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Review

Functional analysis of the TGFβ receptor/Smad pathway through gene ablation in mice

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Many ligands, serine/threonine kinase receptors and intracellular effectors, termed Smads, of the TGFβ superfamily have been identified in vertebrate and invertebrate species. Studies on cells in culture have revealed many aspects of how molecular interactions between these components take place. Functional analysis through gene ablation in mice is now becoming an important part of understanding how these signal transduction pathways function *in vivo*. These studies are demonstrating the importance of these genes in development and disease but are also showing that the molecular models based on experiments in cultured cells are not always providing predictions that are born out *in vivo*. Here we review the current status of loss-of-function of the serine/threonine kinase receptors and Smads in the mouse, in the context of (early) embryonic phenotypes caused by lack of signalling by their putative endogenous ligands.

TGFβ superfamily ligands, receptors and Smads

In mammals, the transforming growth factor β (TGFβ) superfamily consists of about 30 structurally related proteins; these include 3 isoforms of TGFβ itself, 3 forms of activin and over 20 bone morphogenetic proteins (BMPs). These growth factors control a broad range of cellular behaviour. They have been reported to regulate cell growth, differentiation and apoptosis of various cell types *in vitro* and to induce the production of extracellular matrix proteins (Roberts and Sporn, 1990; Kingsley, 1994). Furthermore, they are involved in the specification of developmental fate. This was first elegantly illustrated in genetic studies in the fruit fly, *Drosophila melanogaster* and was followed by a series of studies in the South African clawed toad *Xenopus laevis*, in which ectopic expression of (mutated) ligands and receptors and downstream signalling molecules, confirmed the importance of these factors for vertebrate development (reviewed in Kingsley 1994; Attisano and Wrana, 1998). Here we consider the current view of the function of TGFβ superfamily ligands that signal through a specific set of serine/threonine kinase receptors and Smads in mammals through gene ablation studies in mice.

Abbreviations used in this paper: TGFβ, transforming growth factor β; BMP, bone morphogenetic protein; TβRII, TGFβ receptor type II; TβRIII, TGFβ receptor type III; ALK, activine receptor like kinase; ActR, activin receptor; MH, mad homology; R-Smad, Receptor regulated Smad; I-Smad, Inhibitory-Smad; Co-Smad, common mediator Smad; BmPR, BmP receptor.

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Members of the TGFβ family exert their effect by binding to heteromeric complexes of two different kinds of serine/threonine kinase receptors denoted type I and type II (reviewed in Massagué, 1998; Heldin *et al.*, 1997). For the TGFβs themselves, there is an additional group of cell surface binding proteins e.g. betaglycan (Lopéz-Casillas *et al.*, 1993), also termed TGFβ type III receptor (TβRIII) and endoglin (Gougos and Letarte, 1990) that play a relatively passive role in mediating cellular responses. Upon ligand binding, the constitutively phosphorylated type II receptor kinase trans-phosphorylates and activates the type I receptor in a region known as the GS-box; this initiates downstream signalling (Wrana *et al.*, 1994). Seven different type I receptors (activin receptor-like kinase (ALK)-1 to 7) (ten Dijke *et al.*, 1994a,b) and four different type II receptors have been identified to date. The ligand specificity of these receptors has been determined primarily by their ability to bind a given ligand and activate specific downstream genes. TGFβ family members often bind to more than one type II and type I receptor combination (Fig. 1). TGFβ binds to TβRII in combination with TβRI, also known as ALK-5 (Franzén *et al.*, 1993; Lin *et al.*, 1992). In endothelial cells however, TGFβ can also bind to a TβRII-ALK-1 receptor combination although with much lower affinity (Lux *et al.*, 1999; Oh *et al.*, 2000). Activin can bind to two type II receptors, ActR-IIA and ActR-IIB, predominantly in a complex with ALK-4 or ActR-IB although it can also bind in a complex with ALK-2 (ActR-IA). BMPs bind to BMPR-II, but are also able to bind to the two activin type II receptors in a combination with ALK-2, ALK-3 or ALK-6 (ten Dijke *et al.*, 1994a,b). To date, definitive ligands for ALK-7 have not been identified.

After ligand stimulation, the activated type I receptor transduces the signal by phosphorylating a member of a family of proteins known as Smads (Fig. 1). Eight Smad proteins have been identified in mammals so far and have been divided into three classes based on their structure and function: receptor-regulated Smads (R-Smads), common-partner Smads (co-Smads) and inhibitory Smads (I-Smads). The R-Smads can be further divided in 2 subtypes: those activated by TGFβ and activin receptors (Smad2, -3), and those activated by BMP receptors (Smad1,-5,-8), although data is accumulating which suggests that Smad1,-5,-8 might also act promiscuously with TGFβ receptors (Macias-Silva *et al.*, 1998; Lux *et al.*, 1999; Oh *et al.*, 2000). One co-Smad (Smad4) has been described so far in mammals, but others might exist; two co-Smads were found in *Xenopus laevis* (Howell *et al.*, 1999; Masuyama *et al.*, 1999).

In general, Smad proteins have two large conserved regions, the N-terminal mad homology (MH)1 domain and the C-terminal MH2 domain, which are separated by a less conserved linker region. The R-Smads transiently interact with the ligand-activated type I receptor, become phosphorylated at their C-terminal SSXS sequence and oligomerize with a common partner, Smad4; this heteromeric complex then translocates to the nucleus where it regulates transcription of target genes (reviewed in Verschueren and Huylebroeck, 1999; ten Dijke *et al.*, 2000). Co- and I-Smads lack the C-terminal phosphorylation site and are not phosphorylated upon receptor activation. Interaction of the R-Smads with defined type I receptors determines specificity of the TGFβ family member (reviewed in ten Dijke *et al.*, 2000; Christian and Nakayama, 1999).

To date, two I-Smads have been identified in mammals, Smad6 and Smad7 (Imamura *et al.*, 1997; Nakao *et al.*, 1997; Topper *et al.*, 1997). These Smads have been identified as inhibitors of TGFβ, activin and BMP signalling and might function in negative feedback loops since TGFβ, activin and BMPs are all able to induce their expression (reviewed in Christian and Nakayama, 1999). I-Smads interact stably with activated type I receptors and prevent phosphorylation of the R-Smad by these receptors. An additional mechanism has been proposed for Smad6; Smad6 competes with Smad4 for binding to Smad1, thereby preventing the formation of Smad1/Smad4 complexes (Hata *et al.*, 1998). Smad7 interacts with all activated type I receptors (Souchelnytskyi *et al.*, 1998) and is a general inhibitor of TGFβ superfamily induced responses, whereas Smad6 is thought to inhibit preferentially the phosphorylation of BMP Smads (Itoh *et al.*, 1998) although this is controversial (Imamura *et al.*, 1997). While Smad7 mRNA expression is rapidly upregulated by R-Smads, Smad6 mRNA is induced after several hours and is maintained for 48 hours or more (Miyazono, 1999). This again suggests different mechanisms for the action of the two I-Smads.

Analysis by gene targeting revealed that most of the TGFβ superfamily receptors and Smads are crucial to normal embryonic development (Table 1), although unexpected redundancy has provided new insights into models of signal transduction based largely on experiments on (epithelial) cells in culture. Integrating the results of the multiple knockouts allows identification of those ligands, receptors and Smads that belong genetically to the same signal transduction cascade and has been particularly useful in understanding their function in the regulation of (early) developmental events. In addition it has allowed identification of functionally redundant components of the signal transduction cascades *in vivo*, as will be outlined in the following sections. Several of the mutant mice have abnormal bone formation, that affects both craniofacial development as well as causing skeletal defects (see Table 1), but this aspect of later developmental functions of TGFβ superfamily signalling will not be covered in this review.

Defects in epiblast organisation, initiation of gastrulation and mesoderm formation

In order to understand the phenotypes that will be described in the following sections, the major events that take place during early

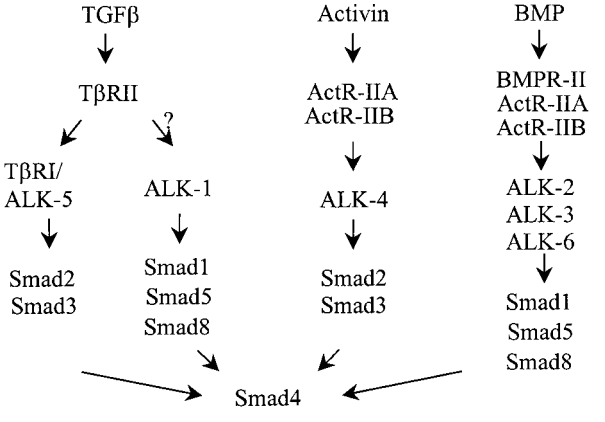

Fig. 1. TGFβ signal transduction. *TGFβ family members signal through a distinct set of type I and type II receptors resulting in the phosphorylation of receptor regulated Smad molecules. Upon activation, the R-Smad forms a complex with Smad4, a co-Smad, and translocates to the nucleus to regulate transcription. Four distinct type II and seven type I receptors have been identified and 5 receptor regulated Smads. ActR: Activin receptor; BMPR: BMP receptor.*

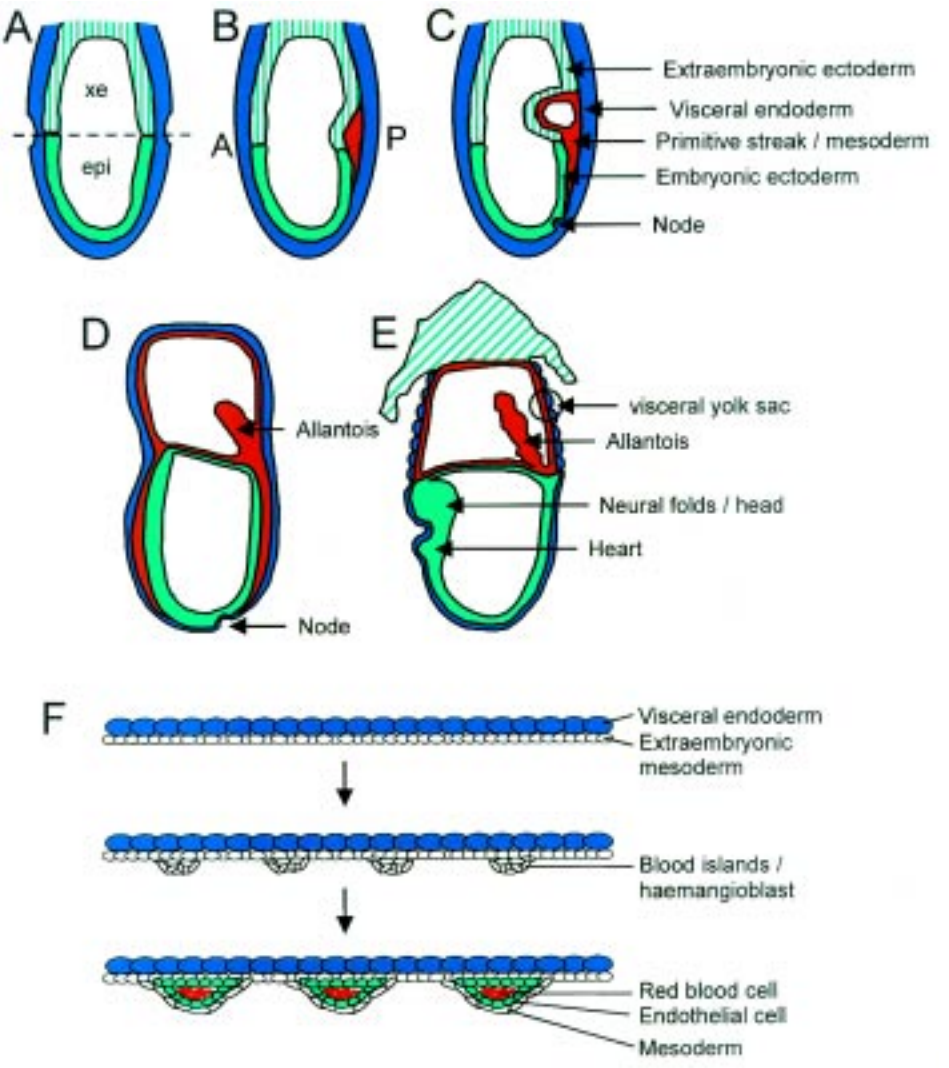

Fig. 2. Schematic representation of early mouse development. A) *Prestreak stage: after implantation in the uterine wall, the ectodermal and endodermal cells of the mouse embryo start to proliferate rapidly and organise into a two-layered structure known as the egg cylinder. By E6.0, a distinct demarcation divides the egg cylinder into an embryonic (epiblast) and an extraembryonic part. Cuboidal cells of the visceral endoderm derived from the initial primitive endoderm surround the entire structure.* **B)** *Early streak stage: at E6.5, cells delaminate from the epiblast and accumulate as a layer of individual cells between the ectoderm and the visceral endoderm. The appearance of this new cell layer, the mesoderm, marks the beginning of gastrulation. The embryo has a clear anterior-posterior-polarity in that the primitive streak marks the posterior side.* **C)** *The primitive streak then elongates through intercalation of newly recruited epiblast cells to regions proximal to the anterior aspect of the streak, which is marked by a structure known as the node.* **D)** *At E7.5, the mesoderm has moved to the anterior side of the epiblast. The extraembryonic mesoderm differentiates and forms the allantois and the mesodermal component of the visceral yolk sac.* **E)** *At E8.5, the allantois is elongated, the neural folds and heart are developing and there is a clear anterior-posterior axis.* **F)** *Schematic representation of the development of the visceral yolk sac. Mesodermal cells in the visceral yolk sac begin to proliferate, forming an irregular rim of thickened mesoderm masses known as blood islands or hemangioblasts. The hemangioblast starts to differentiate and will form primitive nucleated red blood cells that produce haemoglobin and loose their intracellular attachments. The outer cells of the blood islands will flatten, form a primitive plexus and gradually organise into a vascular network. Xe: extraembryonic ectoderm; epi: epiblast.*

post-implantation development of the mouse embryo and the cell and tissue types that form are summarized briefly in the following section and are illustrated schematically in Fig. 2.

Soon after implantation in the uterine wall, the ectodermal and endodermal cells of the mouse embryo start to proliferate rapidly and organise into a two-layered structure known as the egg cylinder (Fig. 2A). Simultaneously, the polar trophectoderm, an extraembryonic tissue in contact with the inner cell mass of the blastocyst prior to implantation, also begins to proliferate and extend into the blastocoelic cavity to give rise to the extraembryonic ectoderm, so that by E6.0, a distinct demarcation divides the egg cylinder into embryonic and extraembryonic parts (Fig. 2B). Cuboidal cells of the visceral endoderm, derived from the initial primitive endoderm, now surround the entire structure, although they flatten distally and become squamous at the most distal tip. At E6.5, cells delaminate from the epiblast and accumulate as a layer of individual cells between the epiblast and the visceral endoderm. The appearance of this new cell layer, the mesoderm, marks the beginning of gastrulation. At E7.0, the primitive streak elongates through intercalation of newly recruited epiblast cells to regions proximal to the anterior aspect of the streak, which is marked by a structure known as the node. The epiblast also gives rise to extraembryonic mesoderm that forms the allantois and the mesodermal component of the yolk sac (Fig. 2D,E).

Defects secondary to loss of signals from extraembryonic tissues

The first TGFβ signal transduction cascade that is important for gastrulation (Fig. 2) is most likely to be that initiated by nodal signalling through activin type II receptors, ALK-4, Smad2 and Smad4 (Table 3). *Nodal* was originally identified as the gene affected by a retrovirally-induced recessive lethal mutation. Subsequent cloning showed that it was a ligand belonging to the TGFβ superfamily (Zhou *et al.*, 1993; Conlon *et al.*, 1994). In pregastrulation embryos, *nodal* is expressed throughout the epiblast but becomes rapidly restricted to the posterior region of the epiblast around the start of gastrulation and then along the primitive streak during its elongation. *Nodal* is also transiently expressed in the primitive endoderm. At the late primitive streak stage (Fig. 2), expression is restricted to a small group of cells surrounding the node region. Nodal deficient embryos arrest just before gastrulation, lack mesoderm and do not form a primitive streak, indicating that nodal may

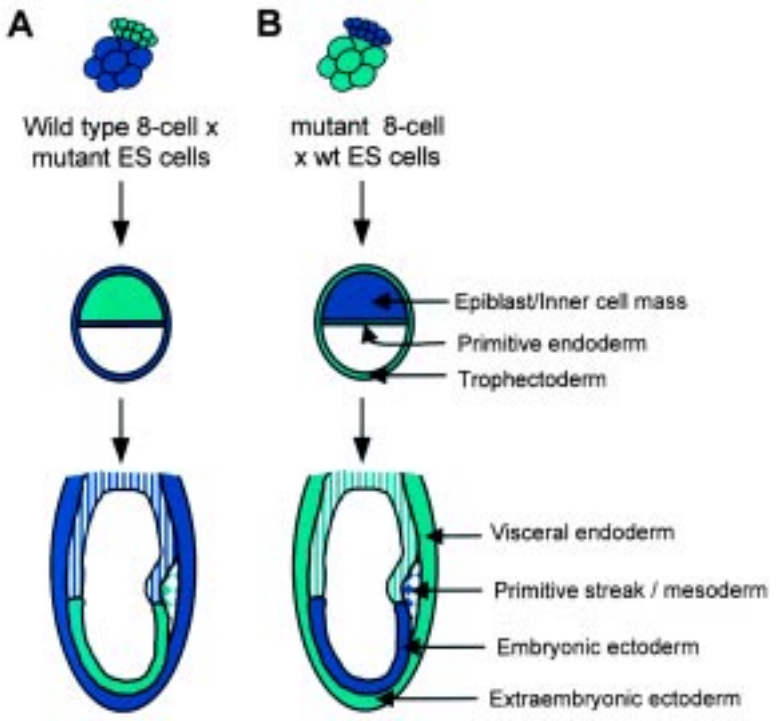

Fig. 3. Chimeric rescue experiments. *Analysis of chimeric embryos generated by incorporation of ES cells into host morulae or blastocysts can reveal the primary site of action of a gene. Combining mutant ES cells with a wild-type morula generates a chimera in which the epiblast that gives rise to the entire embryo as well as the extraembryonic mesoderm will be derived from mutant ES cells. The wild-type morula cells form the primitive endoderm, that gives rise to the entire endoderm of the yolk sac and the trophectoderm, which will form the trophoblast layers of the placenta. The use of a tetraploid (4N) host embryo, generated by electrofusion of the blastomeres of a two-cell embryo, will ensure that the embryo will be almost 100% ES cell derived. Conversely, by combining a mutant embryo with wild-type ES cells, the requirement for the mutated gene in the visceral endoderm or extraembryonic ectoderm in normal development can be monitored.*

play an important role in this process. Chimeric experiments (Fig. 3), in which the extraembryonic tissues were nodal deficient and the embryo proper was derived from wild-type tissues, revealed that the primary function of nodal lies in the extraembryonic tissue, although there were pronounced abnormalities later in development in the anterior neural tube (Table 2; Varlet *et al.*, 1997).

To date, unique functions for individual type II receptors in early murine development, with the exception of TβRII (see below) have not been demonstrated (although note that the BMPR-II ablation data is not yet available). However, Song and co-workers (1999) have generated double mutants for ActR-IIA and ActR-IIB. They reasoned that these receptors might be functionally interchangeable since they are both expressed in early mouse embryos. In addition, data from *Xenopus laevis* suggested a role for ActR-II (an earlier nomenclature for ActR-IIA) in gastrulation (Schulte-Merker *et al.*, 1994, Dyson and Gurdon, 1997). ActR-IIA is expressed from E6.5 in both embryonic and extraembryonic ectoderm and in the visceral endoderm. At E7.5, all 3 germ layers express ActR-IIA. ActR-IIB is ubiquitously expressed in early mouse embryos except for the visceral endoderm (Mummery and van den Eijnden-van Raaij, 1999). Embryos deficient in both ActR-IIA and -IIB are arrested at the egg cylinder stage, with growth of the egg cylinder impaired and no mesoderm formation (Song *et al.*, 1999). Furthermore, the same study demonstrated that one wild-type allele of either ActR-IIA or -IIB was sufficient for egg cylinder growth to proceed normally and for the initiation of gastrulation, although ActR-IIA$^{-/-}$ActR-IIB$^{+/-}$ embryos did display severe gastrulation defects, defects in anterior patterning and died eventually around midgestation. They exhibited defects in primitive streak elongation, very similar to those described for *ALK-2*-deficient mice (see below). ActR-IIA$^{+/-}$ActR-IIB$^{-/-}$ embryos on the other hand, died later in development or shortly after birth, providing genetic evidence for partially redundant functions of the two activin type II receptors.

The most likely type I receptor to participate in this nodal-ActR-II signalling cascade is ALK-4. ALK-4 is ubiquitously expressed in both embryonic and extraembryonic tissues in early embryos (E5.5-7.5), although in visceral endoderm expression is restricted to a few cells at the distal tip of the egg cylinder. ALK-4 is expressed in all 3 germ layers of the embryo proper, with more intense expression near the primitive streak. ALK4$^{-/-}$ embryos are also developmentally arrested before gastrulation (Gu *et al.*, 1998). They do not express *Brachyury T*, a marker of nascent mesoderm and no primitive streak is formed (Wilkinson *et al.*, 1990). In some mutant embryos there are fewer epiblast cells, the epiblast epithelium has lost its usual columnar shape and is thickened. Besides malformations in the embryonic tissues, ALK-4$^{-/-}$ embryos also have impaired visceral endoderm differentiation. The first abnormality in these embryos is observed at E5.5, when the distal visceral endoderm is slightly more vacuolated and detached from the epiblast. At E6.5, only vacuolated columnar visceral endoderm cells are found surrounding the egg cylinder. When the epiblast of chimeric embryos is derived from ALK-4$^{-/-}$ ES cells and the extraembryonic ectoderm and visceral endoderm are from a wild-type host, ALK-4$^{-/-}$ ES cells are able to form mesoderm (Table 2). If more than 80% of the embryo is (ALK-4$^{-/-}$) ES cell-derived, the embryos fail to undergo gastrulation, although they do form a relatively normal egg cylinder. When wild-type ES are injected into ALK-4$^{-/-}$ (host) embryos, development is still abnormal, but a relatively normal visceral yolk sac develops. Together these results indicate that ALK-4 has a function in the formation of both embryonic and extraembryonic tissues.

Targeted disruption of Smad genes has demonstrated that they play crucial roles in mediating signals from the receptor kinases during epiblast formation and gastrulation. Establishing which Smad mediates signalling downstream of each type I receptor is complicated. As will be discussed below, Smad2 and Smad4 are most likely

to be involved in signalling through ALK-4. Both Smads are ubiquitously expressed in both extraembryonic and embryonic tissues from E8.5 (Waldrip *et al.*, 1998; Yang *et al.*, 1998). Smad2-deficient embryos have been generated using a number of different targeting strategies. These include replacing the MH1 domain (Nomura and Li, 1998), inserting a *LacZ* reporter gene in the MH2 domain (Nomura and Li, 1998), deleting the C-terminal part of the MH2 domain (Weinstein *et al.*, 1998) or replacing the first coding exon (Waldrip *et al.*, 1998; Heyer *et al.*, 1999). All of these Smad2 mutations resulted in early embryonic lethality although with different phenotypes. The mutation created by deletion of the translational start site (Waldrip *et al.*, 1998; Heyer *et al.*, 1999) might have resulted in Smad2 truncated in the amino terminal domain since translation could have been initiated from a downstream methionine codon. This truncated protein would actually be expected to increase the Smad2 activity as has been demonstrated by injection of a similar protein in *Xenopus laevis* (Baker and Harland, 1996). Heyer *et al.* have so far not been able to detect any truncated protein.

Nomura and Li (1998) and Weinstein *et al.* (1998) have reported that embryos lacking Smad2 do form an organised egg cylinder

TABLE 1

MAJOR DEFECTS IN TGFβ LIGAND, RECEPTOR AND SMAD DEFICIENT MICE

Model	Phenotype	Refs
TGFβ1	Defective yolk sac vasculogenesis and haematopoiesis. Embryonic lethal (E9.5-11.5)	Dickson *et al.*, 1995
	Inflammation and autoimmunity	Shull *et al.*, 1992; Kulkarni *et al.*, 1993; Letterio *et al.*, 1996
TGFβ2	Cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, urogenital defects. Perinatal lethality	Sanford *et al.*, 1997
TGFβ3	Cleft palate, delayed lung maturation. Mutants die shortly after birth.	Proetzel *et al.*, 1995; Kaartinen *et al.*, 1995
BMP-2	Embryonic lethal (E7.5-10.5)	Zhang and Bradley, 1996.
	Failure of proamniotic canal to close, heart malformation	
BMP-4	Embryonic lethal (E7.5-9.5)	Winnier *et al.*, 1995
	1. Arrest at egg cylinder stage, lack of mesoderm	
	2. Develop until early somite stage with disorganised /truncated posterior structures and reduced extraembryonic mesoderm	
	Defects in PGC and allantois formation	Lawson *et al.*, 1999
BMP-7	Skull, eye and kidney defects	Dudley *et al.*, 1995
	Perinatal lethality	Luo *et al.*, 1995
Activin βA	Cleft pallet, lack of whiskers and incisors	Matzuk *et al.*, 1995
Activin βB	Failure of eyelid fusion. Females show impaired reproductive ability	Schrewe *et al.*, 1994; Vassalli *et al.*, 1994
Activin βA/βB	No additional defects	Matzuk *et al.*, 1995
Nodal	Embryonic lethal (E7.5). Failure in gastrulation and primitive streak formation	Conlon *et al.*, 1994
ALK-1	Embryonic lethal (E10.5-11.5)	Oh *et al.*, 2000
	Defects in angiogenesis and vsmc differentiation	
ALK-2	Embryonic lethal (E7.5-9.5)	Gu *et al.,* 1999
	Failure in primitive streak elongation, delayed mesoderm formation and malformed visceral endoderm	
ALK-3	Embryonic lethal (E7.5-9.5)	Mishina *et al.*, 1995
	Fail to form mesoderm, reduced proliferation of the epiblast	
ALK-4	Embryonic lethal (E7.5-9.5)	Gu *et al.*, 1998
	Defect in epiblast and extraembryonic ectoderm organisation and gastrulation	
ALK-6	Defects in digit formation and fore- and hindlimb development	Yi *et al.*, 2000; Baur *et al.*, 2000
TβRII	Defective yolk sac vasculogenesis	Oshima *et al.*, 1996
	Embryonic lethal (E10)	
ActR-IIA	Skeletal and facial abnormalities in percentage of mice	Matzuk *et al.*, 1995
ActR-IIB	Mice die after birth	Oh and Li, 1997
	Cardiac defects associated with defects in left-right asymmetry	
	Homeotic transformation in the skeleton	
ActR-IIA/ActR-IIB	Embryonic lethal	Song *et al.*, 1999
	Defect in primitive streak formation and gastrulation	
Endoglin	Defective yolk sac vasculogenesis, embryonic angiogenesis and vsmc development.	Li *et al.*, 1999;
	Embryonic lethal (E10.5-11.5)	Arthur *et al.*, 2000;
	Cardiac malformations	Bourdeau *et al.*, 1999
Smad1	Failure in establishing chorion-allantoic circulation. Embryonic lethal (E9.5)	Lechleider *et al.*, Pers. Com.
Smad2	Embryonic lethal (E7.5-E8.5)	Weinstein *et al.*, 1998
	Failure in egg cylinder elongation, gastrulation and mesoderm formation	Nomura and Li, 1998
		Waldrip *et al.*, 1998
Smad3	Metastatic colorectal cancer (4-6 months of age)	Zhu *et al.*, 1998;
	Impaired immunity and chronic infection	Datto *et al.*, 1999; Yang *et al.*, 1999b;
	Accelerated wound healing	Ashcroft *et al.*, 1999
Smad4	Growth retardation, no mesoderm formation, abnormal visceral endoderm.	Sirard *et al.*, 1998
	Embryonic lethal (E7.5-E8.5)	Yang *et al.*, 1998
Smad5	Embryonic lethal (E9.5-10.5)	Chang *et al.*, 1999, 2000
	Defect in angiogenesis, left-right asymmetry, cranialfacial abnormalities and induced mesenchymal apoptosis	Yang *et al.*, 1999a
Smad6	Cardiovascular abnormalities	Galvin *et al.*, 2000
	Defect in endocardial cushion transformation	

and recognisable anterior-posterior-patterning, but are unable to form embryonic mesoderm. *Brachyury T* expression was not detected demonstrating the absence of nascent mesoderm. The boundary between the epiblast and extraembryonic ectoderm is disrupted, and both the epiblast and extraembryonic ectoderm are disorganised (Nomura and Li, 1998; Weinstein *et al.*, 1998). Smad2 mutants were often located outside the yolk sac. In addition and in contrast to the other Smad2 deficient mice described below, Nomura and Li reported that some heterozygous embryos exhibited severe gastrulation defects. Waldrip *et al.* (1998) and Heyer *et al.* (1999) reported the second homozygous null phenotype. Their Smad2 deficient embryos survived one day longer, exhibited transient induction of mesoderm and developed extraembryonic tissues such as the visceral yolk sac, but fail to establish distinct anterior-posterior polarity, as demonstrated by a lack of *Hex-1* (Heyer *et al.*, 1999). Furthermore, the entire epiblast adopted a posterior mesodermal fate expressing *Brachyury T*.

Injection of wild-type ES cells into Smad2$^{-/-}$ blastocysts, did not rescue the phenotype in chimeras, indicating that, as for nodal, activin type II receptors and ALK-4, a Smad2-deficient extraembryonic region does not support wild-type epiblast development to form embryonic mesoderm (Table 2; Waldrip *et al.*, 1998). When Smad2 homozygous ES cells were injected into a wild-type tetraploid embryo so that the extraembryonic tissues, with the exception of extraembryonic mesoderm, are host derived, gastrulation proceeded normally but the embryos did eventually show defects associated with left/right asymmetry and in anterior development (Heyer *et al.*, 1999).

Smad4$^{-/-}$ embryos also arrest early in development (Sirard *et al.*, 1998; Yang *et al.*, 1998). They are growth retarded, have abnormal visceral endoderm and fail to form mesoderm. At E5.5, Smad4$^{-/-}$ embryos are morphologically normal, but at E6.5 they are reduced in size mainly due to smaller extraembryonic regions. There is little or no elongation of the extraembryonic region and no clear boundary between the embryonic and extraembryonic part of the egg cylinder. Furthermore, the proliferation rate of the epiblast cells is decreased. The early mesoderm and gastrulation defects are rescued by aggregation of Smad4$^{-/-}$ cells with tetraploid host embryos (Table 2). The signals provided by the wild-type extraembryonic region are apparently capable of rescuing gastrulation (Sirard *et al.*, 1998). However, there are still substantial anterior defects after gastrulation, which were not rescued by wild-type extraembryonic tissues. Furthermore, Smad4$^{-/-}$ ES cells are capable of forming mesodermal derivatives *in vitro* indicating that there is no cell autonomous requirement for Smad4 in early mesoderm development. The chimeric rescue experiments demonstrate that both Smad2 and Smad4 deficient embryos overcome their gastrulation defect if wild-type extraembryonic tissue is provided, but that both have additional functions later in development (see below for discussion).

These gene ablation and chimeric experiments illustrate accumulating evidence that TGFβ superfamily members play important roles in the extraembryonic region. Nodal synthesis in the visceral endoderm is necessary for patterning of the anterior axis, a Smad2 deficient extraembryonic region does not support the formation of embryonic mesoderm from wild-type epiblast and a wild-type extraembryonic region rescues the gastrulation phenotype of Smad4-deficient embryos. Furthermore, an ALK-4 deficient extraembryonic region does not support wild-type epiblast development. Smad4-deficient embryos show a defect in the differentiation and function of the visceral endoderm (an extraembryonic tissue), as do ALK-4 and Smad2 mutant mice. Although both nodal and ALK-4 are required in both the epiblast as well as the extraembryonic tissues, the primary requirement for Smad2 and Smad4 is in the extraembryonic cells during early development. These results indicate that TGFβ signals are essential for the ability of the extraembryonic region to ensure normal embryonic development and that signals from both the extraembryonic cells and the epiblast are necessary to form embryonic mesoderm. The striking similarity, including a complete lack of mesoderm, between *nodal* knockout embryos and the ALK-4/ Smad2/ Smad4 phenotypes, provide genetic evidence that nodal may be part of this signalling cascade. In summary, nodal, ActR-II, ALK-4, Smad2 and Smad4 may function in the same signalling pathway and be the first essential combination of TGFβ superfamily ligands, receptors and Smads for further development.

TABLE 2

TGFβ DEFICIENT MICE EXHIBITING GASTRULATION DEFECTS

	ALK-2	ALK-3	ALK-4	Smad2	Smad4
Mutant phenotype	No elongation of PS, abnormal VE. Lethal at E9.5	Defect in mesoderm formation and cell proliferation. Lethal at E6.5-9.5	Defect in mesoderm formation. Lethal at E6.5-9.5	Defect in mesoderm formation. Lethal at E6.5-9.5	Defect in mesoderm formation. Lethal at E6.5
WT embryonic (ES wt) Mutant extraembryonic	No rescue		No rescue	No rescue	
Mutant embryonic (ES$^{-/-}$) WT extraembryonic	Early defect rescued, but defect in posterior development at E9.5.		Mesoderm formation rescued if < 80% -/- ES cells contributed to the embryo	Early defect rescued, defect in anterior development and left/right asymmetry at E9.5.	Early defect rescued, but defect in anterior development at E9.5.
In Vitro assays	EBs form no mesoderm Defective VE	Normal growth of blastocyst in culture			EBs form mesoderm
Teratoma		Form mesoderm derived structures, but less than wt	Form mesoderm derived structures, but less than wt	Form mesoderm derived structures, but less than wt	

VE: visceral endoderm, PS: primitive streak, EB: embryoid body; wt: wild-type

Defects due to a loss of BMP signals

In addition to the requirement for an activin/TGFβ type signal (nodal) for the formation of the third (mesodermal) germ layer, activation of the BMP signal cascade is also important for early development (Table 3). Before and around gastrulation, *BMP-4* is expressed in the extraembryonic ectoderm. At later gastrulation stages, *BMP-4* is detected both in the extraembryonic ectoderm, as well as in the mesoderm (Lawson *et al.*, 1999). Embryos homozygous for a null mutation in BMP-4 exhibit a variable phenotype and die between E6.5 and E9.5 (Winnier *et al.*, 1995). Most BMP-4 mutants do not advance beyond the egg cylinder stage, show little or no mesoderm formation and do not express *Brachyury T*. There is a paucity of extraembryonic mesoderm, resulting in a blebby appearance of the yolk sac. BMP-4 deficient embryos contain no primordial germ cells, the cells that ultimately give rise to the gametes, (Lawson *et al.*, 1999) and lack an allantois, that is derived from extraembryonic mesoderm and eventually contributes to the umbilical cord. On certain genetic backgrounds, more advanced BMP4$^{-/-}$ embryos develop to the early somite stage and even undergo turning, but all mutants show disorganised or truncated posterior structures. Chimeric rescue experiments, in which the extraembryonic ectoderm and visceral endoderm is derived from BMP-4 deficient (host) embryos, and the epiblast is formed by wild-type ES cells (Table 2), demonstrated that BMP-4 produced by the extraembryonic ectoderm is required by the epiblast to develop the allantois and allocate primordial germ cells (Lawson *et al.*, 1999).

Among the type I receptor mutants described so far, ALK-3 or BMPR-IA is most likely the endogenous receptor for BMP-4 since the recent description of the ALK-6$^{-/-}$ mutant documents only a postnatal phenotype (Baur *et al.*, 2000; Yi *et al.*, 2000). *ALK-3* is ubiquitously expressed in the egg cylinder of pregastrulation embryos (de Wulf *et al.*, 1995). ALK-3 deficient embryos (Mishina *et al.*, 1995) show an overt phenotype from E7.0 onwards. Morphological and molecular analysis revealed that the egg cylinder was smaller, no mesoderm formed and the embryos failed to undergo gastrulation. Although the growth of ALK-3 $^{-/-}$ blastocysts in culture was indistinguishable from controls, there was a decrease in epiblast proliferation *in vivo* at E6.5, before morphological abnormalities could be observed. By creating teratomas from E7.0 embryos via injection into syngeneic hosts, Mishina *et al.* (1995) demonstrated that ALK-3 mutant cells are capable of forming mesodermal derivatives, although to a lesser extent than their wild-type or heterozygous counterparts. This suggests that the lack of mesoderm in the embryos is not an intrinsic defect in the capacity of the epiblast to form mesoderm but an inability to generate or respond to cues for mesoderm differentiation *in vivo*.

Both BMP-4 and ALK-3 are necessary for normal development through gastrulation, but the primary requirement for Smad4 is in the extraembryonic tissues. Although Smad4 deficient embryos are morphologically identical to ALK-3$^{-/-}$ embryos, cultured ALK-3 deficient blastocysts proliferate and differentiate normally, whereas Yang *et al.* (1998) reported that Smad4$^{-/-}$ blastocysts display a cellular defect in proliferation and endoderm differentiation. This suggests that a Smad4-independent pathway for transducing the TGFβ signal exists downstream of ALK-3 in the early epiblast or alternatively, that there is at least one other co-Smad in mammals which may be involved in early epiblast development, as has recently been described for *Xenopus laevis* (Masuyama *et al.*, 1999; Howell *et al.*, 1999). The non-redundant function of ALK-2, -3 and -4 illustrates the complexity of TGFβ signalling in regulation of morphogenesis during early development.

Defects in anterior development

Chimeric analysis (Table 2) and crossing of several heterozygous mutants have revealed that besides a function in gastrulation and streak formation, an activin-like signal transduction cascade is involved in anterior head development. This function became evident after the early gastrulation defects were rescued in chimeric embryos and development proceeded until E9.5. In chimeric mice, combining nodal (Varlet *et al.*, 1997), ActR-IIA/IIB (Song *et al.*, 1999), Smad2 (Nomura and Li, 1998) or Smad4 (Sirard *et al.*, 1998) -deficient embryonic tissues with wild-type extraembryonic ectoderm and visceral endoderm, the primitive streak was formed but failed to elongate, an effect most likely caused by impaired movement of the mesoderm. At E9.5, the different mutant embryos show anterior abnormalities ranging from absence of mandibles to cyclopia or even complete lack of a forebrain. These experiments demonstrated that a TGFβ signal originating from the anterior visceral endoderm is required to pattern anterior development. This phenotype was not only observed in the different chimeras, but also nodal$^{+/-}$/ActR-IIA$^{-/-}$, ActR-IIA$^{-/-}$IIB$^{+/-}$ and nodal/Smad2 *trans*-heterozygous had craniofacial abnormalities and defects in forebrain development linking these molecules to the same signal transduction cascade

Defects in the progression of gastrulation

Another signal transduction pathway crucial for development that became evident through gene ablation is signalling through ALK-2, probably initiated by BMP-2 or BMP5/7 and leading to

TABLE 3

POSSIBLE SIGNAL TRANSDUCTION CASCADES INVOLVED IN DEVELOPMENTAL PROCESSES

Developmental process affected	Signal transduction cascade
Defects in mesoderm formation and gastrulation	Nodal ▼
Later in development: Defects in left-right asymmetry and anterior development	ActR-IIA/B ▼ ALK-4 ▼ Smad2 Smad4
Defect in epiblast proliferation and initiation of gastrulation	BMP-4 ▼ ALK-3 ▼ Smad4
Defect in the progression of gastrulation	BMP-2, BMP5/7 ▼
Later in development: Defects in left-right asymmetry and anterior-posterior development	ALK-2 ▼ Smad1, Smad5
Defect in vasculogenesis and haematopoiesis	TGFβ1 ▼ TβRII, Endoglin ▼ Alk-1 ▼ Smad5

activation of Smad1 and/or Smad5 (Table 3). These mutants are discussed in the following section.

ALK-2 is expressed in the extraembryonic endoderm at the time of gastrulation. Mouse embryos deficient in ALK-2 were arrested shortly after the onset of gastrulation. The formation and growth of the egg cylinder was apparently normal (Mishina *et al.*, 1999; Gu *et al.*, 1999). The primitive streak initially formed but then failed to elongate correctly, as indicated by a small group of *Brachyury T* positive cells near the embryonic/ extraembryonic boundary. The ALK-2 deficient embryos display a thickening of the embryonic ectoderm, delayed and severely impaired mesoderm formation and they lack an amniotic fold and allantois. Furthermore, the morphology of the proximal visceral endoderm was abnormal. They do, however establish an anterior-posterior axis by E8.5; *HesX1*, an anterior marker, is expressed opposite to *Brachyury T* that is a posterior gene at this stage. The morphological defects in ALK-$2^{-/-}$ embryos suggest a failure of the epiblast cells to intercalate into the early primitive streak. The phenotype was not rescued in chimeras when the epiblast was derived from wild-type ES cells with ALK-$2^{-/-}$ extraembryonic ectoderm and visceral endoderm, suggesting that ALK-2 mediated signalling, like ALK-4, is indirectly involved in mesoderm formation since its signal originates from the extraembryonic tissues (Table 2). ALK$2^{-/-}$ ES cells are able to contribute to all three germ layers. However, embryos that are completely derived from mutant ES cells exhibit a severely reduced posterior region at E9.5. In *in vitro* assays, ALK-$2^{-/-}$ embryoid bodies did not form a mesoderm layer and had defective visceral endoderm differentiation, lacking for example *HNF4* (Gu *et al.*, 1999). ALK-2 most likely mediates BMP signals, as does ALK-3, but it does not affect egg cylinder growth as had been observed in ALK-3 deficient mice.

The BMP ligand signalling via ALK-2 is very likely to be BMP-2. The phenotype of BMP-2 deficient mice resembles that of ALK-$2^{-/-}$ embryos (Zhang and Bradley, 1996; Gu *et al.*, 1999). Both mutants show abnormal development of amnion, chorion, proamniotic canal and the allantois. BMP-$2^{-/-}$ mice showed moreover a defect in cardiac development, manifested by an abnormal development of the heart in the exocoelomic cavity.

More intriguing is the question of which Smad is signalling downstream of ALK-2 in this developmental process. Smad 1 is one of the "BMP" Smads and is expressed firstly in the early embryonic mesoderm and later ubiquitously (Waldrip *et al.*, 1998). *Smad5*, on the other hand, is expressed in the epiblast only and not in the extraembryonic endo- and mesoderm at E6.5 but becomes ubiquitous from E7.5 (Chang *et al.*, 1999).

Smad1 deficient mice die around E9.5 due to a failure in establishing the chorion-allantoic circulation (R. Lechleider, personal communication). The allantois fails to elongate in nearly all Smad1 mutants and no chorio-allantoic placenta is formed. The embryo proper is developmentally retarded, most likely due to a lack of nutrition, but otherwise there are no apparent morphological abnormalities. In this respect, the phenotype of the Smad1 null mice resembles that of the BMP-2, BMP-4 or BMP5/7 (double) knockouts (Solloway and Robertson, 1999). Besides a failure in chorion/allantoic fusion, the BMP5/7 double mutants exhibited retarded heart development. Heart morphogenesis is delayed both through altered growth and differentiation. Heart function was severely compromised and was the likely cause of embryonic lethality around E10.5. The BMP5/7 deficient mice furthermore showed delayed rostral closure of the neural tube, suggesting a regulatory role in the growth of the telencephalon and metencephalon; branchial arch outgrowth was also dramatically reduced. They often displayed abnormalities in somite morphogenesis. The underlying mechanism for all of these defects might be abnormal apoptosis. Increased apoptosis was also observed in Smad5 deficient mice (Yang *et al.*, 1999a) suggesting a common signal transduction pathway. Furthermore, the cardiac and extraembryonic defects in Smad5 deficient mice (Chang *et al.*, 1999) are similar but less severe then those in the BMP-2 knockout suggesting again a common pathway. Together the data suggest that signalling via ALK-2 is mediated by more than one BMP (BMP-2, BMP-5/7) and Smads (Smad1 and/or Smad5).

Defects in cardiovascular system: vasculogenesis, angiogenesis and hematopoiesis

The cardiovascular system is the first functional organ system to develop in vertebrate embryos (Fig. 2). Its development starts with the appearance of discrete blood islands in the yolk sac at E7.5-8.0. Extraembryonic mesoderm cells derived from the epiblast will differentiate into hemangioblasts, bipotential stem cells capable to differentiate into haematopoietic and endothelial cells. The hemangioblasts form a primitive plexus in a process known as vasculogenesis. The emerging vascular plexus undergoes rapid remodelling and pruning to form a mature tree-like network with larger vitelline vessels feeding into smaller capillaries. Angiogenesis involves the differential growth and sprouting of endothelial tubes and recruitment and differentiation of mesenchymal cells into vascular smooth muscle cells and pericytes. Angiogenesis requires extensive interactions of endothelial cells with themselves and the extracellular matrix, pericytes or smooth muscle cells. TGFβ is able to signal in endothelial cells via two pathways (Fig.1). In addition to using the classical type I receptor, TβRI or ALK-5 and phosphorylation of Smad2 and Smad3, it has recently been demonstrated that TGFβ might also be able to use ALK-1 to transduce signals and phosphorylate Smad1or Smad5 (Macias-Silva *et al.*, 1998; Chen and Massagué 1999; Oh *et al.*, 2000). Furthermore, endothelial cells express a specific type III receptor endoglin that is involved in the receptor ligand complex formation. That TGFβ signalling is indeed of importance for vasculogenesis, angiogenesis, haematopoiesis as well as smooth muscle cell differentiation, becomes evident by comparing embryos lacking TGFβ1, endoglin, TGFβ receptor II (TβRII), ALK-1 and Smad5. These embryos can be divided into two groups based on their phenotypes. In one group the formation of the vessels in the yolk sac is affected (TGFβ1, TβRII) and in the other the yolk sac vasculature, as well as the vessels in the embryo and the vascular smooth muscle cells are affected (endoglin, ALK-1, Smad5).

The first report on TGFβ1 deficient mice suggested that the ligand was only required postnatally (Shull *et al.*, 1992; Kulkarni *et al.*, 1993). Later it became evident that on certain genetic backgrounds transplacental and lactational transfer of maternal TGFβ1 from TGFβ1 heterozygous mothers to their ligand deficient foetuses and lactating pups may have been sufficient to rescue their null phenotype until after weaning (Letterio *et al.*, 1994). On a C57BL/6 background however, 99% of all knockout animals died of a preimplantation defect (Kallapur *et al.*, 1999). This was in agreement with independent results in which zygote injection of a

dominant negative (dn) TβRII blocked development beyond the 2-cell stage (Roelen *et al.*, 1998). On the other hand, if a TGFβ1 deficient allele is crossed into a mixed 129 x NIH/Ola x C57BL/6 background, 50% of the mutant embryos and 20% of TGFβ1$^{+/-}$ embryos die around E10.5 due to inadequate yolk sac development (Dickson *et al.*, 1995; Bonyadi *et al.*, 1997). They show a failure in both vasculogenesis and haematopoiesis. There is a defect in terminal differentiation of the endothelial cells in the yolk sac, affecting endothelial tube formation and/or its integrity resulting in insufficient capillary tube formation and weak vessels. However, there were no obvious abnormalities in vascular development within the TGFβ1 deficient embryos themselves at E9.5, although at later stages, the embryos did show secondary defects due to impaired circulation and hypoxia. The defect in haematopoiesis resulted in a reduced number of red blood cells within the yolk sac causing yolk sac anaemia.

TβRII deficient embryos (Oshima *et al.*, 1996) as well as chimeric embryos overexpressing a dominant negative TβRII in the extraembryonic mesoderm of the yolk sac (Goumans *et al.*, 1999) show a failure to remodel the primitive plexus into a vascular network of robust vessels. There is a reduction in cellular adhesiveness between the yolk sac layers, causing separation of the visceral endoderm and the extraembryonic mesoderm. Analysis of the differentiation capacity of ES cells *in vitro* demonstrated that embryoid bodies expressing dnTβRII were defective in endothelial cell differentiation and the formation of haematopoietic cells. Furthermore, they produced lower levels of fibronectin, an extracellular matrix protein normally deposited between the two layers of the yolk sac, which might cause the reduced cellular adhesiveness. Indirect immunofluorescence demonstrated reduced fibronectin staining between the visceral endoderm and mesoderm components of the yolk sac in chimeric embryos derived from dnTβRII-ES cells. This must have resulted from reduced secretion of fibronectin by the mesodermal layer since it is this layer and not the visceral endoderm that expresses *TβRII*, *ALK-5* and *ALK-1* (Goumans *et al.*, 1999).

Endoglin deficient embryos die around E11.5 from defective vascular development (Li *et al.*, 1999; Arthur *et al.*, 1999; Bordeau *et al.*, 1999). They lack vascular organisation and fail to form mature blood vessels in the yolk sac. Although disruption of endothelial organisation occurred between E9.5-E10.5, poor vascular smooth muscle cell development is evident in the endoglin deficient yolk sacs by E8.5. The presence of an immature perineural vascular plexus at E10.5 is indicative of failure of endothelial remodelling not only in the yolk sac, but also in the embryo proper, although to a lesser extent. Although there was extensive endothelial remodelling of the vasculature in wild-type littermates at E10.5, the major vessels including the dorsal aortae, intersomitic vessels, branchial arches, and carotid arteries were atretic and disorganised in some of the endoglin$^{-/-}$ embryos (Li *et al.*, 1999). When embryos were stained with an antibody to α-smooth muscle cell actin, it became clear that there was a significant difference in the development of the vascular smooth muscle cells at E9.5 between endoglin$^{-/-}$ and wild-type embryos, since no smooth muscle cells surrounded the major vessels of the endoglin deficient embryos. This defect in vascular smooth muscle cell differentiation preceded the defect in endothelial remodelling (Li *et al.*, 1999). Besides a defect in angiogenesis, Arthur and co-workers showed that endoglin deficient embryos have reduced levels of haematopoiesis in the yolk sac. In addition, many endoglin$^{-/-}$ embryos show evidence of abnormal cardiac development (Arthur *et al.*, 1999) manifested as enlarged ventrical and outflow tract, abnormal cardiac looping and pericardial effusion, and failure of the endothelial surface of the truncal cushions to organise correctly. Interestingly, heterozygous mice on a 129/ola background exhibited extensive dilated and weak-walled vessels, typical of a disease known as Hereditary Haemorrhagic Telangiectasia (HHT), suggesting that this mouse could be a good model to study this human genetic disorder, since mutations in endoglin or ALK-1 can cause HHT in humans (McAllister *et al.*, 1994; Johnson *et al.*, 1996).

As might then be expected, ALK-1 deficient embryos die around E11.5 and show defects similar to the endoglin mutant mice (Oh *et al.*, 2000). At E8.5, ALK-1$^{-/-}$ embryos were morphologically indistinguishable from their wild-type littermates but by E9.5-10.5, they develop clear defects. Posterior development of the embryo was abnormal, mature vessels in the yolk sac were absent, clusters of blood cells formed and there was pericardial effusion. Furthermore, the vessels in the yolk sac were highly dilated. The embryo proper showed excessive fusion of capillary networks at E9.5, not seen in endoglin deficient mice and dilation of the large vessels. Analysis of the expression of genes involved in vessel formation revealed that ALK-1 *null* embryos have elevated expression of vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2) and urokinase-type plasminogen activator (uPA), which could cause the excessive growth of endothelial cells and fusion of capillary vessels seen in the mutant embryos. By crossing ALK-1$^{+/-}$ mice with transgenic mice containing a *LacZ* transgene driven by a smooth muscle cell marker (SM22α), Oh and co-workers demonstrated that ALK-1 deficient embryos exhibit delayed differentiation of the vascular smooth muscle cells and failure of these cells to localise to the perivascular regions surrounding the endothelium. This study also demonstrated a TGFβ dependent interaction between ALK-1 and Smad1 and -5, thereby linking TGFβ1, via endoglin, TβRII and ALK-1 to Smad1 and -5 downstream signalling cascade.

The Smad gene used in this signalling cascade is most likely Smad5. Smad5 deficient embryos die between E10.5-11.5 caused by a defect in the circulatory system (Yang *et al.*, 1999a; Chang *et al.*, 1999). At E9.0, the yolk sacs of Smad5$^{-/-}$ mice contained red blood cells but although they possessed a primitive plexus, they lacked a well-organised yolk sac vasculature with vitelline vessels and capillary sprouts. Furthermore, as has been described for the TGFβ1 and TβRII deficient embryos, the two layers of the yolk sac were often loosely attached. However, the integrity of the vessels in the embryos proper was also affected, which was not the case in TGFβ1 or TβRII deficient embryos. Smad5 mutants have enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells. At E9.5, there was no capillary network formation in the developing brain, suggesting also a defect in branching of the existing vessels in the embryo proper.

Since all of these phenotypes result from a defect in angiogenesis (Table 3) it is tempting to speculate that there are two TGFβ1 signalling cascades in endothelial cells. TGFβ bound to endoglin-TβRII-ALK-5 gives rise to a signal for endothelial cell proliferation and differentiation, as well as the production of extracellular matrix proteins. On the other hand, TGFβ bound to endoglin-TβRII-ALK-1 and phosphorylation of Smad5 is necessary to induce or maintain the endothelium in a quiescent state by inhibiting endothelial cell proliferation and to stimulate the differentiation of mesenchymal cells to

form a mature vascular wall surrounded by vascular smooth muscle cells. Why endothelial cells developed these two pathways activated in response to the same ligand and what the different downstream effectors are is still unclear; crossing appropriate knockout mice for genes in this pathway may help to unravel the puzzle.

Defects in establishment of left-right asymmetry

Left-right asymmetry is the process in which visceral organs become asymmetrically localised in position or shape along the left-right axis, e.g. the heart is on the left-hand side. Heart looping (leftward instead of rightward) and embryonic turning (clockwise instead of anticlockwise) are the two earliest morphogenetic events that lead to left-right asymmetry. The involvement of signalling by TGFβ superfamily members in the establishment of left-right asymmetry has been illustrated by several mutant mice. For example in *nodal* (Collignon *et al.*, 1996), activin type IIB receptor (Oh and Li, 1997) and Smad2 deficient mouse embryos (Nomura and Li, 1998) left-right asymmetry is abnormal indicating the importance of an activin-like signal. Whereas the expression of *Smad2* and *ActR-IIB* is bilateral in the early embryo, *nodal* is expressed asymmetrically which might result in a signal via ActR-IIB/Smad2 on the right side of the embryo only. That these genes most likely belong to one signalling cascade was demonstrated by Oh and Li (1998) showing that Smad2 and nodal *trans*-heterozygous mice exhibit defects in left-right patterning. Although the ActR-IIB deficient mice were born, defects in left-right asymmetry resulted in e.g. cardiac malformation caused by transposition of the great artery and right pulmonary isomerism (Oh and Li, 1997).

The defects in embryonic turning and heart looping observed in the Smad5 knockout indicates that a 'BMP-signal' is involved in left-right patterning. Although Smad5 is also ubiquitously expressed around the time of the establishment of left-right asymmetry, Smad5 deficient embryos exhibited a defect in heart looping and axial-rotation. *Nodal*, normally only expressed on the right side by E8.5, was found to be expressed bilaterally in Smad5$^{-/-}$ mice (Chang *et al.*, 2000). Mutant mice for BMP-2, a ligand normally not expressed asymmetrically, show similar defects in embryonic turning. This suggests a signalling cascade via BMP-2 to Smad5, most likely through an asymmetrically expressed receptor complex or Smad cofactor to inhibit the expression of *nodal* on the left side.

Gene dosage effect

An increasing body of evidence demonstrates that ligands of the TGFβ superfamily can elicit a spectrum of concentration dependent responses (Ferguson and Anderson, 1992; Green *et al.*, 1992; Gurdon *et al.*, 1994). A gene dosage effect has been observed in mice deficient in ligands such as BMP-4 (Dunn *et al.*, 1997; Lawson *et al.*, 1999) and *nodal*; receptors such as ActR-IIA/B (Song *et al.*, 1999) and downstream effectors like Smad2 (Nomura and Li, 1998).

Lawson *et al.* (1999) for example, demonstrated that in wild-type mice, 45 epiblast cells were allocated to the PGC lineage at E7.2, whereas in BMP-4 heterozygous mice this number was reduced by half. Nomura and Li (1998) reported that 20% of the Smad2 heterozygous embryos exhibited severe gastrulation defects and lacked mandibles or eyes at later stages and almost all mice *trans*-heterozygous for both Smad2 and nodal displayed craniofacial abnormalities, indicating that the gene dosage is critical for signalling.

To demonstrate that the activin type II receptors also function in a gene dosage-dependent manner during early development, Song *et al.* (1999) analysed ActR-IIA$^{-/-}$nodal$^{+/-}$ embryos. These embryos showed the same defects as ActRIIA$^{+/-}$ActRIIB$^{-/-}$ deficient mice, demonstrating a synergistic effect of nodal and activin type II receptors in gastrulation and anterior patterning.

Dose-dependency has also been suggested for TGFβ1 and its function in yolk sac development and haematopoiesis (Dickson *et al.*, 1995; Goumans *et al.*, 1999).

This demonstrates that signalling by at least some members of the TGFβ superfamily in mammals may function with thresholds, regulated at the level of ligand, receptor and downstream targets as was previously demonstrated for activin in amphibians (Gurdon *et al.*, 1994).

Postnatal phenotypes

In the light of the phenotypes of all the above mentioned mice deficient in one of the R-Smads, Smad3$^{-/-}$ mice were, surprisingly, born alive. Although *Smad3* is expressed in the developing mouse embryo, Smad3 mutants die between 1 and 8 months of age (Zhu *et al.*, 1998; Datto *et al.*, 1999; Yang *et al.*, 1999b). They are smaller than wild-type littermates and exhibit leukocytosis and impaired mucosal immunity and die of chronic infection, as has been described for some of the TGFβ$_1$ deficient mice (Shull *et al.*, 1992; Kulkarni *et al.*, 1993). Adult Smad3 mutant mice have also been shown to develop metastatic colorectal cancer on certain genetic backgrounds (Zhu *et al.*, 1998) and have accelerated wound healing (Ashcroft *et al.*, 1999). Gene targeting thus revealed that Smad3 is not able to compensate for the early embryonic defects in Smad2 deficient mice and that Smad3 may have exclusive functions in certain adult tissues. The interchangeability of Smad2 and Smad3 in mediating TGFβ/activin signalling in most assays on cells in culture is therefore not reflected *in vivo* where there is clear functional specificity (Ashcroft *et al.*, 1999).

Summary

During recent years, our understanding of TGFβ signalling through serine/threonine kinase receptors and Smads has increased enormously. Activation of R-Smads by receptor induced phosphorylation is followed by complex formation with co-Smads and translocation to the nucleus, where the transcription of specific genes is affected and ultimately results in changes in cell behaviour. Experimental analysis primarily of epithelial cells in culture has revealed that a number of members of the TGFβ family are interchangeable in the effect they have on growth and differentiation. On the other hand, different ligands of the TGFβ superfamily can result in different responses because of cell type specific expression of other components of the signalling pathway. The relative expression levels of receptors and Smads within the cell is an important determinant of TGFβ induced responses. Functional analysis of genes in the TGFβ superfamily signal transduction cascade *in vivo* in mice either lacking entire genes, or expressing dominant negative forms of particular proteins, are providing profound new insights into the signalling cascades, their

interaction and their specificity (Table 3). For example, by phenotypical comparison and intercrossing different heterozygous mutants, it has become clear that nodal, until recently an orphan protein without receptor/signal complex, probably signals through the activin type II receptor, ALK-4 and Smad2 (Nomura and Li, 1998; Song *et al.*, 1999). Many of the genes of this cascade that have been targeted in the mouse result in early embryonic lethal phenotypes, demonstrating an important function for the BMP and TGFβ/activin-activated pathways in mesoderm formation and differentiation, but masking a possible role in later events. For example mutations in BMP2 and 4 are lethal at or soon after gastrulation so that their putative role in skeletogenesis cannot be studied in mice lacking these genes. The difference in severity of the phenotypes between ligand, receptor and Smad deficient mice suggest that other receptors and ligands may partially compensate for the loss of one protein. Chimeric analysis provides one tool for analysing later developmental functions. By rescuing the early defects it was demonstrated that TGFβ family members have an important function in anterior development and left/right asymmetry. Temporal and spatial specific gene targeting will be a powerful tool for analysing the function of TGFβ family members in for example, bone formation, angiogenesis and carcinogenesis.

Isolation of cells from the different gene targeted mice provides a unique source of material to gain more insight in the biochemical mechanisms of specific pathways. For example, use of cells deficient in Smad2 for biochemical and cell biological assays could give a better view of the function of Smad3. Smad3 deficient mice already demonstrate that there is a clear difference between Smad2 and Smad3 during development. Full descriptions of the remaining gene ablation studies of this signal transduction cascade, namely those for ALK-5, BMPR-II and Smad1 and -7 are eagerly awaited to complete the puzzle.

As more of these superfamily of ligands and their signalling pathways have been functionally dissected, it has become evident that this superfamily of growth factors plays a pivotal role in epiblast formation and gastrulation, signalling from both the epiblast as well as the extraembryonic tissues. Furthermore, it becomes clear that TGFβ is indeed important for proper vessel formation and that it might use endoglin, as well as ALK-1, ALK-5 and Smad5 to mediate this function. Further analyses of these mice should provide a clearer understanding of the mechanism of TGFβ action in vascular development and remodelling.

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Note added in proof: A description of the BmPR-II$^{-/-}$ phenotype has just been published and shown to resemble that of ALK3/BmPR-IA$^{-/-}$ embryos. Beppu, H. et al. (2000) *Dev.Biol. 221:* 249-258

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