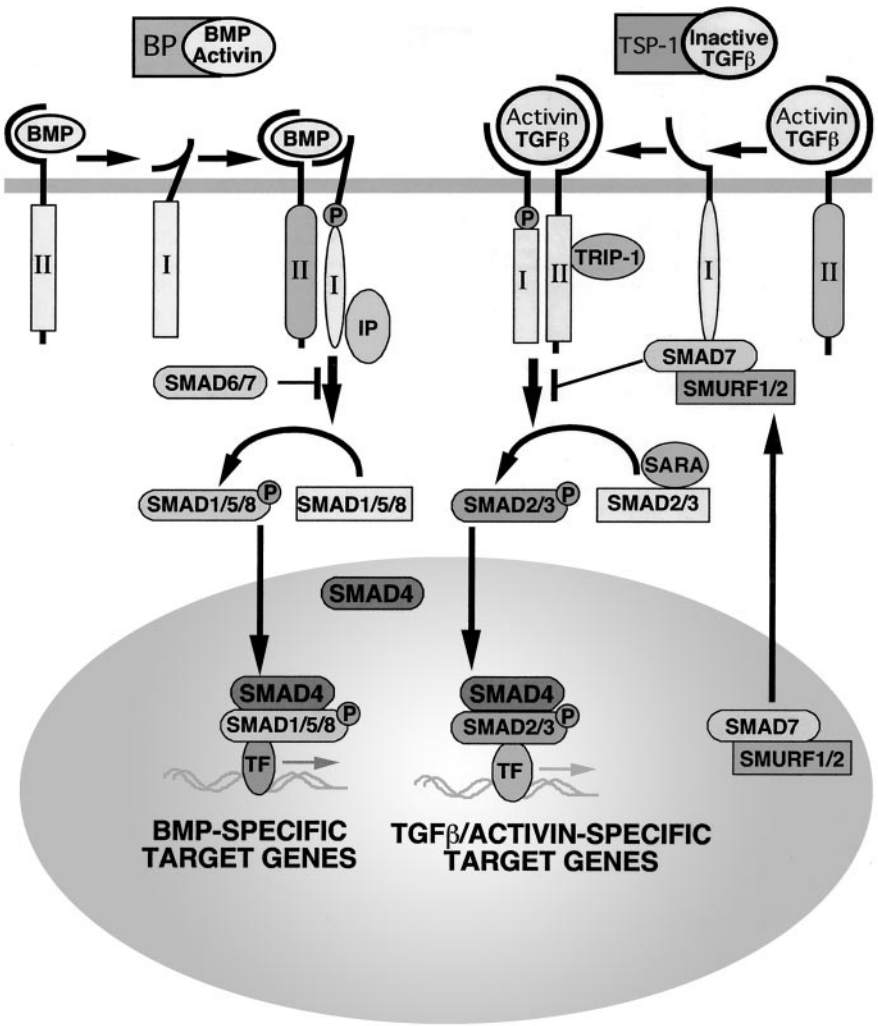

FIG. 2. Regulation of TGF-β superfamily signal transduction. TGF-β superfamily signaling is regulated at multiple levels. BMPs and activin/TGF-βs typically use different signal transduction pathways. BMPs will bind to either of a transmembrane serine/threonine kinase type I or type II receptor to stimulate formations of a ternary complex. The type II receptor will phosphorylate and activate the type I receptor. The type I receptor will then phosphorylate SMAD1, SMAD5, or SMAD8, allowing each to migrate to the nucleus, interact with SMAD4, and transcription factors (TF) or coregulators, and stimulate BMP-specific target genes. Activins and TGF-βs will follow a similar pathway, except that they must first bind type II receptors and their type I receptors will phosphorylate SMAD2 or SMAD3. SMAD6 is a negative regulatory SMAD that interferes with BMP type I receptor phosphorylation of SMADs. Induction of the E3 ubiquitin ligases SMURF1 or 2 will cause SMAD7 to migrate from the nucleus to activin/TGF-β type I receptors where the complex will cause degradation of the type I receptor, thereby inactivating the pathway. SMAD6 and SMAD7 may act similarly to inhibit the BMP pathway, although there is no evidence yet of this mechanism. SARA functions as a linker protein between the type I activin/TGF-β receptors and SMAD2/3 to stimulate SMAD phosphorylation. Multiple type I interacting proteins (IP) and extracellular binding proteins (BP) such as follistatin have been identified. Extracellular TSP-1 will bind to and activate latent TGF-β1.

essential for fin development in zebrafish (26–28). A comparison of the two nomenclatures for the type I and type II receptors is listed in Table 1. Because of the promiscuity of the type I receptors for different TGF-β superfamily ligands in most cases, the ALK nomenclature is probably most appropriate.

TGF-β superfamily ligands activate an intracellular signaling cascade by binding to and bringing together type I and type II receptors. For TGF-βs and activins, ligands directly bind type II receptors and then recruit type I receptors. In these cases, only ligand-bound type II receptors physically interact with type I receptors (22, 29–31). In contrast, both type I and type II BMP receptors bind ligands (32–35). Once an active ligand-type I/type II receptor complex is formed, type II receptors activate type I receptors by phosphorylation, and type I receptors subsequently phosphorylate downstream SMAD proteins that propagate the signal from the cytoplasm to the nucleus (3, 6, 36).

Another distinct type of cell surface receptors for the TGF-β ligands was discovered by ligand cross-linking methods. These receptors were called type III receptors because of the higher molecular weight of these proteins compared with type I and type II receptors (37). Betaglycan and endoglin are two examples of type III receptors. Endoglin is a cell surface protein expressed at high levels in endothelial cells and at lower levels in monocytes, erythroid precursors, and other cell types (38, 39). Betaglycan is a membrane-anchored proteoglycan that can bind TGF-βs and facilitate their interaction with the type II receptor (40, 41). Betaglycan also can bind inhibin and promote its binding to the activin type II receptor, thereby competing with activins at this receptor and antagonizing activin signaling (42). A distinct inhibin receptor [called inhibin-binding protein (InhBP) or p120] has also been identified (43–45). InhBP is a transmembrane member of the Ig superfamily expressed from an X-linked gene. InhBP is expressed in gonadotrophs of the pituitary and Leydig cells of the testes. InhBP levels closely correlate with serum inhibin B levels in female rats. This finding is interesting because InhBP specifically interacts with ALK4, and inhibin B (but not inhibin A) disrupts the association of InhBP, ALK4, and ActRIIA, thereby antagonizing activin signaling *in vitro*. In contrast, inhibin A appears to bind a complex of betaglycan and ActRIIA to disrupt activin signaling through an ActRIIA-ALK4 complex. The significance of these complexes in activin and inhibin actions *in vivo* will have to await gene knockout studies.

TABLE 1. Mammalian TGF-β superfamily receptors and their alternative names

ALK1	ACTVRL1
ALK2	ActRIA, ACVR1
ALK3	BMPR1A
ALK4	ActRIB
ALK5	TβRI
ALK6	BMPR1B
ALK7	N/A
ACVR2	ActRIIA
ACVR2B	ActRIIB
AMHR2	MISRII
BMPR2	BMPRII
TGFBR2	TβR2

N/A, Not applicable.

C. SMAD proteins

SMAD proteins are intracellular components of the TGF-β superfamily signal transduction pathways (Fig. 2). The first member of this family is MAD [mothers against dpp (decapentaplegic)], which was identified from genetic screens in *Drosophila melanogaster* as an enhancer of the weak alleles of *dpp* (46). Other members of this family were identified on the basis of their sequence homology with MAD. Three *Caenorhabditis elegans* homologs of MAD have been named as *sma-2*, *sma-3* and *sma-4*, because mutation of their respective genes causes developmentally arrested, third-stage dauer larva with smaller body sizes than wild-type larva (47). Vertebrate homologs of sma and MAD are called SMAD, as a combination of sma and MAD (48). At least 10 vertebrate SMAD proteins have been identified to date (49). Mutations in *SMAD2*, *SMAD3*, and *SMAD4* have been found in human tumors, suggesting that these genes function as tumor suppressors *in vivo* (3, 50, 51). For example, human *SMAD4*, which is also called DPC4 (deleted in pancreatic carcinoma locus 4), is frequently deleted or mutated in human pancreatic cancers (Ref. 52; Table 2).

SMAD proteins have two highly conserved domains at the N terminus and the C terminus of the protein named as MH1 (MAD homologous region 1) and MH2 (MAD homologous region 2) domain, respectively. The linker region between MH1 and MH2 domains is highly variable (3, 6, 53).

Members of the SMAD family play different roles in TGF-β superfamily signaling (Fig. 2). SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8 can be phosphorylated directly by type I receptors after ligand-induced dimerization of type I and type II receptors, and they are called receptor-regulated SMADs (R-SMADs; Refs. 3, 6, and 49). The phosphorylation of these R-SMADs triggers their localization from the cytoplasm to the nucleus where they can interact with the common SMAD and function to regulate gene transcription. An initial indication of a functional specialization among different SMADs came from frog animal cap assays. SMAD1 and SMAD5 induce ventral mesoderm formation like BMPs (54–56), whereas SMAD2 induces dorsal mesoderm formation similar to activins or Vg-1 (54). Further studies revealed that different Smad proteins are coupled to different receptors. SMAD2 and SMAD3 are phosphorylated and translocated to the nucleus upon stimulation by activin and TGF-β (57–59). SMAD1 and SMAD5 are phosphorylated and translocated to the nucleus upon stimulation by BMPs (*e.g.*, BMP-2, BMP-4, and BMP-7; Refs. 60–62). SMAD proteins are classed into subgroups on the basis of sequence homology. These structural similarities may provide the basis for functional redundancy between different SMAD proteins in one subgroup. SMAD8 (previously known as SMAD9) is structurally very similar to SMAD1 and SMAD5 and may also mediate BMP signals (63).

Common SMADs in vertebrates include SMAD4 or SMAD4β (found only in *Xenopus laevis*; Refs. 64 and 65), sma-4 in *C. elegans* (47), and Medea in *Drosophila* (66–68). Compared with other SMAD proteins, common SMADs have a characteristic insertion in the MH2 domain and lack the C-terminal SSXS motif, the site of phosphorylation by the type I receptors (59). Because of this sequence alteration, common SMADs are

TABLE 2. Human disease-causing mutations in TGF-β superfamily ligands, receptors, and regulatory and signaling proteins

Protein/gene symbol	Disease	Somatic/germline mutations/polymorphisms	Refs.
Activin receptor-like kinase 1 (ACVRL1, ALK1)	Hereditary hemorrhagic telangiectasia	Germline	372
	Gonadotroph tumor; 482V mutation of unknown significance	Somatic	373
	Familial primary pulmonary hypertension	Germline	374
Anti-Müllerian hormone (AMH)	Persistent Müllerian duct syndrome	Germline	323
Anti-Müllerian receptor, type II (AMHR2)	Persistent Müllerian duct syndrome	Germline	323
BMP receptor type 1a (BMPR1A)	Juvenile polyposis	Germline	375
BMP receptor type II (BMPR2)	Familial primary pulmonary hypertension	Germline	174
CBP	Rubinstein-Taybi syndrome; increased cancers	Germline	105, 376
Endoglin	Hereditary hemorrhagic telangiectasia	Germline	377, 196
GFRα4 (GDNF family receptor)	Hallervorden-Spatz syndrome	Somatic	300
GDNF	Hirschsprung disease[a]	Germline	297, 298
GDF5	Brachydactyly C	Germline	270
	Hunter-Thompson chondrodysplasia	Germline	271
	Grebe chondrodysplasia	Germline	272
Neurturin	Hirschsprung disease	Germline	299
Noggin	Multiple synostoses syndrome	Germline	282
	Proximal symphalangism	Germline	282
	Autosomal dominant stapes ankylosis with broad thumbs and toes, hyperopia, and skeletal anomalies	Germline	421
p300	Colorectal and gastric carcinoma	Somatic	106
RET	Papillary thyroid carcinoma, multiple endocrine neoplasia types 2A and 2B, Hirschsprung disease	Somatic and germline	296
SMAD2	Colorectal, lung, hepatocellular	Somatic	57, 378, 379
SMAD4 (DPC4)	Multiple cancers (pancreatic, colorectal, lung, breast, prostate, ovarian, head and neck, esophageal, gastric, bladder, hepatocellular, renal cell)	Somatic	52, 380
	Familial juvenile polyposis	Germline	381
SMADIP1 or ZFHX1B	Hirschsprung disease; other developmental defects	Somatic; haploinsufficiency	118–121
TGIF	Holoprosencephaly	Germline	117
TGFβ1	Camurati-Engelmann disease	Germline	382
	Advanced tumor invasion and metastasis	Somatic	383, 384
	Fibrosis, hypertension, osteoporosis, atherosclerois	Germline	385, 386
TGFβ receptor 1 (TGFBR1)	Cancer (breast, pancreatic, biliary, cervical, chronic lymphocytic leukemia, head and neck)	Germline	371
		Somatic	387, 388
TGFβ receptor 2 (TGFBR2)	Multiple cancers (colorectal, gastric, endometrial, prostate, breast, lung, hepatocellular, lymphoma, pancreatic, cervical, glioma)	Somatic	389–393
	Atherosclerosis	Somatic	394

[a] Possible digenic mode of inheritance with the *RET* gene.

not substrates of the type I receptors. Common SMADs form hetero-oligomers with receptor-specific SMAD proteins and translocate with the receptor-specific SMAD proteins into the nucleus upon activation of the signaling pathways (55, 59, 61, 69). SMAD4 forms a complex with SMAD1, SMAD5, or SMAD8 when BMP pathways are activated and forms a complex with SMAD2 and SMAD3 when activin or TGF-β pathways are activated. Consequently, overexpression of *Smad4* in frog animal caps induces both ventral and dorsal mesoderm as a result of the activation of both BMP and activin pathways, respectively (70, 71).

The inhibitory Smad proteins include SMAD6 and SMAD7 in vertebrates, Dad in *Drosophila*, and possibly Daf-3 in *C. elegans* (6, 49). These Smad proteins contain a characteristic C-terminal MH2 domain, but their N terminus has little similarity with typical MH1 domains (49). The only known function of this group of Smad proteins is to inhibit the signaling activity of R-SMADs. SMAD6 preferentially inhibits BMP signaling, whereas SMAD7 can inhibit both TGF-β and BMP signaling (72–74). SMAD7 inhibits phosphorylation of R-SMADs by occupying the type I receptors for BMPs, activins, and TGF-βs. SMAD6 preferentially inhibits BMP signaling by competing with SMAD4 for binding to receptor-activated SMAD1 and forms an inactive SMAD1-SMAD6 complex. SMAD6 and SMAD7 levels are increased in response to BMP, activin, or TGF-β signaling, suggesting that these SMADs function as negative feedback controls for different pathways.

D. Other components in the TGF-β superfamily signaling cascade

Signaling of the TGF-β superfamily members is highly regulated at multiple levels. Extracellular proteins such as follistatin, noggin, and chordin function as antagonists of many TGF-β ligands to alter the signaling process. Follistatin can bind to activin and prevent the binding of activin to its cell surface receptors (75, 76). Follistatin can also bind to BMP-7 at lower affinity than activin and may antagonize BMP signals *in vivo* (77). Noggin and chordin bind to BMP-4 and prevent its interaction with receptors (78, 79).

TSP-1 is a large homotrimeric protein secreted by many cell types (80, 81). In cell-free systems, TSP-1 binds to and activates both small and large latent forms of TGF-β1 (15, 82, 83). TSP-1 is likely a major activator of TGF-β1 because mice lacking TSP-1 highly resemble the mutant phenotypes observed in young TGF-β1 null mice with regard to lung, pancreas, liver, kidney, stomach, testes, skin, bone, and heart development (84). Furthermore, systemic treatment with a peptide that blocks the activation of TGF-β1 by TSP-1 can induce similar lung and pancreas phenotypes. Lung and pancreatic abnormalities of TSP-1 null mice reverted toward wild type when treated with a peptide derived from TSP-1 that could activate TGF-β1 (84).

Intracellularly, FKBP12 can interact with the cytoplasmic domain of type I receptors (32, 85, 86). FKBP12 was initially thought to be a negative regulator of TGF-β signaling (86, 87). However, no detectable effects were observed on TGF-β superfamily signaling or cell growth in *Fkbp12* null fibroblasts (on processes such as Müllerian duct regression or FSH regulation by activins) or by disrupting FKBP12/TβRI (TGF-β type I receptor) binding with FK506 or rapamycin (88–90). However, another group surprisingly showed that fibroblasts that they isolated from the same *Fkbp12* null mice grew more slowly than controls (91). Presently, it is unclear why such apparently conflicting data have been generated.

TRIP-1 was identified as a TβRII (TGF-β type II receptor)-interacting protein, and its interaction with TβRII is ligand-independent (92). TRIP-1 functions as a modulator of TGF-β signaling by receptor-dependent and -independent mechanisms (93). Lastly, SARA (SMAD anchor for receptor activation) interacts with SMAD2 and SMAD3 and appears to function as a linker to recruit SMAD2 to the TGF-β receptor (94). The functions of TRIP-1 and SARA *in vivo* are unknown.

TAK1 (TGF-β-activating kinase 1), a member of the MAPK kinase kinase family (95), is another protein found to be involved in TGF-β superfamily signaling. It participates in regulating TGF-β-induced gene expression, as well as BMP-induced mesoderm formation and embryonic patterning (95–97). TAB1 (TAK1-binding protein 1) is an activator of TAK1 so that an association between the kinase domain of TAK1 and the C-terminal portion of TAB1 triggers activation of phosphorylation-dependent TAK1 (98–100). XIAP (human X-chromosome-linked inhibitor of apoptosis protein) was isolated as a TAB1-binding protein that also binds to BMP receptors in mammalian cells; XIAP may function as a positive regulator of the BMP signaling pathways by linking the BMP receptors and TAB1-TAK1 (101).

Although SMAD proteins bind directly to DNA, work from several groups suggests that high-affinity binding requires interactions with other DNA binding proteins such as coactivators (for review, see Ref. 102). Two important coactivators are the paralogous proteins, cAMP-response element binding protein (CREB) binding protein (CBP) and p300, which function in multiple transcriptional pathways by acting as scaffolding proteins and promoting histone acetylation-dependent chromatin relaxation and acetylation of transcription factors. In TGF-β signaling, CBP and p300 cooperate with SMAD2-SMAD4 and SMAD3-SMAD4 in activating TGF-β/activin-mediated gene transcription (103, 104). Humans with heterozygous mutations at the *CBP* locus have Rubinstein-Taybi syndrome (105), which is characterized by multiple craniofacial, skeletal, and cardiac defects, as well as growth retardation, severe mental retardation, and increased risk of cancer. Biallelic mutations of the p300 gene in humans have been found in colorectal and gastric carcinomas (106). Mice homozygous for a truncated CBP protein (amino acids 1–1084 out of 2441 amino acids) died between embryonic day (E)9.5 and E10.5 due to defects in neural tube closure, hematopoiesis, and yolk sac vascular development (107, 108); in a few cases, hematological malignancies including lymphocytic and myelogenous leukemia and histiocytic sarcomas occur (109). Homozygous mutant mice lacking p300 also die at midgestation due to defects in neural tube closure, enlarged heart cavities, poor vascular development within the yolk sac, and overall developmental retardation, possibly due to decreased cell proliferation (110). Mice heterozygous for both Cbp and p300 (*i.e.*, $Cbp^{+/-}$ $p300^{+/-}$) also died at midgestation with defects similar to the single homozygous mutants (110). This suggests important redundancy between these two related proteins, as might be expected. Some of these developmental defects in the *Cbp* and *p300* knockouts could be attributed to abnormalities in TGF-β superfamily signaling cascades (see *Section III.B–D).*

Another important SMAD DNA-binding protein is FoxH1 (forkhead or winged helix DNA-binding protein 1, also known as FAST2). FoxH1 was initially isolated in *X. laevis* as a protein that bound to an activin response element in the *Mix.2* gene (111). FoxH1 can bind to complexes of SMAD4/phosphorylated SMAD2 or SMAD3, suggesting that these have an important role in activin signaling pathways in mammals. Knockout of the *FoxH1* gene results in early embryonic lethality due to defects in anterior primitive streak and node formation (112), consistent with a function in the activin/Nodal-Smad signaling pathway (see *Section III.A*).

In addition to binding to DNA with transcriptional coactivators such as *FoxH1*, SMADs also associate with a number of transcriptional corepressors. This group of corepressors includes SnoN and Ski, members of the Ski family of oncogenes, as well as SMAD nuclear interacting protein 1 (SNIP1; Ref. 113), 5′ transforming growth 3′ interacting factor (TGIF; Ref. 114), TGIF2 (115), and SMAD-interacting protein 1 (SMADIP1; Ref. 116). SNIP1, which interacts with SMAD1, SMAD2, SMAD4, and CBP/p300, appears to modulate TGF-β signaling mainly through preventing the association of SMAD4 and CBP/p300. TGIF and TGIF2 interact with TGF-β-activated SMADs to repress TGF-β-responsive transcription. TGIF and TGIF2 also recruit histone deacetylase, and TGIF (but not TGIF2) binds CBP and competes with p300

for binding to a SMAD2 complex. Both TGIF and TGIF2 bind to a specific TGIF binding site via their unique DNA-binding homeodomains. Interestingly, mutations in the human *TGIF* gene cause holoprosencephaly (117), presumably by interfering with NODAL/TGF-β signaling through SMAD2 at specific loci, thereby affecting the development of midline structures in mammals (see *Section III.E.4*). Lastly, SMADIP1, a member of the δEF1/ZFH-1 family of two-handed zinc finger/homeodomain proteins, interacts with SMADs after receptor-mediated activation and also binds to 5′-CACCT sequences in several promoters. A knockout of the mouse *Smadip1* gene has not been created. However, heterozygous mutations in the human *SMADIP1* gene cause syndromic Hirschsprung disease with a number of other findings, including mental retardation, hypospadias, and agenesis of the corpus callosum (118–121). Thus, altering the dosage of SMADIP1 in humans is sufficient to cause a complex developmental disorder. Other genes involved in TGF-β superfamily signaling and mutated in humans are presented in Table 2.

SnoN and Ski have also been shown to act as repressors by recruiting histone deacetylases. However, they appear to function in other ways as well, and their regulation in response to TGF-β is very interesting. In particular, SnoN has been shown to antagonize TGF-β signaling by binding to SMAD2, SMAD3, and SMAD4 (122, 123). SnoN is rapidly degraded in response to TGF-β. SMAD2 and SMAD3 mediate this SnoN degradation using different pathways. TGF-β induces a complex of SMAD2 and the E3 ubiquitin ligase, which stimulates ubiquitin-mediated degradation of SnoN (124). TGF-β also promotes the formation of a complex containing SMAD3, the anaphase-promoting complex, and SnoN, which leads to SnoN ubiquination and degradation (125). Thus, TGF-β stimulation results via both transcription activation and degradation of a corepressor. In *Drosophila*, DSmurf can bind MAD (the SMAD1/5 homolog) and promote its proteolysis, thereby restricting the BMP signaling pathway (126). Likewise, in mammals SMURF2 can target SMAD1 for ubiquination and proteosome-mediated degradation (127), thus favoring a TGF-β/activin signaling pathway over the BMP signaling pathway. Interestingly, SMURF2, which is normally a nuclear protein, can complex with SMAD7, be exported into the cytoplasm, complex with the TGF-β receptor, and cause ubiquitin-mediated degradation (down-regulation) of the receptor complex (128). In this way, ubiquitin-mediated proteolysis acts bifunctionally first to rapidly stimulate TGF-β superfamily signaling and subsequently to halt further TGF-β signaling. Similar to SMURF2, SMURF1 is also an E3 ubiquitin ligase that has been shown in *X. laevis* to specifically interact with BMP pathway-specific SMADs to trigger their ubiquination and degradation (129). In mammalian cells and parallel to SMURF2, SMURF1 interacts with SMAD7 to cause translocation of the complex to the cytoplasm, in which the complex will bind to the TβRI where it enhances ubiquination of both TβRI and SMAD7, thereby inducing turnover of these two proteins (130). On the basis of this data, there might be functional redundancy of SMURF1 and SMURF2 *in vivo*. The *in vivo* functions of the TGF-β superfamily transcriptional corepressors SnoN, SNIP1, and TGIF2 and the E3 ubiquitin ligases, SMURF1 and SMURF2, are not yet known. However, significant *in vivo* functional data have been accumulated on SKI. Mice lacking the *Ski* proto-oncogene have multiple craniofacial defects, including exencephaly, midline facial defects, eye abnormalities (*e.g.*, iris defects that range from aniridia to coloboma), decreased muscle mass, and extra digits (postaxial polydactyly; Refs. 131 and 132). When challenged with carcinogens, *Ski* heterozygous mutant mice show an increased incidence of tumors (133). In humans, absence of *SKI* may be in part causal for the craniofacial and muscle findings observed in individuals with the 1p36 deletion syndrome. These findings suggest that SKI may function in multiple TGF-β signaling pathways. In fact, the neural tube defects (exencephaly) may be secondary to elevated BMP-4 levels. Thus, multiple ligands, receptors, binding proteins, and downstream proteins may modulate TGF-β superfamily signaling cascades.

III. TGF-β Superfamily Signaling and Development

A. Early postimplantation mouse embryonic and extraembryonic development

An early postimplantation mouse embryo consists of three cell lineages: the extraembryonic ectoderm, the primitive endoderm, and the epiblast. The extraembryonic ectoderm gives rise to the placenta and the ectodermal component of the chorion, the primitive endoderm forms the endoderm of the parietal and visceral yolk sacs, and the epiblast gives rise to the embryo proper and extraembryonic mesoderm. The process of gastrulation converts the epiblast into the three definitive germ layers of the embryo, as well as generating the extraembryonic mesoderm that participates in the formation of the visceral yolk sac, amnion, chorion, and allantois. Gastrulation also results in the establishment of the primary body axes, *i.e.*, the anterior-posterior, dorsal-ventral, and left-right axes. Coordinated growth and morphogenesis of the embryonic and extraembryonic cells are required for normal development of an embryo (134, 135).

Genetic evidence indicates that TGF-β family members regulate both embryonic and extraembryonic development of an early postimplantation mouse embryo. Mice null for *Bmp4*, *Nodal*, *lefty2*, *Alk2*, Alk3 (*Bmpr1a*), *Alk4* (ActRIB), *Bmpr2*, *Smad2*, or *Smad4* either fail to initiate gastrulation or have defects in mesoderm differentiation. Other mutant mice that have defects in components of the TGF-β superfamily signaling cascade are described in Table 3.

Nodal and lefty-2 are two TGF-β superfamily ligands that may have opposite roles during mesoderm differentiation. Mutant mice lacking Nodal fail to develop a primitive streak and do not have mesoderm differentiation (136), whereas mice deficient in lefty-2 have an expanded primitive streak and form excess mesoderm. Furthermore, the lefty-2 mutant phenotype is partially suppressed by heterozygosity for the Nodal mutation, suggesting that Nodal and lefty-2 may function as antagonists of each other in normal mesoderm differentiation (137).

The defects in *Alk2*-mutated embryos are less severe than those of mouse embryos lacking either ALK3 (BMPR1A) or ALK4 (ActRIB) receptors. Embryos homozygous for an *Alk2*

TABLE 3. Mouse TGF-β superfamily ligand, binding protein, processing protein, receptor, or signaling protein mutants

Knockout mouse model	Phenotype	Refs.
TGF-β superfamily ligands		
Activin/inhibin βA (*Inhba*)	Perinatal lethal; craniofacial defects (*i.e.,* cleft palate and lack of whiskers, incisors, and lower molars)	213, 228
Activin/inhibin βB (*Inhbb*)	Viable; eyelid defects and reproductive abnormalities in females	240, 241
Activin/inhibin βC	Viable and fertile; no obvious abnormalities	395
Activin/inhibin βE	Viable and fertile; no obvious abnormalities	395
Artemin	Viable and fertile; ptosis; widespread defects in the migration of the sympathetic nervous system	303
BMP-2	Embryonic lethal; failure to close proamniotic canal; defective cardiac development; role in allantois and PGC development	146, 156
BMP-3	Viable; increased bone density in adult mice	277
BMP-4	Embryonic lethal; most show no mesoderm differentiation; haploinsufficient in C57BL/6 genetic background; no PGCs	335, 396, 397
BMP-5 (*short ear*)[a]	Viable; skeletal and cartilage abnormalities; role in allantois development and fusion with the chorion along with BMP-7	148, 157, 398
BMP-6	Viable; slight delay in ossification of sternum; role in cardiac cushion formation and septation along with BMP-7	158, 161
BMP-7	Perinatal lethal; kidney dysgenesis and anophthalmia; skeletal patterning defects; role in cardiac cushion formation and septation along with BMP-6; role in allantois development and fusion with the chorion along with BMP-5	148, 159–161
BMP-8A	Viable; some male infertility due to germ cell degeneration; epididymal epithelial degeneration in a small percentage of males	351, 362
BMP-8B	Viable; male infertility due to germ cell depletion; defects in PGC and allantois development	147, 350
BMP-15 (*FecX*^{I} or *FecX*^{H})[a]	Viable; female subfertility; interacting role with GDF-9 in ovarian physiology	361
GDF-1	Embryonic and postnatal lethal; majority die between E14.5 and P2; left-right asymmetry defects	185
GDF-5 (brachypodism)[a]	Viable; bone defects in the limbs	260
GDF-7	Viable; abnormal seminal vesicle development results in male infertility	399
GDF-8 (myostatin)	Viable; increased muscle mass via muscle cell hypertrophy and hyperplasia	284
GDF-9	Viable; female infertility; folliculogenesis arrested at one layer primary follicle stage	354–356
GDF-10	Viable; no obvious defects	400
GDF-11/BMP-11	Viable; homeotic transformation of the axial skeleton; posterior displacement of hindlimbs	219
GDF-15	Viable; no obvious defects	401
GDNF	Neonatal lethal; required for proper kidney development including heterozygotes; lack enteric nervous system	288–290
Inhibin α	Tumor suppressor role for inhibin; gonadal and adrenal tumors	342, 343
Lefty-1	Majority of litter die embryonically; partial loss of left-right asymmetry	184
Lefty-2	Embryonic lethal; expanded primitive streak and excess mesoderm	137
MIS (anti-Müllerian hormone or AMH)	Viable males develop uteri; Leydig cell hyperplasia and infertility in males; premature disappearance of primordial follicles in females	313, 318
Nodal (413-d mutant)[b]	Embryonic lethal; arrest at early gastrula stage; no primitive streak; role in formation of anterior-posterior axis; role in left-right asymmetry and craniofacial development	136, 145, 180, 403, 404
Neurturin	Viable and fertile; defects in the enteric nervous system; decreased parasympathetic innervation; loss of GFRα2-positive cells in trigerminal and dorsal root ganglion	291
Persephin	Viable; normal development and behavior; hypersensitive to focal cerebral ischemia or stroke	302
TGF-β1	>50% die during embryogenesis from yolk sac defects; survivors develop inflammatory disorders and die typically within 1 month	167, 190, 192
TGF-β2	Perinatal lethal; various craniofacial defects, axial and appendicular skeletal defects; retinal hyperplasia; heart defects; renal defects in a majority of females	169
TGF-β3	Perinatal lethal; cleft palate; delayed lung development	205, 206

TABLE 3. Continued

Knockout mouse model	Phenotype	Refs.
TGF-β receptors		
ALK1	Embryonic lethal; defective vascular development due to abnormalities in angiogenesis and arteriovenous malformations	198, 199
Activin receptor type IA (ALK2)	Embryonic lethal; gastrulation defective; abnormal visceral endoderm; defective mesoderm formation	138, 139
BMP type IA receptor (ALK3 or BMPR1A)	Embryonic lethal; no mesoderm formation; role in formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb	141, 278
Activin receptor type IB (ALK4)	Embryonic lethal; disrupted primitive streak	405
ALK5	Embryonic lethal; defective vascular development	202
BMP type IB receptor (ALK6; *FecB*)[a]	Viable; defects of chondrogenesis in the limb; female infertility due to defects in ovarian cumulus expansion	365, 406
Activin receptor type IIA (ACVR2)	25% die perinatally from craniofacial defects; males have delayed fertility; females are infertile	142, 407
Activin receptor type IIB (ACVR2B)	Perinatal lethal; left-right asymmetry and anteroposterior axis defects	143, 144
BMP type II receptor (BMPR2)	Embryonic lethality; arrest at gastrulation	140
GFRα1 (GDNF family receptor)	Neonatal lethal; agenesis of the kidney; lack enteric nervous system	293, 294
GFRα2	Viable; severe growth retardation; defects in parasympathetic nervous system	295
GFRα3	Viable and fertile; ptosis; widespread defects in the migration of the sympathetic nervous system	303
c-RET (GDNF family receptor)	Neonatal lethal; required for proper kidney development including heterozygotes; lack enteric nervous system	292
MIS type II receptor (AMHR2)	Phenocopy of MIS ligand-deficient mice; males develop uteri; Leydig cell hyperplasia and infertility in males	320
TGF-β receptor type II (TGFBR2)	Phenocopy of lethal TGF-β1-deficient embryos; disrupted hematopoiesis and vasculogenesis in the yolk sac	193
TGF-β ligand or receptor binding proteins or ligand processing proteins		
Biglycan (TGF-β type III receptor)	Viable; slower growth; reduced bone mass similar to osteoporosis	408
Endoglin	Embryonic lethal at E11.5; defective vascular development	197
FKBP12	Majority of mice die between E14.5 and birth due to cardiomyopathy and neural tube defects	90
Follistatin	Perinatal lethal; shiny skin; craniofacial defects, rib defects	216
Noggin	Perinatal lethal; cartilage hyperplasia; abnormal joint formation	279
Subtilin-like proprotein convertase 1 (SPC1 or Furin)	Embryonic lethal at E10.5 to E11.5; defective vascular development; severe ventral closure defects; failure of heart tube to fuse; abnormal embryonic turning; phenotype similar to *Smad5* mutant mice suggesting that it may function in BMP processing	410
SPC4/PACE4	Homozygous viable and non-Mendelian frequency; ~25% lethality during embryogenesis due to anterior CNS patterning and L/R axis formation defects (*i.e.*, situs ambiguous with left pulmonary isomerism and/or craniofacial malformations including cyclopia); role in Nodal processing and activation of BMPs	409
Thrombospondin 1 (TSP-1 or *Thbs1*)	Viable; increased monocytes and eosinophils; older mice (4 wk) develop extensive acute and organizing pneumonia	84, 411
SMAD proteins, SMAD-interacting proteins, or specific downstream targets		
SKI	Perinatal lethality; craniofacial defects including exencephaly and midline facial clefts, eye abnormalities including iris defects, postaxial polydactyly, and skeletal muscle decrease; heterozygotes have increased tumor susceptibility	131–133
SMAD1	Embryonic lethality; death prior to E10.5 due to extraembryonic (allantois formation defect and chorion overproliferation) defects; dramatically reduced or absent PGCs	151, 152
SMAD2	Embryonic lethal; no embryonic mesoderm formation; lack anteroposterior axis; haploinsufficient	145, 412, 413
SMAD3	Viable; reduction in body size; reduced litter size from homozygous matings; mice develop metastatic colorectal tumors; defective immune response	414–417
SMAD4 (DPC4)	Embryonic lethal; defective visceral endoderm leading to gastrulation defect	418, 419

TABLE 3. Continued

Knockout mouse model	Phenotype	Refs.
SMAD5	Embryonic lethal; allantois shortening; defective vascular development, ventral closure, cardiac development and craniofacial development; abnormal heart looping and embryonic turning; defective PGC development	149, 150, 172, 337
SMAD6	Partial postnatal lethal; defective cardiovascular development including hyperplasia of the cardiac valves and outflow tract septation defects; aortic ossification and elevated blood pressure in viable mutants	173
FoxH1 or FAST2	Embryonic lethality; death prior to E10.5 due to failure to pattern the anterior primitive streak and form the node, prechordal mesoderm, notochord, and definitive endoderm; Smad DNA-binding partner that responds to activin and Nodal signaling	112
PTX3	Viable; female subfertility due to periovulatory cumulus-oocyte complex defects; downstream target of GDF-9 and BMP-15 signaling pathways	363
p300	Embryonic lethality; midgestation defects in neurulation, cell proliferation, yolk sac vasculature, and heart development; heterozygotes also exhibited embryonic lethality	110
CBP	Embryonic lethality; neural tube, hematopoiesis and yolk sac vasculature defects; hematologic malignancies in a few percent of heterozygotes	109, 420

[a] *Short ear* and *brachypodism* are induced mutations that have been identified as mutations in the mouse Bmp5 and Gdf5 genes, respectively; *FecX*^{I}, *Fecx*^{H}, and *FecB* are spontaneous mutations that arose in sheep.
[b] The 413-d mutant is a retrovirally induced recessive lethal mutation in the Nodal gene.

null mutation are arrested at early gastrulation with abnormal visceral endoderm morphology and severe disruption of mesoderm formation (138, 139). In Alk3 null or *Alk4* null embryos, the formation of the primitive streak and mesodermal cells is completely blocked. Although ALK3 probably functions as the type I receptor for BMP-4 to regulate epiblast cell proliferation and mesoderm formation, ALK4 appears to mediate a Nodal or an activin-like signal that is essential for egg cylinder organization and primitive streak formation.

BMPRII transduces signals for BMPs through heterodimerization with ALK2, ALK3, or ALK6. *Bmpr2* null embryos are arrested at the egg cylinder stage before gastrulation (140), very similar to *Alk3* null embryos (141). This suggests that the essential BMP signaling pathway in this early developmental period involves heterodimerization of BMPRII and ALK3.

Several other key TGF-β family signal mediators, including ALK4 (ActRIB), SMAD2, and SMAD4, have been shown to function in extraembryonic cells during early mouse development. Although ALK4 (ActRIB) and SMAD2 function in both epiblast and extraembryonic cells, their roles in the extraembryonic cells are required for the formation of the embryonic rather than the extraembryonic mesoderm. Similar to ALK2/ActRIA, SMAD4 is required for the differentiation and function of the visceral endoderm, and inactivation of SMAD4 in the extraembryonic cells blocks embryonic growth and mesoderm formation. It is possible that ALK2/ActRIA and SMAD4 function in the same signaling pathway during early mouse development.

Genetic studies have shown that embryos lacking either *Acvr2* (142) or *Acvr2b* (143) develop to term with no gross defects in mesoderm formation. However, mutant embryos homozygous for both *Acvr2* and *Acvr2b* are arrested at the egg cylinder stage and do not form mesoderm (144), demonstrating functional compensation between these two receptors. Furthermore, *Acvr2*$^{-/-}$ *Acvr2b*$^{+/-}$ embryos fail to form an elongated primitive streak, causing disruption of the mesoderm formation. These gastrulation defects are similar to the defects in *Nodal*$^{+/-}$ *Acvr2*$^{-/-}$ (144) and *Nodal*$^{+/-}$ *Smad2*$^{+/-}$ (145) double mutants, suggesting that Nodal, the type II activin receptors, and *Smad2* function in the same genetic pathway to regulate mouse gastrulation (144). Other craniofacial findings in these mutant mice will be discussed below.

In addition to the above defects, BMP signaling is also important for extraembryonic development. In mice lacking *Bmp2*, *Bmp4*, or *Bmp8b*, there are extraembryonic defects. *Bmp2* null embryos demonstrate shortened and delayed allantois development, respectively (146, 147). About 50% of the *Bmp2* null embryos fail to undergo normal chorioallantoic fusion. In contrast, *Bmp4* null embryos completely lack an allantois. Lastly, *Bmp5/Bmp7* double mutant embryos demonstrate developmentally delayed and smaller allantois and also demonstrate failure of the allantois to fuse with the chorion in most cases (148). Knockouts of *Smad1* and *Smad5*, which encode proteins downstream of BMP stimulation, also have defects in extraembryonic tissue development. Both *Smad1* and *Smad5* knockout mice die at midgestation before E10.5 (149–152). Both models have defects in allantois formation, this being more dramatic in the *Smad1* knockout mice. These findings suggest that there is redundancy of these two SMADs in allantois development (see *Section IV.B*). In *Smad5* knockout mice, there is mislocation of allantois tissue that is also found in the amnion (149). In the *Smad1* knockout, there are defects in fusion of the allantois and chorion (similar to the *Bmp2* null embryos and the *Bmp5/Bmp7* double mutant embryos) and overproliferation of the chorion (151, 152). Thus, BMP-2, BMP-4, BMP-5, BMP-7, and BMP-8b signaling through SMAD1 and SMAD5 appear to play important functions in the integrity of the allantois and chorion.

In summary, the TGF-β superfamily is essential for several important steps in early postimplantation development, including organization and growth of the egg cylinder, development of the extraembryonic membranes, formation of the primitive streak, and differentiation of the mesoderm. One recent report suggests BMP-2 signal is important for the spacing of embryo implantation (153). Functional importance of TGF-β superfamily signaling in preimplantation embryo development is less clear.

B. Heart development

During mouse embryogenesis, the heart is the first organ to differentiate and function (154). The heart initially appears as two cardiac primordia that then fuse together to form a linear heart tube consisting of an inner endothelium surrounded by an outer layer of myocardium. Between these two layers lies a complex layer of extracellular matrix known as cardiac jelly secreted mainly by the myocardium. At later developmental stages, loops and turns break the symmetry of the linear heart tube. In addition, endocardial cells respond to signals from the overlying myocardium and undergo an epithelial-to-mesenchymal transformation to invade the intervening extracellular matrix and form the cardiac cushion. The mature heart valves and septa derived from the cardiac cushions ultimately divide the heart into four functional chambers (154). For more details on heart development and congenital heart diseases, please refer to a recent review by Harvey (155).

Genetic studies have shown that TGF-β superfamily signaling is essential for heart development. BMP-2 is required for the initial formation of cardiac primordium, because *Bmp2* null mice either do not have a heart or develop a very retarded and malformed heart (156). BMPs are also likely to be involved in later septa and valve formations as suggested by their expression patterns. In the midgestation mouse heart, BMP-2 and BMP-4 are expressed in the atrial-ventricular (AV) canal and AV cushion, respectively, whereas BMP-5, -6, and -7 are expressed more homogeneously in the myocardium, and BMP-10 is expressed exclusively in the trabeculae. Gene inactivation analysis indicates functional redundancy among 60A subgroup of BMPs, namely BMP-5, BMP-6, and BMP-7. Heart development is normal in embryos lacking BMP-5 (157), BMP-6 (158), or BMP-7 (159, 160). However, mice carrying mutations in both *Bmp5* and *Bmp7* genes die around E10.5 with multiple defects in heart development, involving the AV cushion, septum, free wall, and trabeculae (148). Mice deficient for both BMP-6 and BMP-7 have delayed cardiac cushion formation in the outflow tract, which results in subsequent valve and septation defects. These embryos die due to cardiac insufficiency (161).

In addition, several TGF-β isoforms may control epithelial-mesenchymal transformation in the AV canal of the heart (162–164). During murine endocardial cushion formation, TGF-β1 is expressed in endothelial/mesenchymal cells, whereas TGF-β2 and TGF-β3 are expressed in the myocardium (162, 165–168). Although TGF-β1 knockout mice do not seem to have any heart abnormalities, TGF-β2 knockout mice have specific defects in the development of valves and septa of the heart (169). In addition, both TGF-β type II and type III (betaglycan) receptors are expressed in AV endothelial cells. At least in avian explants, antibodies against either TGF-β type II or type III receptors inhibit epithelial-mesenchymal transformation and mesenchymal cell migration after transformation (162–164). Further studies on TGF-β ligand signaling can be expected to provide important information for understanding normal heart development and congenital heart diseases.

Two type I receptors, ALK3 and ALK5, have also been shown to be involved in heart development. ALK3, also known as BMP type IA receptor, is essential for mesoderm formation as revealed by conventional knockout mice (141). Conditional loss of ALK3 in midgestation mouse myocardium results in defects in AV cushion formation and subsequent abnormalities in cardiac septa and valves (170). The cardiac defects of ALK3 conditional knockout mice resemble those of *Bmp5*, *Bmp7* double knockout mice (148), consistent with the biochemical data that ALK3 is upstream of BMPs. TGF-β2 seems to be downstream of ALK3 during cardiac cushion formation, as suggested by the finding from ALK3 conditional knockout mice that TGF-β2 expression in the myocardium adjacent to the AV canal is greatly reduced when ALK3 is absent from the myocardium. This finding explains the resemblance of the cardiac defects between TGF-β2-deficient mice and ALK3 conditional knockout mice. It also suggests that a cascade of TGF-β superfamily signaling is required for normal cardiac cushion formation. ALK5, a receptor for TGF-β isoforms, may be required for heart looping, because directed expression of a constitutively active form of ALK5 (L193A, P194A, T204D) in the mouse myocardium arrests heart looping (171).

Both *Smad5* and *Smad6* knockout mice have defects in heart development. *Smad5* knockout mice (172) display defects in heart looping due to abnormalities in left-right axis determination (see *Section III.C*). In contrast to the *Smad5* knockout mice, which die during embryogenesis, the *Smad6* null mice live to birth, but a majority of null pups die before weaning (173). Analysis of the expression of a *LacZ* reporter in the *Smad6* locus reveals high specificity of the reporter in the developing outflow tract and AV cushion of the heart between E9.5 and E13.5, expansion of the expression pattern to the vascular endothelium of larger vessels during late embryogenesis, and a restriction of the expression to the cardiovascular system postnatally. Consistent with this expression pattern and a role of SMAD6 negatively regulating TGF-β signaling during the endocardial cushion transformation, *Smad6* null mice show hyperplasia of the cardiac valves and septal defects in the outflow tracts. *Smad6* null mice that live to the adult stage display aortic ossification and elevated blood pressure. Thus, SMAD6 inhibitory function is key to normal development of the cardiovascular system.

In summary, heart development is regulated by both BMPs and TGF-β isoforms at multiple developmental stages, including cardiac primordia specification, heart looping (discussed further in *Section III.C*), and cardiac cushion formation, and later, in septum and valve formation. The finding that TGF-β2 is dramatically down-regulated when ALK3 is absent from the heart suggests genetic connections among

signaling pathways of different TGF-β superfamily members (170). Mutations in BMPRII have been found in the congenital heart disease, primary pulmonary hypertension, implying functional conservation of TGF-β signaling in mammals (174). Future studies on conditional knockout mice as well as double knockout mice will provide us with more information on the roles of TGF-β superfamily signaling in cardiac development and congenital heart diseases.

C. Left-right asymmetry

During mouse embryogenesis, morphological asymmetry of the left-right axis first occurs around E8.0, when the embryonic heart tube loops toward the right (154). This is followed by a leftward axial rotation of the embryo at the 9–10 somite stage (154). Several TGF-β family ligands, such as Nodal, lefty-1, and lefty-2, are expressed asymmetrically before or around the appearance of the morphological asymmetry, and these ligands appear to be involved in normal left-right axis formation (175–178).

1. Nodal. Expression of *Nodal* is first detected at E5.5 in the primitive ectoderm (136). At gastrulation, the highest level of Nodal expression is maintained at the posterior region of the epiblast and marks the site of future primitive streak formation. *Nodal* is also expressed transiently in the visceral endoderm before and during early streak formation (136). The roles of Nodal in primitive streak formation and subsequent mesoderm differentiation during mouse embryogenesis are confirmed by the lack of primitive streak development in mice deficient in *Nodal* (136, 179, 180).

Besides its early roles in primitive streak formation, Nodal is also involved in left-right asymmetry establishment. At E7.5, *Nodal* is expressed in cells around the node, and this expression becomes asymmetric at E8.0, with the left side being greater. When the embryo develops three to five pairs of somites (E8.0), *Nodal* is expressed on the left side of the lateral plate mesoderm (LPM), but not on the right side (2). This asymmetric expression of *Nodal* precedes the appearance of morphological asymmetry. In mouse strains carrying mutations causing defects in left-right asymmetry, the expression pattern of *Nodal* is changed according to the direction of heart looping and embryonic turning. For example, in *iv* (inversus viscera) mice, heart looping and embryonic turning are randomized, and the expression of *Nodal* in the LPM is also randomized (*i.e.*, Nodal is expressed on the left side, the right side, or bilaterally, or it is absent). In *inv* (inversion of embryonic turning) mice, in which situs is completely inversed, *Nodal* is expressed only on the right side of the LPM (2, 181). In addition, ectopic expression of *Nodal* on the right side of the LPM in chicken embryos randomizes the direction of heart looping (182). Furthermore, genetic evidence also reveals roles for Nodal in left-right asymmetry establishment. Mice heterozygous mutant for both *Nodal* and *Smad2* develop defects in left-right asymmetry, including transposition of the great arteries, right pulmonary isomerism, and right-sided stomach (145).

2. Lefty-1 and lefty-2. Lefty-1 and lefty-2 are two divergent members of the TGF-β superfamily with highest homology to each other (183). Their names are based on their embryonic expression patterns. *Lefty-1* is expressed strongly on the left side of the putative ventral floor plate (PFP) and weakly on the left side of the LPM at E8.0 to E8.5 (183). *Lefty-2* is expressed strongly on the left side of the LPM and weakly on the left side of the PFP at E8.0 to E8.5 (183). *Nodal* and *lefty-2* expression patterns in knockout mice lacking lefty-1 are normal at early somite stages (three to five pairs of somites) but become bilateral at later developmental stages (six to eight pairs of somites). In accordance with these expression patterns, earlier events in left-right asymmetry, such as heart looping and embryonic turning, are normal in *lefty-1* knockout mice, whereas later events, such as the position and differentiation of visceral organs, are abnormal (184). The most common defect in *lefty-1* knockout mice is pulmonary left isomerism in which both lungs of the mutant mice have only one lobe in contrast to wild-type mice in which the left lung has one lobe and the right lung has four lobes (184).

Two conclusions can be drawn from these studies. First, left-right asymmetry is established sequentially. Early events (such as embryonic turning and heart looping) are related to an early phase of *Nodal* and *lefty-2* expression, whereas later events (such as the differentiation of the visceral organs) are related to a late phase of *Nodal* and *lefty-2* expression. Second, lefty-1 functions as a negative regulator of late phase *Nodal* and *lefty-2* expression, but not the early phase of *Nodal* and *lefty-2* expression.

The effect of lefty-2 on left-right axis formation cannot be evaluated because lefty-2 has an essential role in primitive streak formation, and *lefty-2* knockout mice fail to develop to a stage in which defects in the left-right axis can be observed (137). Analysis of conditional knockout mice lacking lefty-2 at later developmental stages may provide direct evidence for the role of lefty-2 in left-right axis formation.

3. GDF-1. In contrast to Nodal, lefty-1, and lefty-2, *Gdf1* is expressed symmetrically around the time of left-right asymmetry establishment (185). *Gdf1* is initially expressed throughout the embryo proper at E7.5 and later becomes restricted to cells around the node, cells in the ventral neural tube, and cells in the proximal and LPM (185). In late-stage embryos and adult mice, the expression of *Gdf1* is restricted to the nervous system (186). A minority of knockout mice lacking GDF-1 die between E14.5 and birth. About two thirds of the mutant mice live to birth and die within 48 h of severe cardiac defects. A wide range of left-right axis defects are obvious in *Gdf1* knockout mice, including visceral situs inversus, right pulmonary isomerism, and cardiac anomalies (185). Furthermore, the absence of *lefty-1*, *Nodal*, and *lefty-2* expression in the PFP and LPM of *Gdf1* knockout mice suggests that GDF-1 is an upstream factor required for the asymmetric expression of these genes (185). The receptors and SMADs through which GDF-1 signals are unknown at this time.

4. Other components. Although several TGF-β superfamily ligands have been implicated in left-right axis formation in mice, little is known about their receptors and downstream SMADs. ACVR2B is the only receptor shown to be essential for left-right asymmetry. *Acvr2b* knockout mice develop pulmonary right isomerism and other defects related to left-

right axis formation (143), similar to *Nodal/Smad2* double heterozygous mutants (145). Although activins A and B bind to ACVR2B *in vitro*, the physiological ligands that bind to ACVR2B and regulate left-right axis development in mammals are not known.

Direct evidence for the involvement of SMAD2 in left-right asymmetry is lacking because mice deficient in *Smad2* fail to initiate gastrulation. However, mice double heterozygous for both *Nodal* and *Smad2* mutations develop variable mutant phenotypes, including gastrulation defects, complex craniofacial abnormalities (see *Section III.E*), and defects in left-right patterning. These data indicate that SMAD2 may mediate Nodal signaling in these developmental processes (145).

Smad5 knockout mice have shown that a signaling pathway through SMAD5 is involved in left-right asymmetry (172). At early stages, the mutant embryos fail to initiate or complete turning properly, a nearly completely penetrant phenotype that is obvious at E9.5. In addition, there are defects in heart looping in these mutants. In wild-type embryos at E9.5, the rostral part of the heart loops toward the right, whereas the caudal part twists to the left. In the *Smad5*$^{-/-}$ embryos, the rostral part of the heart either fails to loop (6 of 17), loops randomly (9 of 17), or elongates and fails to show any looping. At the molecular level, *lefty-1* was essentially undetectable, and *Nodal*, *lefty-2*, and *Pitx2* were expressed bilaterally. This suggests that a SMAD5 signaling cascade is upstream of these genes and is essential for left-right axis determination.

D. Vasculogenesis and angiogenesis

Vasculogenesis and angiogenesis are two processes leading to the formation of new blood vessels (187). Vasculogenesis refers to the primary *in situ* differentiation of endothelial precursor cells from mesoderm and their subsequent organization into a primary capillary plexus. Angiogenesis is the formation of new vessels from the preexisting vessel plexus through splitting and sprouting (187, 188). During embryogenesis, vasculogenesis first occurs in the extraembryonic yolk sac when blood islands differentiate from the extraembryonic mesodermal cells. Blood islands contain hemangioblasts capable of forming both endothelial cells and hematopoietic cells. A primary blood plexus is formed in the yolk sac and then connects with the primary blood plexus in the embryo proper. The earliest circulatory system is established when the embryonic heart starts to beat (154). Primary embryonic vasculature is remodeled at later developmental stages through vasculogenesis and angiogenesis.

TGF-β signaling pathways play important roles in vasculogenesis and angiogenesis (Fig. 3; Ref. 189). During mouse embryogenesis, TGF-β1 is expressed in many tissues, including endothelial and hematopoietic precursor cells (165). Targeted disruption of TGF-β1 in mice results in midgestation lethality in half of the homozygotes and about a quarter of the heterozygotes (167, 190–192). The primary causes of death are defects in the yolk sac vasculature and hematopoietic system. Although initial differentiation of mesodermal precursors into endothelial cells occurs, subsequent differentiation of endothelial cells into capillary-like tubules is defective, resulting in vessels with decreased wall integrity.

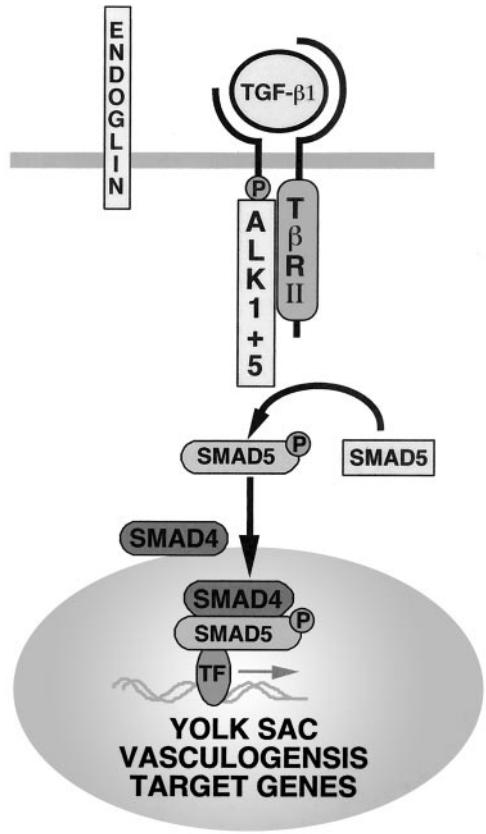

FIG. 3. TGF-β superfamily signaling in the yolk sac. Vasculogenesis in the yolk sac requires a unique TGF-β superfamily signal transduction pathway, as demonstrated by knockout mice. Endoglin, TGF-β1, TβRII, ALK1, ALK5, and SMAD5 have been shown to play a role in this pathway. Mice with a knockout of these signaling components display a similar defect in the development of the vascular network in the yolk sac and will subsequently die at midgestation. Some TGF-β1 mutant mice will bypass this block, perhaps due to compensation by TGF-β2 or TGF-β3.

TβRII-deficient mice have also been generated and demonstrate a similar mutant phenotype (193).

The decreased vessel wall integrity in mutant mice deficient in TGF-β1 or TβRII is strikingly similar to the vascular lesions that occurred in human patients with hereditary hemorrhagic telangiectasia. The earliest detectable change in the telangiectatic lesions is dilation of postcapillary venules in the upper dermis (194). Two genes have been cloned that cause hereditary hemorrhagic telangiectasia, and one of these genes encodes endoglin, a type III receptor for TGF-β (38, 195, 196). The other gene is *Alk1*, which encodes a type I receptor for TGF-β ligands. Endoglin can bind to TGF-βs (195) and is likely involved in TGF-β1 signaling *in vivo* because endoglin knockout mice die at midgestation due to defective vascular development (197), similar to half of the TGF-β1 knockout mice (167) and mice lacking TGF-β type II receptor (193). The endogenous ligands of ALK1 remain to be identified. Consistent with functions in vascular development, mice lacking endoglin or ALK1 die from defective angiogenesis at midgestation (197–199). ALK1 is also required for establishing distinctions between arteries and veins during early vascular development (199).

SMAD5, originally thought to be a signal transducer for BMPs, appears to mediate TGF-β1 signaling through ALK1 and TβRII. ALK1 can interact with TβRII or ActRII and phosphorylate SMAD1 and SMAD5 (200, 201). Constitutively active ALK1/Q201D can phosphorylate SMAD1 and SMAD5 but not SMAD2 and SMAD3 (198), suggesting that ALK1 transduces signals through SMAD1 and/or SMAD5. ALK1, however, is not the only type I receptor for TGF-β1 signaling during vascular development.

Consistent with the biochemical data that SMAD5 is downstream of ALK1, vascular lesions in *Smad5* knockout mice highly resemble those in TGF-β1, TβRII, and ALK1 knockout mice (149, 150). *Smad5* knockout mice die between E9.5 and E11.5. After E9.0, the yolk sacs of *Smad5* mutants contain red blood cells but lack well organized vasculature.

ALK1, however, is not the only type I receptor for TGF-β1 signaling during vascular development. Mice deficient in ALK5 also show severe vascular lesions reminiscent of TGF-β1 knockout mice (202). Endothelial cells derived from *Alk5* null mice show enhanced proliferation, improper migratory behavior, and impaired fibronectin production *in vitro* (202). ALK5 appears to signal through Smad2 in endothelial cells and induces the expression of PAI-1, a known proteinase inhibitor, to prevent degradation of extracellular matrix proteins around nascent vessels (203). Therefore, the possible function of the TGF-β/ALK5 pathway during angiogenesis is to inhibit cell migration and proliferation.

Therefore, in endothelial cells, TGF-β signals through both ALK1 and ALK5, and the two receptors phosphorylate different sets of downstream Smad proteins (ALK1 phosphorylates Smad1 and Smad5, ALK5 phosphorylates Smad2 and Smad3). The TGF-β/ALK5 pathway leads to inhibition of cell migration and proliferation, whereas the TGF-β/ALK1 pathway induces endothelial cell migration and proliferation (203). The activation state of each pathway is determined by the dose of TGF-β present (203). The fate of the endothelium may thus be dependent on the balance of ALK1 *vs.* ALK5 activation. These genetic studies solved a puzzle in the field for many years, namely, how TGF-β can function as both an inhibitor and a promoter of angiogenesis.

In summary, TGF-β signaling is essential for vascular development. A genetic pathway composed of TGF-β1, endoglin, TβRII, ALK1, ALK5, and SMAD5 (Fig. 3) determines several important aspects of angiogenesis, including maintenance of the integrity of vessel walls, recruitment of smooth muscle cells, deposition of extracellular matrix, as well as differentiation of arteries and veins.

E. Craniofacial development

1. Cleft palate. A variety of craniofacial malformations have been associated with mutations in TGF-β superfamily members. Isolated cleft palate is a relatively common human birth defect that is generally remedied by surgical correction within the first few months to years of life, depending on its severity. It is considered a complex trait in humans, and presumably the malformation reflects deleterious effects on multiple gene products. This is in contrast to many mouse models in which the knockout of a single gene is sufficient to cause clefting in 100% of homozygous null mutants (204–206). Palate formation is a complex process that requires the growth, elevation, and fusion of palatal shelves, followed by a loss of the midline epithelial seam and subsequent differentiation of overlying and adjacent mesenchymal cells (207). During embryogenesis, many TGF-β superfamily members, their receptors and their associated regulatory proteins are expressed in the developing palate (Refs. 208–212 and Fig. 4). TGF-β1, TGF-β2, and TGF-β3 are all expressed in the palate, but their spatiotemporal expression patterns differ. All are thought to contribute to palate shelf elongation and fusion. At E13.5, TGF-β3 expression is restricted to the medial edge epithelium (MEE) as the palatal shelves move from a vertical to horizontal position. One day later, TGF-β1 and TGF-β3 are expressed in the MEE, whereas TGF-β2 expression is restricted to mesenchymal cells. The type I TGF-β receptor (*Tgfbr1*) is expressed in cells comprising the palate, oral, and nasal epithelia, as well as the medial edge seam (212). The type II TGF-β receptor (*Tgfbr2*) is highly expressed in the MEE seam but is only weakly expressed in oral and nasal epithelia (212).

Knockouts of the *Tgfb2* and *Tgfb3* genes have revealed their importance in palate development; however, *Tgfb1* knockout mice demonstrate no palate defects (190, 192), illustrating that expression patterns alone are not reliable predictors of the functional requirement of a gene product in a specific biological process. Twenty-three percent of TGF-β2 knockout mice have complete anteroposterior cleft secondary palate, extending to the soft palate. This defect is due to the failure of palatal shelves to elevate into a horizontal position for apposition and fusion. In contrast, 100% of TGF-β3 knockout mice develop cleft palate of variable severity, and although the palatal shelves reach juxtaposition, they fail to fuse (205, 206). This is likely due to a failure of the cells at the MEE to form normal filopodia. Filopodia are finger-like projections at the cell surface that increase cell surface area. In addition, the filopodia help to maintain the surface concentration of cell adhesion molecules and free cell surface charge energy. Their presence also correlates with cell migration and mitogenic activity. It has been suggested that some or all of these functions may be compromised in TGF-β3 knockout mice (212).

Activin/inhibin βA (*Inhba*) is another member of the TGF-β superfamily that is highly expressed in the developing palate, oral, and nasal mesenchyme (210). Similar to TGF-β2 knockout mice, activin βA null mutants have incomplete penetrance of cleft palate, ranging from 29–33%, depending on the background strain (213). The severity of the palate defect ranges from complete anteroposterior clefting to a membranous hard palate devoid of ossified tissue. Although the precise mechanism for the clefting has not been determined, the ossification failure may reflect important roles for activin βA in facilitating palate chondrogenesis and subsequent ossification, as well as palate fusion (213).

Activin receptor type II (*Acvr2*) null mutants also have cleft palates. However, the clefting is likely secondary to the hypoplastic mandibles that are present in 22% of these mice, although primary effects on palatogenesis cannot be excluded. This phenotype is similar to the Pierre Robin sequence in humans, in which the development of a hypoplastic mandible results in a posterior and superior

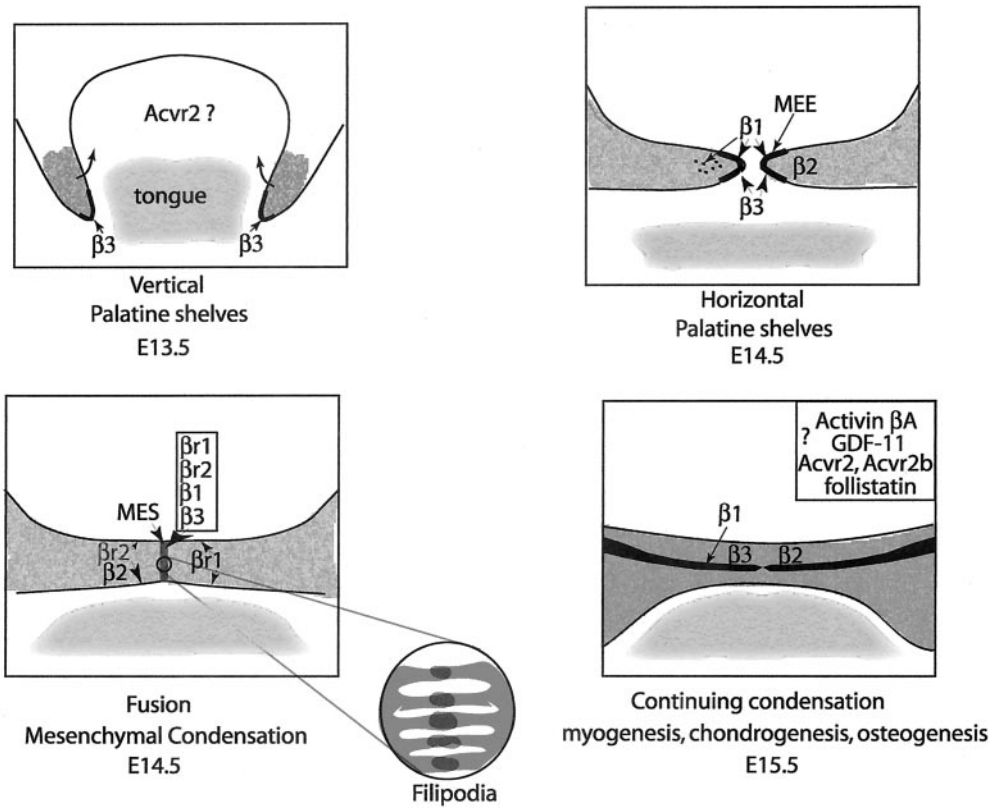

FIG. 4. TGF-β superfamily and palate development. The developing palatal shelves undergo a variety of morphological changes as they progress through phases of growth, elevation from a vertical to horizontal position, elongation toward the midline, and fusion, forming the medial epithelial seam (MES). Disappearance of the seam coincides with mesenchymal condensation and the completion of palate differentiation. Several TGF-β superfamily members participate at various stages and, in some cases, are restricted to specific cell populations (*e.g.*, surface or MEE, or mesenchyme). Intact TGF-β signaling is essential to maintain many of these processes. Filipodia, foot-like processes at the junction of the MES, aid in palate fusion, as demonstrated in TGF-β3 knockout mice whose palates fail to fuse in the absence of these structures. Concomitantly, mesenchymal condensation and myogenesis, chondrogenesis, and osteogenesis lead to the formation of the fully differentiated palate. Activin βA, GDF-11, their receptors, and follistatin also play important roles in these processes. β1, TGF-β1; β2, TGF-β2; β3, TGF-β3; βr1, TGF-β receptor type I; βr2, TGF-β receptor type II.

positioning of the tongue during craniofacial development. This results in a mechanical obstruction to palate fusion. Like the *Acvr2* null mutant mouse, the severity of the Pierre Robin sequence ranges from relatively mild retrognathia (*i.e.*, posteriorly placed mandible) with or without clefting, to severe micrognathia (*i.e.*, small, hypoplastic mandible) and vestigial tongue and airway compromise (214). In contrast to the *Acvr2* null mutant mouse, however, the Pierre Robin sequence occurs sporadically.

Follistatin was initially described as an activin binding protein (215); however, on the basis of studies from follistatin null mutant mice (216), transgenic mice overexpressing the protein (217), and studies in *Xenopus* embryos (77, 218), it is clear that follistatin modulates the effects of other TGF-β superfamily members as well (218). Follistatin homozygous null mutant mice die in the perinatal period, and some of the craniofacial features of these mice are strikingly similar to those of the activin βA knockout mouse (216). Twenty-one percent of follistatin null mutant mice lack a hard palate, consistent with its role in modulating the effects of activin signaling.

In addition to skeletal patterning defects, *Gdf11* null mutant mice have cleft palates, consistent with *Gdf11* expression pattern during embryogenesis (219, 220). Some functions of GDF-11 are hypothesized to be transmitted through the activin receptor, ACVR2B (see *Section III.F*). In summary, several TGF-β superfamily members participate in the process of palatogenesis. The TGF-βs, activins, GDF-11, TGF-β and activin receptors, and follistatin have all been shown to play a role in mouse models. However, the failure of activin βA to rescue the palate fusion defects of *Tgfb3* null mutant mice (212) suggests that alternative signaling pathways may contribute synergistically to the complex process of palatogenesis. It is also likely that there are major players and minor players in the game, and the relative importance of each is reflected by the penetrance of the null phenotype. This model is consistent with the multifactorial nature of this complex biological process.

2. Tooth development. The development of teeth in mammals is a complex process involving at least five well characterized developmental signaling pathways [BMP, activin A, fibroblast growth factor (FGF), Hedgehog, and WNT]. The expression of more than 200 genes has been examined in teeth, and the expression patterns of many of these are now available in a web-based graphical format (221). The process of

tooth development first requires the definition of a tooth region (*i.e.*, it must first be determined where a tooth will grow). Next, tooth identity must be established (*i.e.*, incisor, canine, premolar, or molar in humans; incisor or molar in mice). Both processes are influenced by antagonistic effects of BMP and FGF signaling and rely on reciprocal, temporally regulated signals between the inductive epithelium and its underlying mesenchyme. Also, the same signals are used repeatedly at various stages as tooth identity is established. (For excellent reviews on mammalian tooth morphogenesis, please refer to Refs. 222 and 223.) Within the TGF-β superfamily, both BMP-4 and activin βA have been shown to play important roles in tooth morphogenesis. BMP-4 has been the most extensively studied (224–226). During the determination of the tooth region, BMP-4 inhibits and FGF-8 stimulates the expression of *Pax9* (which encodes a paired box transcription factor) in the mesenchyme (227). PAX9 and other transcription factors are likely required for the progression of tooth morphogenesis beyond the bud stage. BMP-4 has also been shown to play several important roles in determining tooth identity. Exposure of E9–E10 mouse mandibles to Noggin (a BMP antagonist) causes an incisor to molar transformation (225) due to the inability of BMP-4 to inhibit BARX1, a homeobox-containing transcription factor expressed in molar mesenchyme. Expression of mesenchymal BMP-4 (induced by epithelial BMP-4; Refs. 224 and 226) and activin βA (induced by epithelial FGF-8) at E10–11 (228), precede the budding of epithelium at the sites where teeth will form. This observation makes these two TGF-β superfamily members candidate signals for the initiation of tooth bud formation. Activin βA also plays an important role in dental patterning, because homozygous null mutant mice lack incisors and mandibular molars but maintain normal maxillary molar development (228, 229). Comprehensive expression studies of the tooth buds of these mice using *in situ* hybridization show that none of the genes thought to influence the early stages of tooth development (including BMP-4) are affected in the knockout mice. However, follistatin, normally expressed in the tooth epithelium, was absent in the mutants. Interestingly, follistatin null mutants have essentially the same tooth phenotype as activin βA null mutants (216). These observations suggest that activin βA in the tooth mesenchyme normally induces follistatin expression in the overlying epithelium. This induction is possibly mediated through activin type II and IIB receptors, because double heterozygous mutant mice for these receptors have a similar tooth phenotype as activin βA and follistatin knockout mice (228). It has been suggested that follistatin acts as a sink during tooth morphogenesis, removing activin from the local environment, thereby resetting the activin signaling cascade (228).

BMP-4 is absent from the dental mesenchyme of mice with homozygous null mutations for the transcription factors LEF1, MSX1, or PAX9 (224, 230, 231), all of which show arrest of tooth development at the bud stage. However, progression can occur in tooth buds from *Msx1* mutants *in vitro* if BMP-4 is added to the medium (232). Thus, BMP-4 is also a good candidate for a mesenchymal factor that signals the transition from the bud to cap stage. Later, the apoptosis of enamel knots (structures on the apical surface that ultimately give the tooth its rough contour) is associated with BMP-4 expression in the knot cells (233), suggesting that this protein is important at essentially all stages of tooth morphogenesis. In summary, both BMP and activin signaling play important roles in normal tooth development. Furthermore, BMP expression is controlled by a variety of transcription factors that have their effects at the earliest stages of this process.

3. Eye development. Several TGF-β superfamily members are involved in eye development. Mice homozygous for an activin βA null mutation have reduced rod photoreceptor cells in the neural retina, suggesting that activin A promotes progenitors to differentiate into photoreceptors (234). The presence of ALK2 protein in facial sensory organ primordia, including the eye area, also points to a role for activin signaling in the development of eye (235). BMP-4 and BMP-7 seem to be involved in earlier stages of eye development (236, 237). *Bmp4* is expressed strongly in the optic vesicles and weakly in the surrounding mesenchyme and surface ectoderm. In *Bmp4* null mice, lens induction is absent. Exogenous BMP-4 added to optic vesicles in explant cultures rescues the lens induction defect of the *Bmp4* null mice. Interestingly, *Pax6* expression is not affected in *Bmp4* null mice, and *Bmp4* expression is not affected in *Pax6* null mice. These observations suggest that BMP-4 is a critical factor for lens induction and functions in a pathway independent of PAX6. BMP-7 is another TGF-β superfamily ligand required for lens induction. BMP-7 protein is present in the head ectoderm of mouse embryos at the time of lens induction, and when BMP-7 antagonists are added to *in vitro* cultures during the period of lens induction, lens formation is inhibited (237). Furthermore, mice deficient in BMP-7 have eye abnormalities ranging from anophthalmia to microphthalmia (159, 160, 238, 239). *Tgfb2* null mice also have eye defects, including a thin corneal stroma, and hypercellularity of the posterior chamber (169). Histologically, a hamartomatous mix of melanocytes, neuronal cells, and mesenchymal cells with vascular elements is observed. In addition, there is hyperplasia of the inner and outer neuroblastic layers of the retina. It is unclear whether the hypercellularity of the posterior chamber and retina represents aberrant cell proliferation or a failure of apoptotic programs. Nevertheless, these observations suggest an important role for TGF-β2 in normal eye remodeling.

Activin/inhibin βB (*Inhbb*) homozygous null mutant mice have variable defects in eyelid closure that are evident at birth, suggesting a possible role for this activin in facilitating the proliferation and migration of the peridermal cells involved in this process (240, 241). No other craniofacial anomalies are observed in these mice. Because mutations in other genes (*e.g.*, epidermal growth factor, TGF-α, integrins; Refs. 242–245) also result in open-eye phenotypes, it is likely that many genetic determinants acting in concert contribute to the process of eyelid fusion in mice. In summary, the development of most major eye structures (retina, lens, cornea, posterior chamber, and eyelid) is influenced by members of the superfamily, including the activins, BMPs, and TGF-βs. Moreover, at least the BMPs exert their effects through pathways independent of PAX6.

4. Other craniofacial malformations. Holoprosencephaly is the most severe of a spectrum of disorders caused by the ab-

normal establishment of midline craniofacial structures. Aberrant midline division of the embryonic forebrain results in poor distinction of the cerebral hemispheres, which results in severe neurological abnormalities, and, frequently, death. Anatomically, holoprosencephalies have been subclassified into lobar, semilobar, and alobar forms, in increasing order of severity. Abnormalities are not restricted to the forebrain, because the midline facial structures are also affected. The severity of the condition is highly variable and can include dramatic craniofacial features, such as midline fusion of the developing eye structures (cyclopia), union of the olfactory placodes to form a tube-like structure that projects from the forehead (proboscis), or midline clefting of the lip and palate. Defects may also be as subtle as an abnormally formed nasal septum, narrowly spaced eyes (hypotelorism), or a single central incisor (214, 246). Holoprosencephaly is genetically heterogeneous and is observed as a feature in many genetic disorders (214). Conversely, in rare familial cases, the condition does not breed true, (*i.e.*, the same mutation within a family can give rise to the full spectrum of clinical severity).

Within the context of the TGF-β superfamily, holoprosencephaly has been linked to deficiencies in Nodal signaling in humans and in several experimental model systems. $Nodal^{+/-}$, $Smad2^{+/-}$ double heterozygous mice have a wide range of abnormal phenotypes that include gastrulation defects, errors in left-right axis determination, cyclopia, and truncation of the anterior head structure (145). Similar conditions are observed in $Nodal^{+/-}$, $Acvr2^{-/-}$ mice (144). These findings strongly suggest that normal forebrain development and the formation of midline facial structures require an intact Nodal signaling pathway and that the signal is mediated at least in part by the ACVR2. This hypothesis is further supported by the zebrafish mutants, *cyclops*, *squint*, and *one-eyed pinhead*, which all have cyclopia; the mutated genes all encode proteins that are components of the Nodal signaling pathway. Finally, some holoprosencephaly patients have mutations in TGIF (117). This factor can act as a transcriptional corepressor with SMAD2 *in vivo* (114, 247). Mutations in *TGIF* are hypothesized to result in holoprosencephaly by disrupting the delicate balance between transcriptional activation and repression by SMAD2 complexes during forebrain development (117, 248). Because a nearly identical phenotype has been documented in patients with mutations in the human sonic hedgehog (*SHH*) gene, it has been suggested that compromised Nodal signaling disrupts the normal juxtaposition of SHH-expressing cells and developing cells of the forebrain during gastrulation, placing SHH downstream of the Nodal signaling pathway (117). In summary, holoprosencephaly, the extreme manifestation of disrupted midline development, can occur through perturbation of ligands (Nodal), receptors (ACVR2), and downstream components of Nodal signaling (SMAD2, TGIF). Moreover, disruption of normal Nodal signaling has adverse effects on either cells expressing SHH or SHH signaling.

The short ear (*se*) phenotype in the mouse was described over 50 yr ago and can result from a variety of mutations that affect expression of the *Bmp5* gene (157). These mice have very short external ears, a wide cranium, and a short nose, similar to the craniofacial features of a vole. These and the other skeletal anomalies in these mice (see *Section III.F*) are thought to be related to defects in mesenchymal condensation (an important early event in skeletal morphogenesis that is essential for normal skeletal patterning), because mesenchymal condensations in these mice are either missing or are abnormally formed (249, 250). Clinically, ear-patella-short stature syndrome has been proposed as the human counterpart of the mouse short ear phenotype (251), although this has not been proven at a molecular level.

In addition to cleft palate, *Tgfb2* null mutant mice have other craniofacial anomalies that include abnormally large fontanels (spaces that occur between the skull bones before fusion), defects in bone growth of the occipital region of the skull, and variable mandibular defects (169). These features are consistent with the proposed roles of TGF-β2 in osteoblast differentiation and in osteoblast and osteoclast functioning (252). Other skeletal malformations are observed in some of the aforementioned knockout mice and will be discussed in more detail below.

F. Skeletal morphogenesis

Skeletal morphogenesis requires normal skeletal patterning, cell differentiation, and cell function. Skeletal patterning is broadly defined as the specification of the number, shape, position, and arrangement of skeletal elements. Chondrocytes, osteoblasts, and osteoclasts are the three major cell types that comprise the skeleton. The differentiation of these cell types and the maintenance of their normal function are critical for both the construction and the maintenance of the functional integrity of the skeleton. (For an excellent review on the genetics of skeletogenesis, please see Ref. 253).

TGF-β superfamily members participate in skeletal morphogenesis and can induce ectopic formation of bone *in vivo* and/or stimulate osteogenic transformation of cells *in vitro* (254–257). Many mouse models have been described with skeletal defects resulting from mutations of TGF-β superfamily members, their receptors and binding proteins, alone or in combination (Fig. 5). Although most play important roles in skeletal patterning, recent studies have shown that some affect cell differentiation and/or function.

1. TGF-β superfamily ligands. The BMPs and GDFs have been extensively studied for their roles in skeletal morphogenesis. Similar to short ear (*Bmp5*) mutants, brachypodism (*bp*) is a spontaneously occurring mouse mutation described approximately 50 yr ago (258, 259). The phenotype was later attributed to mutations in the *Gdf5* gene (260). GDF-5 is expressed in a dynamic fashion in developing limbs and is capable of inducing the formation of cartilage, bone, tendon, and ligament when implanted sc (261–264). The *bp* mouse exhibits shortening of several long bones and abnormal joint formation within the limbs and sternum, including absence of some distal interphalangeal joints; this is consistent with an important role for GDF-5 in promoting cartilage formation and normal joint development. Results from ectopic expression and functional inactivation studies suggest that GDF-5 is important for regional specification, support of chondrogenesis, and restricting the expression of joint-specific gene products (265). A subset of these functions is likely carried out by signaling through the BMP type IB (ALK6) receptor (266).

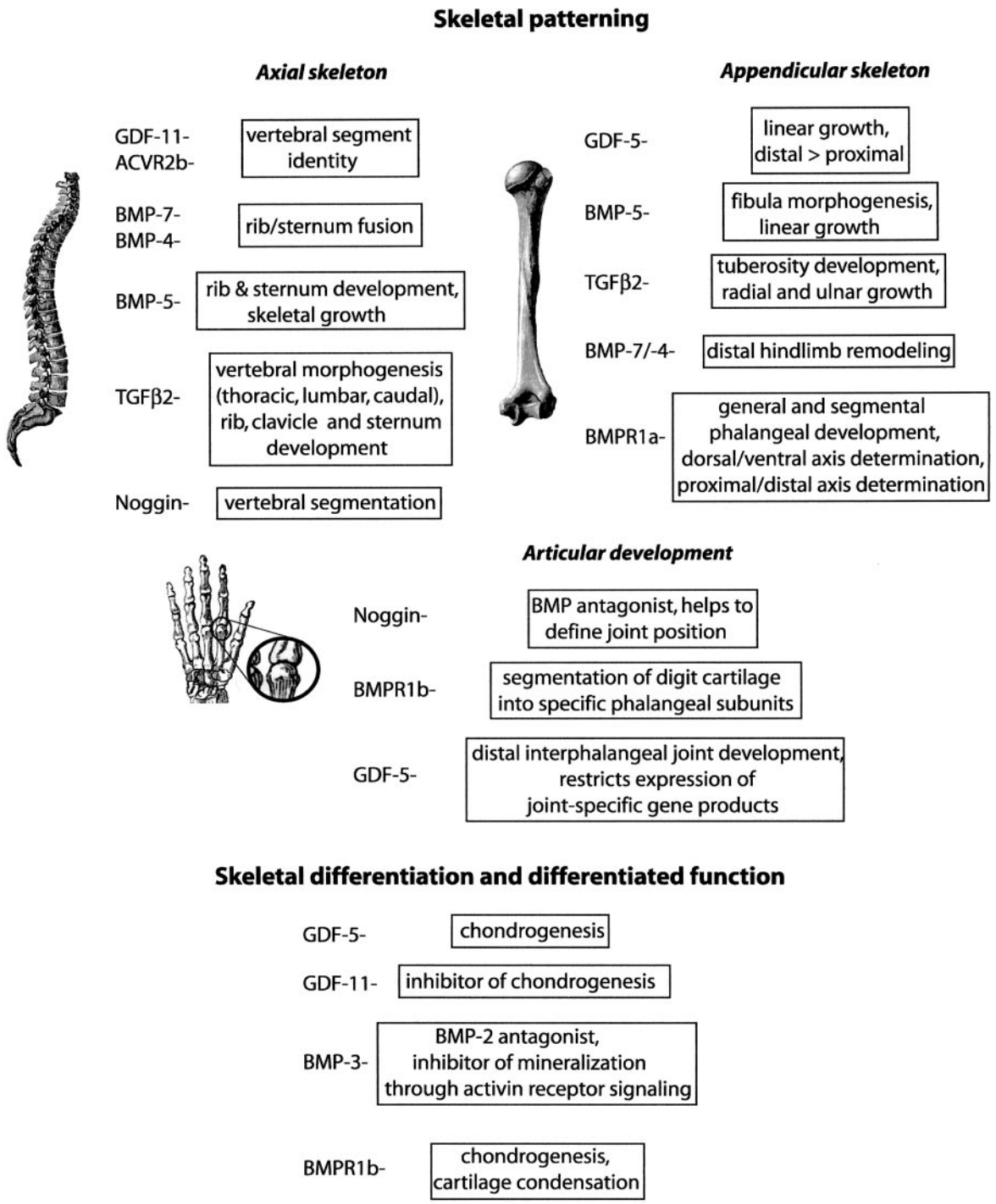

FIG. 5. TGF-β superfamily in skeletal morphogenesis. Skeletal patterning, differentiation, and function are all affected by members of the TGF-β superfamily, their receptors, and binding proteins. Mutations in genes encoding these proteins result in a variety of skeletal malformations including complete absence of skeletal structures, formation of additional skeletal elements, failure of segmentation of a structure, undergrowth, or abnormal morphology. Combinatorial signaling (*i.e.*, one receptor binds to more than one ligand and one ligand binds to more than one receptor) is an important paradigm for TGF-β superfamily signaling in skeletal morphogenesis.

Mutations in the human ortholog of *Gdf5* (also called cartilage-derived morphogenetic protein or CDMP1) lead to at least three different skeletal dysplasias that have provided considerable insight into the mechanisms by which mutations in TGF-β superfamily members might contribute to human diseases. Brachydactyly type C (267), Hunter-Thompson acromesomelic chondrodysplasia (HTC; Ref. 268), and Grebe chondrodysplasia (269) are allelic conditions that have in common a shortening of the extremities that is more severe distally than proximally. Brachydactyly type C is an autosomal dominant condition that results in modest shortening of the phalanges due to underdeveloped middle

phalangeal segments. This is caused by a mutation that introduces a premature termination codon within the mature domain of the *CDMP1* gene (270). HTC is an autosomal recessive chondrodysplasia that causes a relatively severe malformation of the extremities. The bone length and joint defects are more severe in the hands, feet, ankles, and wrists, but the knee joints are also affected. The tibiae, fibulae, radii, and ulnae are short, more so in their distal regions. The humeri are relatively spared. Mutations in the *CDMP1* gene result in truncation of the *CDMP1* protein, thereby producing a null allele, similar to the *bp* mouse (271). Grebe chondrodysplasia is interesting in that it, too, is an autosomal recessive condition caused by a missense mutation in the *CDMP1* gene that results in a single amino acid substitution in the CDMP1 protein. However, the phenotype is more severe in Grebe chondrodysplasia, despite the complete absence of *CDMP1* protein in HTC. Patients with Grebe chondrodysplasia have an amino acid substitution in the *CDMP1* protein that causes impaired secretion of *CDMP1* protein from cells (272). In addition, the mutation results in the heterodimerization of the mutated *CDMP1* proteins with coexpressed TGF-β superfamily members (BMP-2, BMP-3, and BMP-7) thereby impeding their release from the cell (272). Thus, when considering how mutations of TGF-β superfamily members might contribute to human disease, it is important to understand the cellular context of the mutation (*i.e.*, the possibility that other superfamily members within the same cell will also be affected).

Although activin βA and activin βB homozygous knockout mice have no apparent appendicular or axial skeletal malformations, there is compelling evidence to suggest that the activins play important roles in digit formation. Activin βA expression has been demonstrated in the developing joints of both mouse and chick embryos (210, 273). When beads soaked in activin A, B, or βAβB heterodimers are implanted into the interdigital regions of developing chick limbs, ectopic digit formation occurs (273). Conversely, administration of exogenous follistatin, an activin antagonist, blocks both normal and activin-induced digit formation (273). Thus, absence of a limb phenotype in activin βA/βB double knockout mice is likely due to functional redundancy provided by closely related members of the superfamily. The activins have been shown to exert their effects on digit formation by inducing BMP receptor type IB, rendering the digit anlagen responsive to the effects of BMPs and other superfamily ligands (273).

Bmp7 homozygous null mutant mice exhibit asymmetric microphthalmia or anophthalmia (see *Section III.E*), dysplastic kidneys, hydroureters, and skeletal patterning defects. The skeletal malformations are mild, manifesting as preaxial hindlimb polydactyly (extra digits on the medial aspect of the paw) in 25% of homozygous mutants and failure of the seventh rib pairs to fuse to the sternum in 50% of the mutants (159, 160, 238). Interestingly, double heterozygous mutants for *Bmp7* and *Bmp4* have a similar skeletal phenotype as *Bmp7* null mutants, with no obvious defects in eye and kidney development (274), whereas *Bmp7*, *Bmp2* and *Bmp7*, *Bmp5* double heterozygous mutant mice have no obvious defects. These results suggest that BMP-4 and BMP-7 might be acting in the same signaling pathways during skeletal development, that this process is more sensitive to the dosage of BMPs than eye and kidney development, and that *Bmp4* and *Bmp7* are attractive candidate genes in human conditions that have both preaxial polydactyly and rib anomalies.

In addition to the craniofacial features described above, the *Bmp5* mouse has missing ribs, a malformed xiphoid process, and a generalized deficiency of skeletal growth (249). Double mutant studies with *Bmp5* and *Gdf5* mutant mice suggest that BMP-5 also plays an important role in the morphogenesis of other sternal segments and the fibula (263). The skeletal abnormalities are thought to be due to delayed formations of mesenchymal condensations that precede development of mature skeletal elements (275). Consistent with this contention, *Bmp5, Bmp7* double homozygous mutant mice die at E10.5 from multiple defects that reflect deficiencies in the proliferation and maintenance of cell populations in which these proteins are coexpressed (148).

Tgfb2 null mutants have multiple congenital anomalies that affect the development of the heart, lungs, eyes, urogenital system, and skeleton (169). In addition to the craniofacial manifestations described in *Section III.D*, mice lacking TGF-β2 have joint laxity, absent tuberosities from the humeri and femora, short radii and ulnae, structural defects extending from thoracic to caudal vertebrae, and rib, sternum, and clavicle defects (169). Both endochondral and intramembranous ossifications are abnormal, and both the size and the morphology of individual skeletal elements are affected. These observations indicate that TGF-β2 participates in many of the steps of skeletal morphogenesis. Although the differentiation of all skeletal cell types occurs, the size deficiency and abnormal morphology of specific skeletal elements is consistent with important roles for TGF-β2 in directing mesenchymal condensation and normal skeletal patterning.

Gdf11 is an intriguing member of the superfamily because it encodes the first known secreted growth factor to affect skeletal segment identity. *Gdf11* homozygous null mice have an abnormality in the number of vertebral segments such that the number of thoracic vertebrae and associated ribs increases from 13 (normal) to 18 (219). Similarly, the number of lumbar vertebrae increases from six to between seven and nine. The sacral and caudal vertebrae fuse, resulting in mice with short and stubby or absent tails. The total number of somites is normal in *Gdf11* knockout mice, suggesting that the phenotype is due to a true homeotic transformation (*i.e.*, a change from the normal segment identity rather than the formation of additional skeletal elements). The function of GDF-11 has been linked to well known pathways of segment identity by confirming the segmental misexpression of *Hoxc6*, *Hoxc8*, *Hoxc10*, and *Hoxc11* in the *Gdf11* mutants (219). Interestingly, heterozygous mutants also exhibit homeotic transformations that are milder than those of the homozygotes, reflecting a dosage sensitivity to GDF-11 signaling.

In addition to its roles in vertebrae development, GDF-11 is potentially an important regulator of myogenesis and chondrogenesis, as demonstrated by bead implantation studies in the developing chick limb (276). Implantation of GDF-11-soaked beads results in shortening and widening of all limb structures due to negative effects on myogenic and chondrogenic cell differentiation. Hoxd11, Hoxd13, and fol-

listatin were all induced by GDF-11, suggesting that GDF-11 plays important roles in regulating late distal *Hox* gene expression, as well as the expression of the antagonist follistatin.

Bmp6 null mutant mice have a very mild skeletal phenotype involving delayed ossification of the sternum (158). It is likely that compensation by other members of the superfamily limits deleterious effects of functional loss of the BMP-6 protein.

Bmp3 is one of two examples of a TGF-β superfamily member that has an effect on differentiated skeletal function *in vivo*. Originally purified from bone and named osteogenin, it was described for its osteoinductive properties. Recombinant BMP-3 protein, however, does not have this activity. Considerable insight into the functions of BMP-3 was provided by recent *in vitro* experiments and by the knockout mouse (277). BMP-3 is the only example thus far of a BMP that antagonizes the activities of osteogenic BMPs. Its antagonism of BMP-2 was confirmed in several *in vitro* assays and was shown to be mediated by signaling through activin type II receptors (277). *Bmp3* homozygous null mutant mice have an increased bone density, approximately twice that of normal controls (277). Similarly, an increased cortical thickness associated with progressive sclerosis of bone is observed in the autosomal dominant human condition, Camurati Engelmann disease. This disease is caused by heterozygous missense mutations (C225R, R218H, R218C) within the LAP region of the TGF-β1 gene (277a). The mechanism by which these mutations result in abnormal remodeling of bone is unclear; however, all three mutations are predicted to have adverse effects on the normal dimerization of the LAP, subsequently compromising TGF-β1 effects on bone remodeling. These findings illustrate the complexity of signaling that is involved in the regulation of skeletal morphogenesis and the maintenance of differentiated function.

2. Receptors and binding proteins. Activin receptor type IIB (*Acvr2b*) knockout mice have a phenotype that varies significantly in its severity from viable and fertile mice to perinatal lethality (143). However, 100% of *Acvr2b* homozygous null mutant mice have additional thoracic vertebrae. The number of vertebrae is increased from 13 to 16 in 58 of 62 mice, and the remaining mice have 17 thoracic vertebrae. In contrast to *Gdf11* null mutants, however, the number of lumbar vertebrae is normal. It is unclear from these studies whether the total number of vertebrae is increased in *Acvr2b* homozygous null mice or whether this represents a milder homeotic transformation than that observed in *Gdf11* null mice. Nevertheless, the boundaries of expression for *Hoxc6*, *Hoxc8*, *Hoxc9*, and *Hoxc10* are all shifted in a posterior direction (143), similar to the observation in *Gdf11* null mutants. This observation suggests that the ACVR2B functions as the type II receptor for GDF-11 in this context.

BMP receptor type IA (*Bmpr1a* or ALK3) signaling is critical for normal development of both the proximal-distal and dorsal-ventral axes of the limb. Conditional disruption of the *Bmpr1a* gene in limb ectoderm using a *loxP*-Cre recombinase strategy results in a spectrum of malformations that include complete agenesis of the hindlimbs, dorsal transformation of ventral limb structures, extra digits in the forelimbs, and missing metacarpals and proximal phalanges (278). Coincident with these findings, disrupted gene expression patterns are observed in both the apical ectodermal ridge (a structure critical for proximal-distal axis determination) and along the dorsal-ventral axis.

Insertional mutagenesis of the BMP receptor type IB (*Bmpr1b* or ALK6) gene has resulted in an allele ($Bmpr1b^{tg}$) in which expression is conditionally inactivated in the limb. This is due to disruption of an alternative promoter element (266). These mice have demonstrated a critical role for this BMP receptor in initiating cartilage condensation and differentiation in the distal limb and in regulating the segmentation of digit cartilage into phalangeal subunits (266). Homozygous $Bmpr1a^{tg/tg}$ mice have absent proximal (P1) and middle (P2) phalangeal segments but essentially normal growth of the distal phalangeal segment (P3) and normal growth of the metacarpals and metatarsals. Some of these deficiencies are mediated by interactions of the receptor with GDF-5, although other superfamily ligands are also involved, as demonstrated by double mutant studies with $Gdf5^{-/-}$, $Bmpr1b^{tg/tg}$ mice (266). In contrast to single mutants, $Gdf5^{-/-}$, $Bmpr1b^{tg/tg}$ double mutants have severe shortening of the metacarpals and maintain a vestigial phalangeal structure comprised of fused P1-P2 segments in addition to a normal-appearing P3 segment. These studies suggest that combinatorial signaling occurs (*i.e.*, that multiple superfamily ligands bind to the BMP type IB receptor and GDF-5 binds to multiple receptors) to accomplish digit formation and growth.

Follistatin null mutant mice have more subtle homeotic defects in axial patterning. The 13th rib pair is either absent or vestigial in these mice, and five rather than the normal six lumbar vertebrae are often present (229). These defects may reflect impaired regulation of GDF-11 or other superfamily members that activate activin type IIB receptors in the developing vertebrae.

Noggin is a dimeric BMP binding protein and an antagonist of BMP signaling. Functional inactivation of noggin results in several axial and appendicular skeletal anomalies (279). Failure of joint formation and vertebral fusion are the predominant manifestations, resulting in synostosis of multiple skeletal structures such as the humeri and radii at the elbow. The long bones are also shorter and broader than normal. Some of these features are likely to be related to a failure to express GDF-5 in specific skeletal structures of the mutants. The severity of the noggin null phenotype greatly exceeds that of the *Gdf5* null mutation (brachypodism), however, and so it is likely that the unchecked activities of other BMPs contribute significantly.

In support of an important role for noggin in skeletal development in mammals, two allelic autosomal dominant human conditions, proximal symphalangism and multiple synostoses syndrome, are due to missense mutations in the noggin gene (280–282). Both conditions result in the fusion of specific joints of the hands, ankles, hips, and cervical spine. The specific mechanism by which missense mutations in one allele encoding a BMP binding protein results in disease phenotypes is unclear; however, haploinsufficiency, ineffi-

cient homodimerization of the noggin protein, or selective deficiency in BMP binding have all been suggested as possibilities.

G. Body composition and growth

The ability of a TGF-β superfamily member to influence body weight distribution was established with the studies of GDF-8 function (also called myostatin). Expression of GDF-8 is restricted to the myotome compartment of somites in the embryo and is also expressed in adult skeletal muscle (283). Skeletal muscle mass of *Gdf8* knockout mice is approximately twice that of controls, with an overall 30% increase in body weight of the knockout mice (283). A similar phenotype is observed in Belgian Blue cattle, which have considerably increased muscle mass secondary to mutations in the bovine *Gdf8* gene (284). The increased skeletal muscle mass is attributed to both cellular hyperplasia and hypertrophy, consistent with the role of GDF-8 as a negative regulator of skeletal muscle growth. Interestingly, follistatin, a protein that binds and regulates the activities of many TGF-β superfamily members (77, 215, 218), also seems to be important for the maintenance of skeletal muscle mass. Follistatin knockout mice have underdeveloped diaphragms and intercostal muscles at birth that lead to respiratory failure and perinatal lethality (216). Furthermore, follistatin has been shown to inhibit the mesoderm-inducing properties of GDF-11 in *Xenopus* animal cap assays (218). This finding is significant because of the strong sequence similarity between GDF-11 and GDF-8 (90% in the mature domains of the proteins), which suggests that follistatin might also play an important role in regulating the function of myostatin. This suspicion was confirmed recently in transgenic mice that overexpress follistatin specifically in skeletal muscle (285). These mice have an even more dramatic muscle phenotype than *Gdf8* knockout mice, suggesting that other follistatin-binding proteins within the superfamily, such as GDF-11, may also be playing roles in the control of skeletal muscle growth. A similar phenotype was also observed in mice overexpressing a dominant-negative form of the activin type IIb receptor in muscle, suggesting that GDF-8 signals through this receptor *in vivo* (285). Conversely, transgenic mice that ectopically express mouse GDF-8 exhibit a cachexia (wasting) phenotype (286).

Recent work has demonstrated that TGF-β superfamily members can also play important roles as positive growth regulators *in vivo*. Activin βA null mutant mice have congenital anomalies that result in the inability to suckle effectively. As a result, these mice die shortly after birth (229). The malformations and perinatal lethality can be rescued, however, by a targeted insertion of the most closely related family member, activin βB, into the activin βA locus (287). However, this knock-in allele functions as a hypomorph, restoring some, but not all, function to the locus. The restored viability of the knock-in mice has revealed important growth effects for activin βA on the gonads, genitalia, and hair. In addition, activin βA is an important factor for the maintenance of normal somatic growth and survival.

H. Nervous system development

Many members of the TGF-β superfamily signal transduction cascade are expressed in the central and peripheral nervous system. However, few of these factors have been shown to be essential for neural development in knockout mice or humans. The exception is the glial cell line-derived neurotrophic factor (GDNF) family. There are four ligands in this family: GDNF, neurturin, persephin, and artemin. These ligands are distant members of the TGF-β superfamily that signal through a unique receptor system that includes a common signaling component (RET) and a high-affinity glycosylphosphatidyl inositol-linked binding component (GFRα1-GFRα4).

Gdnf knockout mice die in the neonatal period due to renal agenesis and severe intestinal aganglionosis (288–290). The heterozygotes also display kidney development defects. These mice also lack an enteric nervous system. In contrast, neurturin knockout mice are viable (291). However, neurturin knockout mice have defects in enteric, parasympathetic, and sensory neurons. These knockout mice demonstrate decreased density of neuron fibers and size in the enteric nervous system and concomitant abnormalities in gut motility. In addition, GFRα2-expressing neurons in the dorsal root and trigeminal sensory ganglion and GFRα2-expressing parasympathetic neurons in the ciliary ganglion and submandibular salivary gland are lost. Interestingly, neurturin and GFRα2 are coexpressed in adjacent and specific sites of the body (*e.g.*, neurturin is produced by the salivary gland parenchyma, whereas GFRα2 is expressed in the parasympathetic ganglia). Thus, neurturin is the major physiological ligand for GFRα2 and is an essential trophic paracrine factor for postmeiotic enteric neurons and specific populations of sensory and parasympathetic neurons.

RET, the common receptor for the four GDNF family ligands, and GFRα1, which is more specific for GDNF, have also been knocked out in mice (292–294). Mice lacking RET, GFRα1, and GDNF phenocopy each other. Similar to *Gdnf* knockout mice, the *Gfra1* and *Ret* knockout mice die in the early neonatal period due to agenesis of the kidneys and severe defects in the enteric nervous system. Thus, these findings indicate that GDNF is the major ligand for GFRα1 and that GDNF signaling through a complex of GFRα1/RET is most essential for postnatal survival in the mouse.

GFRα2 appears to be the major receptor for neurturin in addition to other ligands of the family. Both *Gfra2* and neurturin knockout mice are viable and display defects in the parasympathetic nervous system (291, 295). In addition, *Gfra2* knockout mice display severe growth retardation, suggesting that other GDNF family ligands relay through this receptor. Consistent with the above findings, these ligands and receptors play important roles in human development (296). Mutations in these genes have been identified in cases of Hirschsprung disease, a congenital malformation associated with aganglionosis of the gastrointestinal tract. *RET* mutations account for approximately 50% of familial cases and 10–20% of sporadic cases. Mutations in the *GDNF* and neurturin genes play a lesser role but are important (297–299). Mutations in *RET* also cause multiple endocrine neoplasia 2A (MEN2A) and 2B (MEN2B) and papillary thyroid

carcinoma (296). Lastly, mutations in the human *GFRA4* gene are believed to play a role in the neurodegenerative Hallervorden-Spatz syndrome (300). Because GFRα4 has been shown to be a receptor for persephin (301), mutations in the persephin gene in mice and humans were expected to phenocopy this syndrome. However, persephin null mice are viable and demonstrate normal brain development and behavior (302). The only defect in persephin knockout mice is hypersensitivity to focal ischemia (*i.e.*, increased cerebral infarction after focal ischemia). Lastly, mice lacking artemin and GFRα3 are viable, demonstrate ptosis (~30% of adults), and show similar defects in migration and axonal projection patterns of the entire sympathetic nervous system (303). These and other included studies suggest that artemin functions as a guidance (chemoattractant) molecule for sympathetic neurons and their axons by signaling specifically through a complex that includes the GFRα3 receptor. Thus, GDNF family ligands and receptors play key roles in multiple nervous system and kidney development processes in mammals.

I. Other organ systems

TGF-β superfamily members are critical proteins in the development of other organ systems. *Inhbb* plays a critical role in the normal development of the mammary gland. *Inhbb* homozygous null mutant mice are unable to nurse their young effectively, resulting in early death of the litters (240, 241). Histological analysis of the mammary glands of *Inhbb* knockout mice demonstrates defective mammary duct elongation and alveolar morphogenesis (304). Tissue transplantation experiments demonstrated that transplanted intact glands from *Inhbb* knockout mice to wild-type mice failed to develop normally, whereas glands from wild-type mice implanted into *Inhbb*-deficient mice grew normally. However, epithelial cells from *Inhbb*-knockout mice grew normally when implanted into fat pads of wild-type mice, suggesting that ductal elongation and epithelial differentiation in the mammary gland during pregnancy requires activin signaling from stromal cells (304).

In almost every developmental and physiological process, TGF-β signaling proteins are involved (1, 10, 305–309). Examining the signal transduction pathway of TGF-β superfamily members will continue to provide important information for understanding fundamental developmental processes, as well as genetic causes of human diseases.

IV. TGF-β Superfamily Signaling and Reproduction

A. Function of MIS signaling pathways in sexual differentiation

Sexual determination is a complex process requiring the precise regulation of multiple events. Transgenic mice have been instrumental in defining and confirming the key gene products required for this process in mammals (for review, see Ref. 310). Both Wolffian and Müllerian ducts, the anlagen for the male and female reproductive tracts, respectively, are initially present in mouse embryos of both sexes. The Wolffian duct regresses in females, and the Müllerian duct regresses in males as sexual differentiation proceeds. In males, testosterone produced by the fetal testis induces the differentiation of the Wolffian duct. MIS [or anti-Müllerian hormone (AMH)] is a TGF-β superfamily ligand secreted by the Sertoli cells of the fetal and adult testis and by granulosa cells of the postnatal ovary (for review, see Refs. 311, 312, and 312a). MIS produced by the fetal testis leads to the regression of the Müllerian ducts, an essential process in male sex differentiation (313). Postnatally, MIS inhibits the proliferation and differentiation of immature Leydig cells in rats (314) and inhibits testosterone synthesis in adult rats (315). Genetic evidence has confirmed the *in vivo* role of MIS in male sex differentiation. Male mice null for MIS develop as pseudohermaphrodites with complete male reproductive tracts as well as oviducts and uteri (313, 316). Ninety percent of MIS null male mice are infertile because the superimposed female reproductive tract blocks sperm transit through the male conduit.

Initially, it was hypothesized that MIS null females would also be infertile. This was based on studies with transgenic female mice, overexpressing MIS during embryogenesis and postnatally, that are born without uteri or oviducts and eventually demonstrate loss of oocytes (317). However, MIS null females are fertile (313). Upon closer inspection, the MIS null females have increased primordial follicle recruitment, resulting in a decreased number of primordial follicles at 4 and 13 months and increased number of preantral and small antral follicles at 25 d and 4 months compared with controls (318). In fact, this more rapid recruitment of primordial follicles in the absence of MIS at earlier time points results in almost no primordial follicles or subsequent stage follicles at 13 months, resulting in an earlier menopause, a finding that could have important implications for prolonging a woman's reproductive life span. Follow-up studies with MIS null and FSH null females showed that MIS also inhibits FSH-stimulated follicle growth, and primordial follicle recruitment is enhanced in the absence of both MIS and FSH but not FSH alone (319). Whether MIS plays a direct or indirect role in regulating primordial follicle recruitment is unknown at this time.

Similar to other family members, MIS functions through type II and type I receptors. The MIS type II receptor is expressed in the mesenchymal cells adjacent to the Müllerian duct epithelium and in Sertoli cells and granulosa cells of male and female mice, respectively (320). Many type II receptors in the TGF-β superfamily appear to bind multiple ligands. However, genetic analysis in mice confirmed that MIS is apparently the only essential ligand for the MIS type II receptor because MIS type II receptor knockout male mice develop as pseudohermaphrodites, a phenocopy of the MIS knockout mice (320, 321). In addition, both MIS ligand and receptor knockout mice develop Leydig cell hyperplasia (see *Section IV.C*). Persistent Müllerian duct syndrome in dogs and human males (322) often causes infertility due to testicular degeneration because of secondary cryptorchidism. Forty-five percent of the persistent Müllerian duct syndrome cases in humans are attributed to mutations in the MIS (ligand) gene, and 39% are due to mutations in the receptor gene (type II; Ref. 323). In half of these cases, there are homozygous mutations; in most of the remaining cases, there are compound heterozygous mutations (*i.e.*, each allele en-

coding either the ligand or receptor has a different mutation). Interestingly, 16% of human persistent Müllerian duct syndrome cases do not have mutations in either the MIS ligand or receptor genes (323), suggesting that defects in other components upstream or downstream in the MIS signaling cascade can also cause this condition.

In contrast to the type II receptor, the MIS type I receptor is not unique to the MIS signaling pathway. Recent *in vitro* biochemical studies have demonstrated that MIS signals via a BMP-like pathway that includes the type I receptor ALK2 (324, 325). However, other ligands must also signal through ALK2 during embryogenesis because ALK2 knockout mice die at the gastrula stage (138, 139). Surprisingly, tissue-specific ablation of *Bmpr1a* (ALK3) in the mesenchymal cells of the Müllerian ducts results in retention of oviducts and uteri in males (325a). These findings suggest that ALK3 is directly or indirectly downstream of MIS *in vivo.* Other signaling data and determination of the expression patterns of the SMADs suggest that *Smad5* (and possibly *Smad1*) are downstream in this pathway.

Other studies have also focused on genes that regulate MIS expression. Earlier studies had shown that a 180-bp genomic fragment upstream of the transcription start site of the MIS promoter is active in Sertoli cells *in vitro*, and that this fragment can direct expression of a reporter gene in an MIS-specific pattern in transgenic mice (326). A nuclear hormone receptor half-site within this region was also shown to specifically bind the orphan nuclear receptor steroidogenic factor-1 (SF-1). In addition, a putative SOX9 binding site also lies upstream of this SF-1 binding site. To further define the functions of SOX9 and SF-1, precise mutations were made in these putative binding sites in the endogenous mouse promoter using homologous recombination in embryonic stem (ES) cells (327). Although homozygous mutations in the SOX9 binding site blocked MIS transcription and resulted in pseudohermaphroditism in males (similar to MIS ligand and receptor knockouts), the subtle mutation in the SF-1 site reduced MIS transcription but had little phenotypic consequence in males. These studies demonstrate that SOX9 is required for MIS transcription, and SF-1 is a likely quantitative regulator of MIS transcription. It is not clear whether mutations in the ALK2, *Smad5*, SOX9, and SF-1 genes could be responsible for some of the undiscovered cases of persistent Müllerian duct syndrome in humans.

B. Primordial germ cell development

Primordial germ cells (PGCs) are the precursor cells of gametes, responsible for transmitting genomic information from one generation to the next (328). During mouse embryogenesis, PGCs are first detectable at E7.5 as a cluster of alkaline phosphatase positive cells posterior to the primitive streak (329, 330). The PGCs migrate from their site of origin to the genital ridges through the hindgut endoderm (331) and proliferate along this route of migration (332, 333).

Several investigators have now defined a TGF-β superfamily signaling pathway important in PGC development (Ref. 334; Fig. 6). Elegant studies performed in knockout mice deficient in BMP-4 revealed that BMP-4 is an early factor essential for PGC differentiation (335). BMP-4 is expressed in

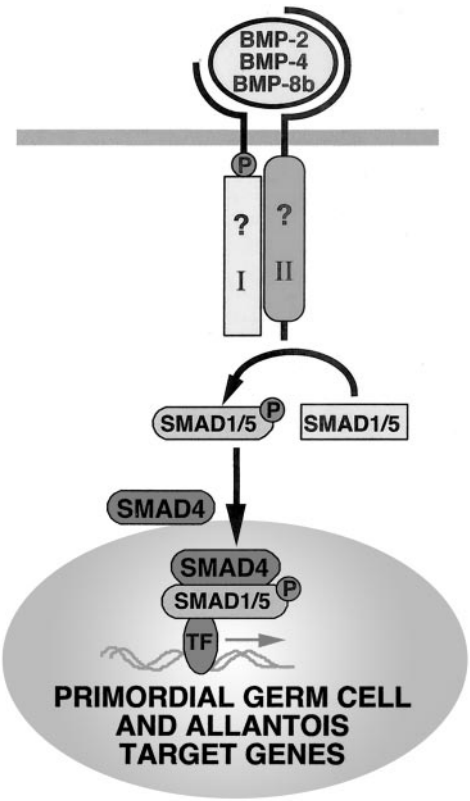

FIG. 6. TGF-β signaling in PGC and allantois development. For proper PGC and allantois development, several BMP family ligands, as shown, signal through unknown receptors to phosphorylate SMAD1 and SMAD5. Loss of any of these results in a decrease or absence of PGCs and an abnormal or absent allantois or a failure of allantois to fuse with the chorion. Mice lacking both BMP-5 and BMP-7 (data not shown) also demonstrate a smaller allantois. TF, Transcription factor.

the extraembryonic ectoderm cells adjacent to the proximal epiblast cells before gastrulation. After gastrulation initiates, BMP-4 is also present in the extraembryonic mesodermal cells. Defects in PGC development can be rescued in chimeric embryos generated by injecting *Bmp4* null ES cells into wild-type blastocysts, but not in chimeric embryos generated by injecting wild-type ES cells into *Bmp4* null blastocysts. When ES cells are injected into the blastocysts, the ES cells preferentially proliferate into the epiblast, which gives rise to all three embryonic germ layers and extraembryonic mesoderm, but not the extraembryonic ectoderm or primitive endoderm. The chimera studies therefore indicate that BMP-4 produced from extraembryonic ectoderm but not from extraembryonic mesoderm is essential for PGC development.

BMP-8b, another TGF-β superfamily member, is also expressed in the extraembryonic ectoderm before gastrulation and is required for PGC development (147). In *Bmp8b* knockout mice, PGCs are greatly reduced or absent, similar to *Bmp4* knockout mice. It is not clear how these two BMPs interact with each other at early stages of PGC development.

BMP-2 is a close relative of BMP-4. During mouse embryogenesis, *Bmp2* transcripts are detected in the endoderm of pregastrula and gastrula mouse embryos (146, 336). The endoderm expression of *Bmp2* is more pronounced in

the midportion of the embryo, at the boundaries between the epiblast and the extraembryonic ectoderm. Mice deficient in *Bmp2* have a shorter allantois and reduced PGC number compared with wild-type littermates (146). Furthermore, mice double heterozygous for *Bmp2* and *Bmp4* have more severe reduction in PGC number compared with mice heterozygous for either *Bmp2* or *Bmp4*, indicating an additive effect of BMP-2 and BMP-4 on PGC generation. In contrast, there is no additive effect of BMP-2 and BMP-8b. Likewise, there is no additive effect between *Bmp8b* and *Bmp4* because mutant mice double heterozygous for *Bmp8b* and *Bmp4* mutation do not have more severe PGC loss than mice heterozygous for *Bmp8b* alone (147). These data suggest that visceral endoderm-derived BMP-2 and extraembryonic ectoderm-derived BMP-4 signal through the same receptors in the process of PGC generation, whereas extraembryonic ectoderm-derived BMP-8b signals through a distinct pathway.

Smad5 is expressed uniformly in the epiblast before gastrulation and later on in all three germ layers and their derivatives (149). Mice deficient in *Smad5* also have defects in allantois development and have a greatly reduced number of PGCs (337), similar to mice lacking *Bmp2*, *Bmp4*, or *Bmp8b*. Furthermore, ectopic PGCs and allantois-like cells are found on the amnion of *Smad5*-deficient mice, suggesting that SMAD5 is also involved in sorting cells that will become PGCs and allantois from those that will become the extraembryonic ectoderm layer of the amnion during development (337). Similar to *Smad5* knockout mice, *Smad1* knockout mice have defects in allantois and PGC development (151). Regression analysis indicates that BMP-2, BMP-4, BMP-8b, SMAD1, and SMAD5 are involved in PGC generation instead of PGC proliferation. Interestingly, at E6.5, expression of *Smad1*, *Smad5*, and *Smad8* mRNAs overlap in most sites. This suggests that the incomplete penetrance of the PGC or allantois phenotypes in mice lacking *Smad1* or *Smad5*, as compared with the *Bmp4* knockout, is because of redundancy of these downstream SMADs. Double mutants of *Smad1* and *Smad5* are expected to more closely phenocopy the *Bmp4* knockouts, and because *Smad8* knockout mice have not yet been generated, it is unclear whether SMAD8 contributes to these processes.

In contrast to the positive roles of BMPs in PGC proliferation, TGF-β1 and activin negatively regulate the proliferation of PGCs *in vitro* (338). The presence of TGF-β1 and activin in the genital ridge at the time of germ cell colonization and the expression of their receptors in the PGCs suggest that these proteins may also have roles in mediating PGC proliferation *in vivo*. However, studies on knockout mice lacking activins or TGF-β1 have not demonstrated an essential role of these proteins in PGC development.

C. Gonadal development

In addition to their functions in embryonic regression of the Müllerian duct and proliferation of PGCs, components of the TGF-β superfamily signal transduction cascade have also been implicated in gonadal growth and differentiation postnatally. FSH has been shown to be essential for stimulation of the growth and division of granulosa cells and Sertoli cells of the ovary and testis, respectively (339). Activins (βA:βA, βB:βB, βA:βB) are known to positively regulate FSH synthesis and secretion, whereas the related inhibins (α:βA, α:βB) inhibit FSH synthesis and secretion (340). Inhibins and activins regulate FSH by acting as local autocrine and paracrine factors, as well as endocrine factors. Activins were confirmed to play an important *in vivo* role by signaling through the type II receptor ACVR2. Mice lacking ACVR2 have suppressed pituitary and serum FSH levels that result in gonadal growth defects (142). Female ACVR2 mice are infertile due to a block at the antral stage of ovarian folliculogenesis (142). The gonadal defects are very similar to the FSHβ knockout phenotype (339), suggesting that absence of activin signaling through ACVR2 mimics FSHβ loss of function. Interestingly, double homozygous mutant male mice for ACVR2 and FSHβ have no further reduction of testis size relative to single homozygous mice; however, sperm counts and litter sizes were decreased in the double mutants as a result of a reduction in type A and I spermatogonia. These findings confirm that an ACVR2 signaling pathway plays an important direct role in spermatogenesis (*i.e.*, independent of FSH effects; Ref. 341).

Other recent studies suggest that activin signaling affects gonadal growth. Activin βB knock-in mice that express activin βB instead of activin βA from the activin βA locus have a dramatic reduction in the size of the gonads, despite increased FSH levels (287). Even with a 50% reduction in testicular volume, however, male mice maintain their fertility. Female fertility, in contrast, is severely compromised (287). These observations confirm that activin βA is a critical gonadal growth factor. Moreover, the activin βA growth signal in testis appears to be most important during late embryogenesis or in the early postnatal period because activin βA mRNA levels are essentially undetectable in the mouse by postnatal day 12 (Ref. 287 and C. W. Brown and M. M. Matzuk, unpublished observations). In contrast, activin βB expression in the testis continues through adulthood in both wild-type and activin βB knock-in mice, suggesting that this protein alone is not sufficient to maintain normal testicular growth. Also, activin βB knockout mice have normal testicular size, suggesting that activin βA provides a sufficient activin signal to support normal testicular growth (240, 241). Interestingly, mice that overexpress follistatin (an activin binding protein) at high levels in the testis have deficiencies of testicular growth (217), and activin $\beta A^{+/-}$ mice also have modest, though significant, testicular growth deficiencies (C. W. Brown and M. M. Matzuk, unpublished observations).

Likewise, a knockout of the inhibin α subunit gene has defined essential roles of inhibins in mammals (342, 343). As expected, these male and female mice lacking both inhibin A (α:βA) and inhibin B (α:βB) have increased FSH levels. In addition, sex cord-stromal (granulosa and/or Sertoli cell) tumors develop as early as 4 wk of age in male and female inhibin α knockout mice. These tumors advance rapidly and cause an activin-induced cancer cachexia-like (wasting) syndrome that kills 95% of the males by 12 wk and 95% of the females by 17 wk. The activins secreted from the tumors cause pathological defects in the liver (apoptosis of hepatocytes around the central vein) and stomach (blocks in differentiation of four major gastric mucosal lineages) by acting directly through ACVR2 (343–345). Furthermore, more re-

cent studies suggest that inhibins avert tumor growth via a pathway involving the cyclin-dependent kinase inhibitor $p27^{Kip1}$ or a related (and/or synergistic) cell cycle suppression pathway (346). Our group is continuing to define the relationship of inhibin and cell cycle control in the adrenal cortex, which develops cancer at high penetrance when inhibin α knockout mice are gonadectomized (343). Using a mifepristone-inducible system, postnatal overexpression of inhibin A in the inhibin α null background suppresses testicular tumorigenesis (347), confirming that inhibins act as tumor suppressors.

Although human mutations in the inhibin receptor (inhibin binding protein) or inhibin subunits have not yet been studied in human granulosa cell tumors, mutations have been identified in cases of premature ovarian failure (348). One patient with premature ovarian failure had a polymorphic mutation (1032 C>T transition) in the *INHBA* (inhibin/activin βA) gene that did not change the amino acid sequence of this subunit. Three of 43 patients (7%) with premature ovarian failure had a 769 G>A transition resulting in an alanine to threonine substitution. Only 1 of 150 control patients (0.7%) had a similar change. These findings suggest a significant association of the *INHBA* variant with premature ovarian failure, which should be examined in a larger group of affected patients.

Male mice lacking either MIS ligand or type II receptor also develop gonadal defects. In these knockout models, Leydig cell hyperplasia is seen with rare Leydig cell tumor development (313, 320). Interestingly, intercrossing inhibin α mutant and MIS ligand or receptor mutant mice results in Leydig cell tumors as early as 1 wk of age (349). These findings demonstrate an important synergism between the MIS and inhibin signaling pathways that control Leydig cell proliferation. This is interesting because MIS inhibits the proliferation and differentiation of immature Leydig cells (314), and one of the inhibin receptors (inhibin binding protein; Ref. 43) is expressed highly in Leydig cells. It is possible that MIS and inhibin act directly to negatively regulate Leydig cell growth and function.

TGF-β superfamily members not only affect the somatic cells of the testis but also affect postnatal germ cell development in the testis. In addition to its role in PGC proliferation in both male and female embryos, *Bmp8b* and its chromosomally linked homolog *Bmp8a* are important for adult male fertility (147, 350, 351). In contrast to other members of the TGF-β superfamily that are expressed in the somatic cells, *Bmp8a* and *Bmp8b* mRNAs are expressed in the testicular germ cells during specific stages of spermatogenesis (352). Highest levels of these mRNA species are seen in round spermatids. Interestingly, *Bmp8b* knockout mice show earlier defects and more dramatic effects on fertility than the *Bmp8a* knockout males. Although both *Bmp8a* and *Bmpb* knockouts demonstrate significant apoptosis of spermatocytes, the *Bmp8b* knockout males also show a decrease in proliferation of germ cells and delayed germ cell differentiation. These defects eventually lead to adult infertility in 100% of the *Bmp8b* knockouts, but less than half of the *Bmp8a* mutants show these defects, and most *Bmp8a* knockouts continue therefore to be fertile. BMP-8a and BMP-8b likely act as autocrine factors to stimulate a predicted SMAD1, SMAD5, or SMAD8 pathway in the testis. It will be interesting in the future to determine the viability of mice lacking both *Bmp8a* and *Bmp8b* (via generation of linked mutations) and, if the mice are viable, to determine the redundant functions of these two growth factors in germ cell physiology in the postnatal testis.

In addition to the granulosa cells of the ovary, which express inhibins and activins, other TGF-β superfamily members are expressed in the theca cells (*i.e.*, BMP-4 and BMP-7) and the oocytes (*i.e.*, GDF-9, BMP-15, and BMP-6). Because *Bmp4* and *Bmp7* knockouts die during embryogenesis and at birth, respectively, their functions in the ovary are unknown. *Bmp6* knockout mice are viable and have minor skeletal defects, but they do not show any ovarian defects and are fertile (158). In contrast, GDF-9 and BMP-15 appear to play several different functions in the ovary to regulate somatic cell function and female fertility. *Gdf9* and *Bmp15* mRNAs have identical patterns of expression in mouse oocytes; primordial follicles do not express these mRNAs, but after recruitment into the active pool, oocytes from the early primary stage through ovulation express these mRNAs (353). These findings suggest that GDF-9 and BMP-15 could theoretically function at any point in folliculogenesis and in the oviduct. In the case of *Gdf9* null mice, females are infertile due to a block at the primary follicle stage (354). These ovaries demonstrate a block in growth and proliferation of granulosa cells, lack of thecal layer development, and oocyte defects, which include oocyte meiotic competence abnormalities and larger than normal growth of the oocyte (354–356). The theca and oocyte defects are likely secondary to the absence of signaling of GDF-9 from the oocyte to the surrounding granulosa cells; for example, absence of GDF-9 causes a dramatic up-regulation of kit ligand in the granulosa cells of primary follicles leading to an apparent increase in stimulation of the KIT signaling pathway in the oocytes and subsequently increased oocyte growth (356). Oocytes are known to secrete multiple factors (357), and in the preovulatory follicle, granulosa cells in close proximity to the oocyte (*i.e.*, cumulus cells) have different gene expression profiles as compared with the granulosa cells farthest away (*i.e.*, the mural cells which are closest to the theca; Ref. 358). Using recombinant GDF-9 and cells from preovulatory follicles, our group has shown that GDF-9 can regulate most of these genes. For example, GDF-9 can stimulate hyaluronan synthase 2, cyclooxygenase 2, prostaglandin E2, the EP2 prostaglandin PGE2 receptor, and progesterone, as well as cumulus expansion, but suppresses LH receptor and urokinase plasminogen activator (359, 360). Thus, GDF-9 functions at multiple stages of folliculogenesis to regulate key somatic cell functions necessary for female fertility.

The *Bmp15* gene was discovered as an X-linked gene that encodes a homolog of GDF-9, sharing 52% amino acid identity to GDF-9 in the mature regions (9). Although BMP-15 expression is identical to that of GDF-9, it did not compensate for absence of GDF-9 in the knockout. *Bmp15* knockout mice are subfertile, demonstrating reduced litter sizes and litters per month (361). Folliculogenesis proceeds fairly normally beyond the primary follicle stage, but there are reduced ovulation and fertilization rates with hormone treatment. Double mutant female mice that are heterozygous for the

Gdf9 null allele and homozygous for the *Bmp15* null allele (*i.e.*, $Gdf9^{+/-}$, $Bmp15^{-/-}$ mutants) demonstrate complete infertility on a 129/SvEv inbred background and subfertility and infertility on a mixed C57Bl/6/129/SvEv genetic background. Whereas eggs isolated from the oviducts of wild-type females are embedded in a resilient three-dimensional extracellular matrix containing cumulus cells, the cumulus cells of $Bmp15^{-/-}$, $Gdf9^{+/-}$ mice readily detach. Thus, these studies demonstrate important dosage-sensitive synergistic interactions between GDF-9 and BMP-15 in the periovulatory period in the mouse. Interestingly, sheep heterozygous for *BMP15* mutations (fecundity × Hanna or fecundity × Inverdale) are more fertile than normal, whereas *BMP15* homozygous mutant sheep phenocopy the findings seen in *Gdf9* null mice (*i.e.*, primary ovarian follicle block and infertility; Ref. 362). Because of the high sequence divergence between mouse, sheep, and human BMP-15 orthologs, it is likely that BMP-15 monomers or GDF-9/BMP-15 heterodimers are more bioactive than GDF-9 homodimers in sheep, whereas the reverse appears to be true in mice. It is unclear which ligand dimer is the most relevant in humans, although X-chromosome deletions that include the region encoding the BMP-15 gene (Xp11.2) are associated with premature ovarian failure.

Signaling in granulosa cells is a complex process; type II receptors for activins (*e.g.*, ACVR2 and ACVR2B), BMPs (*e.g.*, BMPR2), and MIS (*i.e.*, AMHR2) are expressed in granulosa cells, whereas the ligands are expressed in theca (*e.g*, BMP-2 and BMP-7), granulosa cells (*e.g.*, activins and inhibins), and oocytes (*e.g.*, GDF-9 and BMP-15). Multiple synergistic and antagonistic actions of these ligands at every step of folliculogenesis and within various compartments (*e.g.*, normal *vs.* cumulus compartments) will define a gene expression profile in each cell that determines its growth and differentiation.

In an effort to identify additional genes downstream of GDF-9 and BMP-15 in the periovulatory period that could play a role in cumulus cell physiology, we used GeneChip technology. Pentraxin 3 (*Ptx3*) was identified as a GDF-9-induced gene (363). PTX3 is a member of the large pentraxin family of acute phase proteins (364). Our studies show that *Ptx3* mRNA is expressed in the preovulatory follicle after the LH surge and only in cumulus granulosa cells, an expression pattern similar to hyaluronan synthase 2 and cyclooxygenase 2. Knockout studies in our laboratory have shown that *Ptx3* homozygous mutant females are subfertile due to a cumulus cell adherence defect, a defect that phenocopies the $Gdf9^{+/-}$ $Bmp15^{-/-}$ mutant phenotype. Thus, absence of the GDF-9/BMP-15 downstream protein PTX3 is a major reason for the cumulus cell adherence defects in a $Gdf9^{+/-}$ $Bmp15^{-/-}$ mutants. This also suggests that GDF-9 and BMP-15 signal through the same receptor on cumulus granulosa cells.

Although the GDF-9/BMP-15 receptors are not yet defined, recent studies suggest that the type I receptor ALK6 may be involved in cumulus cell physiology. In mice, a null mutation in the ALK6 (*Bmpr1b*) gene results in female infertility (365). Oocytes from ALK6 knockout mice can be fertilized *in vitro* but are defective in their *in vivo* fertilization because of abnormalities in cumulus expansion. In sheep, a point mutation in the kinase domain of ALK6 has been shown to segregate with the Booroola fecundity gene (*FecB*) phenotype (366). The *FecB* mutation (Q249R) is dominant, and in contrast to the null mutation in mice, this mutation in sheep causes increased ovulation rates and litter sizes. Because this mutation is dominant, it is likely to be an activating mutation; further *in vitro* and *in vivo* studies will be required to determine how the mouse and sheep mutations cause these phenotypes and which ligand pathway is affected by the mutations. Thus, TGF-β superfamily signaling pathways affect multiple cell types within the mammalian gonads. It will be important in the future to determine how these various pathways interrelate to precisely regulate mammalian reproductive function.

V. Perspectives and Future Directions

A. Promiscuity within the family

Studies of the TGF-β superfamily have contributed substantially to our understanding of a variety of biological processes. Ligands, their regulatory proteins, and downstream signaling elements play essential roles in embryogenesis, reproduction, growth, tumor suppression, and the maintenance of appropriate body composition. A few themes emerge from observations in mutant animal models. Promiscuity is a term often used to describe the interaction of superfamily ligands and their receptors because the number of TGF-β superfamily ligands greatly exceeds the numbers of type I and II receptors. This observation and results from the numerous studies described above lead to the conclusion that most type II receptors likely bind to more than one ligand. However, this is not a confused or indiscriminate mixing of elements as the term promiscuous implies, but rather a well orchestrated series of events, controlled temporally and spatially within a developing organism. In some cases, multiple ligand-receptor binding events within the same structure or even cell type are required to maintain the normal developmental process (266), and in some cases, heterodimerization of ligand monomers may also play a role (272). These variables contribute to the tremendous complexity of the system, and so an ontogeny map of all the TGF-β superfamily ligands, receptors, binding proteins, and regulatory proteins would be extremely useful to evaluate global patterns of expression and potential interactions.

A second emerging paradigm is the importance of dosage in TGF-β superfamily signaling. Signaling thresholds have been clearly demonstrated in *X. laevis* animal cap assays for the activins (367), and roles for morphogen gradients of dpp have been established for dorsal-ventral patterning in the *Drosophila* embryo and in directing wing development (368, 369). More recent evidence has suggested a role for Nodal gradients in *X. laevis* mesoderm induction (370). In addition, many mouse models with mutated TGF-β superfamily members exhibit gene dosage effects. These manifest as milder phenotypes in single heterozygous mice relative to homozygotes (*e.g.*, *Gdf8*, *Gdf11*, *Gdnf*, activin βA knockouts, and βB knockins), or as more severe phenotypes in double mutant mice (heterozygous, heterozygous) or (heterozygous, homozygous) relative to either of the single heterozygous or homozygous mutants (*Nodal/Acvr2*, *Bmp4/Bmp7*, *Nodal/Smad2*, *Gdf5/Bmpr1b*). Introducing an allele at the activin βA

locus that encodes a hypomorphic protein can augment this effect as we have shown (287). These findings suggest that a proper level of signaling is tightly maintained for some members of the superfamily and that a number of steps including ligand processing, the accessibility to receptors, affinity of ligand for receptor, and the modulation of intracellular signaling are all important potential or proven sites of regulation. It also raises the intriguing possibility that polymorphisms within human genes that affect the level of expression of TGF-β superfamily members may contribute to a dosage-dependent variation in normal physical characteristics such as muscle mass with *Gdf8/Acvr2*, or linear growth with activin βA and its associated receptors and downstream elements. By extension, such a process could also result in disease phenotypes such as skeletal patterning defects associated with *Gdf11* mutations.

B. Intracellular signaling

Tremendous progress has been made over the past few years in dissecting the signals that are downstream of TGF-β superfamily receptor activation. Studies of the SMADs and their associated regulatory proteins have revealed a myriad of potential therapeutic targets, particularly for newer generation antineoplastic agents. Although it seems clear that many other TGF-β superfamily ligands will use these common downstream elements, the signaling pathways for most other ligands need better characterization. This information will become essential as we gain a better understanding of the contribution of the superfamily at large to human disease. Evidence is also growing to support the contention that crosstalk occurs between many different signaling pathways, and dissecting the signaling mechanisms will be essential to understand these interactions fully.

C. Superfamily signaling and human disease

The number of diseases associated with mutations in TGF-β superfamily members continues to expand as discussed earlier and in Table 2. The role of TGF-β signaling in cancer has been well established and has been reviewed recently (371). Considering the large number of developmental processes in which members of the superfamily participate, it seems likely that many more mutations will be identified. Candidate diseases include those in which defects in skeletal morphogenesis is a feature (common to many of the mutants described above). The specific characteristics of the mouse mutants should aid substantially in determining which genes are involved. The link between NODAL signaling and holoprosencephaly has already been established, making that pathway an attractive place to look for mutations in affected individuals. Multifactorial conditions such as hypertension and atherosclerosis are also influenced by TGF-β superfamily signaling, illustrating the nearly ubiquitous influence of this complex, yet well orchestrated family of proteins. The next decade of functional analysis will be an exciting one.

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Address all correspondence and requests for reprints to: Martin M. Matzuk, M.D., Ph.D., The Stuart A. Wallace Chair and Professor, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. E-mail: mmatzuk@bcm.tmc.edu

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H.C., C.W.B., and M.M.M. contributed equally to this review.

Present address for H.C.: Cardiovascular Research Center, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129.

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