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Developmental tumours, early differentiation and the transforming growth factor β superfamily

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ABSTRACT Embryonal carcinoma and embryonic stem cells have been very useful models for identifying some of the factors that regulate differentiation in early mammalian development. Here, we present a brief history of their original isolation and characterization and of their later introduction into the Hubrecht Laboratory. We illustrate in a review their contribution to our current understanding of the function of transforming growth factor β and ligands binding to the receptors of a related factor, activin, in development with some of our own work.

KEY WORDS: *embryonal carcinoma cells, TGFβ, mouse embryo, gene expression*

Embryonal carcinoma cells as a model system for early differentiation

Germ cell tumours have been fascinating pathologists and developmental biologists for decades. Current belief is that germ cell tumours originate from defective germ cell development during embryogenesis and since most germ cells reside in the gonads, germ cell tumours are predominantly located in the testis and ovary. These tumours, also known as teratocarcinomas, usually only occur in man and in certain inbred strains of mice. They may be composed of many kinds of cells and tissues in various stages of maturation that lack organisation (Stevens and Hummel, 1957) and include an undifferentiated stem cell population, known as embryonal carcinoma or EC cells, which accounts for the malignancy of the tumour. In mice, EC cells resemble in morphology and developmental potency the pluripotent cells of the inner cell mass of a blastocyst stage embryo. When injected into blastocysts they become incorporated into the inner cell mass and participate in normal development, contributing to the formation of all tissues, although exceptionally the germ line itself is populated at low frequency, possibly because of genetic abnormalities associated with their tumorigenic origin (Papaioannou, 1981). EC cells can be continuously propagated in culture and retain their capacity to differentiate, often to derivatives of all three germ layers, over prolonged periods (reviewed in Martin, 1980). Teratocarcinomas can also be experimentally induced in some strains of mice by grafting male genital ridges from fetuses 12.5 days post coitum (dpc) or pre- and early postimplantation embryos (6.0-7.5 dpc) to extrauterine sites, such as the testis or kidney capsule, in adults (Stevens, 1968; Damjanov *et al.*, 1971; Solter and Damjanov, 1982). EC cell lines in culture derived from these experimentally-induced teratocarcinomas have provided excellent experimental access to the study of fundamental processes in development, such as the regulation of proliferation and the initiation and direction of differentiation; they have in particular facilitated the analysis of signal transduction pathways activated by polypeptide growth factors in embryonic cells, where biochemical assays require at least an order of magnitude more material than is available from early mouse embryos (examples reviewed in Mummery and van den Eijnden-van Raaij, 1990).

The first EC cell lines isolated were dependent on somatic feeder cells for maintenance of their undifferentiated state; removal of the feeder cells resulted in changes to a more flattened, slowly proliferating phenotype that was no longer malignant. The spectrum of cell types that developed in culture was greatly enhanced by growing the cells as aggregates in suspension; these formed "embryoid bodies", structures that initially resemble the egg cylinder of an early post implantation embryo with an outer (primitive- then visceral) endoderm-like cell layer and an inner epiblast. These later cavitate, become cystic and upon replating in a culture dish form cell types that include skeletal and beating heart muscle, fibroblasts and various neural cells. Driving this differentiation programme in a particular direction has proved much more difficult; the discovery of retinoic acid, a vitamin A derivative, as an effective inducer of differentiation towards endoderm, particularly in combination with dibutyryl cyclic AMP (Strickland and Mahdavi, 1978) or

Abbreviations used in this paper: EC, embryonal carcinoma; TGFβ, transforming growth factor β; ES, embryonic stem; ICM, inner cell mass; LIF, leukemia inhibitory factor; ALK, activin receptor-like; BMP, bone morphogenetic protein; FN, fibronectin.

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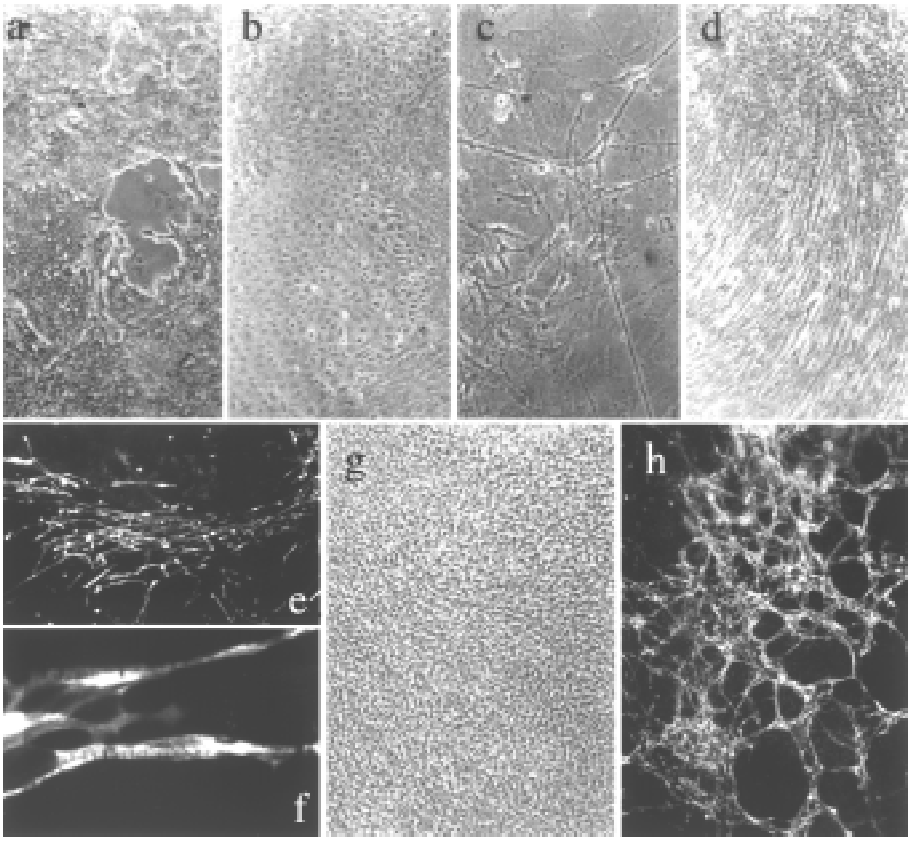

Fig. 1. Differentiated derivatives of ES cells in culture following growth as embryoid bodies in suspension culture and replating on a substrate. (a) *Undifferentiated ES cells;* **(b)** *endoderm-like cells;* **(c)** *neural cells;* **(d)** *skeletal muscle cells;* **(e)** *skeletal muscle cells stained with anti-muscle myosin antibody, MF-20;* **(f)** *higher magnification of* **(e)**, *showing striations;* **(g)** *culture after 21 days, phase contrast;* **(h)** *as* **(g)** *but stained by PE-CAM antibody to endothelial cells organized into tubular vessel structures. From Slager et al., (1993a), (a to f); Goumans et al. (1999). (g,h).*

other agents that elevate intracellular cAMP levels (van de Stolpe *et al.*, 1993) was an important breakthrough in this respect. The ability of retinoic acid also to direct differentiation of EC cells to other lineages, often in a dose dependent manner and particularly in combination with embryoid body formation (Hogan *et al.*, 1981; McBurney *et al.*, 1982; Mummery *et al.*, 1991) was an early indication of subsequent importance of retinoid gradients in development (Tickle *et al.*, 1985; Thaller and Eichele, 1987; Durston *et al.*, 1989). With the advent of techniques to generate cell lines stably expressing selected genes ectopically, has it now become possible to drive embryonic cells in culture to differentiate even more homogeneously to derivatives of other lineages (den Hertog *et al.*, 1993).

In the early 1980's, the perseverance and expertise of two independent groups (Evans and Kaufman, 1981; Martin, 1981) led to a second source of cells with properties similar to those of the inner cell mass (ICM). Reasoning that it should be possible to isolate cell lines directly from blastocyst stage mouse embryos without the intervening teratocarcinoma, these groups isolated feeder-dependent embryonic stem (ES) cell lines which were not only capable of contributing to somatic lineages when injected into host blastocysts, but also contributed to the germ line. These cells were truly totipotent. Using procedures essentially similar to those described, we also isolated several mouse ES cell lines (Tsung and Mummery, 1990). The introduction of molecular biology to the growth factor group by W. Kruijer in 1985 in combination with ongoing research on paracrine/autocrine growth regulation in normal and tumour cells by E.J.J. van Zoelen allowed us to compare mechanisms of growth control in differentiating EC and ES cells (van Zoelen *et al.*, 1989; Mummery *et al.*, 1990a,b; van den Eijnden-van Raaij *et al.*, 1991, 1992). Together these studies demonstrated that both EC and ES cells from mice were insensitive to exogenous growth control by the majority of the common polypeptide growth factors prior to differentiation . These included epidermal growth factor (EGF), platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ) although they did respond to leukemia inhibitory factor (LIF) and, under serum-free growth conditions, insulin-like growth factors (IGFs) (reviewed in Mummery and van den Eijnden-van Raaij, 1990). Later studies also showed that when grown on fibronectin, an extracellular matrix protein, they were also sensitive to mitogenic stimulation by fibroblast growth factors (FGFs; Mummery *et al.*, 1993). In most cases, responsiveness was correlated with the expression of (mRNA encoding) cell surface binding proteins; this in turn essentially reflected the phenotype of cells of the inner cell mass from which ES cells are derived and of their derivatives in the epiblast. Following an inventory of their differentiation capacity *in vitro* (Mummery *et al.*, 1990c; Slager *et al.*, 1993a; Thorsteinsdóttir *et al.*, 1999), we concluded that mouse EC and ES cells are useful model systems for studying growth control in early embryonic cells; examples of various differentiated derivatives of ES cells generated in culture are shown in Figure 1. Of note is that in all cases the cell populations are relatively heterogeneous. Since the useful-

TABLE 1

EXPRESSION OF TGFβ AND ITS RECEPTORS DURING PREIMPLANTATION DEVELOPMENT (FROM GOUMANS, 1999).

	zygote	2-cell	4-cell	8/16-cell	morula	blastocyst
TGFβ1	$+^{b}$,$+^{a}$	$+^{b}$, $-^{a}$	$+^{b}$, $+^{a}$	$+^{b}$, $+^{a}$	$+^{b}$, $+^{a}$	$+^{b}$, $+^{a}$
TGFβ2	nd,$+^{a}$	$+^{b}$, $-^{a}$	nd,$+^{a,c}$	nd,$+^{a,c}$	nd,$+^{a,c}$	nd,$+^{a,c}$
TGFβ3	nd,$+^{a}$	nd, $-^{a}$	nd,$+^{a}$	nd, $+^{a}$	nd,$+^{a}$	nd, $+^{a}$
TβRI	$+^{d}$, $+^{a}$	$+^{d}$, nd	$-^{d}$, nd	$-^{d}$, nd	$-^{d}$, nd	$+^{d}$, $+^{a}$
TβRII	$+^{d}$, $+^{a}$	$-^{d}$, nd	$-^{d}$, nd	$-^{d}$, nd	$-^{d}$, nd	$+^{d}$, $+^{a}$
TβRIII	$+^{d}$, $+^{a}$	$-^{d}$, nd	$-^{d}$, nd	$-^{d}$, nd	$-^{d}$, nd	$+^{d}$, $+^{a}$

a: Paria *et al.,* 1992; b: Rappolee *et al.,* 1988; c: Slager *et al.,* 1991;

d: Roelen *et al.,* 1998. The order is mRNA, Protein.

The discrepancy between mRNA and protein at the two cell stage is most likely due to the switch from maternal to zygotic gene transcription and the degradation of maternal proteins.

ness of the system would be greatly enhanced if differentiation could be driven homogeneously to (precursor) cells of selected lineages, application of information on the genetic control of differentiation is currently providing the greatest contribution to further exploitation of ES cells for research (see Klug *et al.*, 1996; Li *et al.*, 1998).

The loss of malignant phenotype as mouse EC cells differentiate has inevitably coupled our research to the tumorigenic behaviour of human EC cells and implications for therapy of teratocarcinoma of the testis in humans (Mummery *et al.*, 1987; Weima *et al.*, 1988, 1989, 1990; Caricasole *et al.*, 1998). More recently, now that the striking similarity between human EC cell lines in culture and human ES cells (Shamblott *et al.*, 1998; Thomson *et al.*, 1998) has been documented, these cells have become an even more important model for studying the maintenance of pluripotency. Not only do human ES cells share a common profile of antigenic plasma membrane markers with human EC cells but they are independent of LIF for maintenance of pluripotency; feeder-dependent human EC cells that have been described (Pera *et al.*, 1989) are also not rescued from differentiation by LIF, in contrast to feeder-dependent mouse EC and ES cells. Some of our current research involves identification and (expression) cloning of this differentiation inhibiting activity for the human cells (Caricasole, Mummery, Pera, unpublished).

The transforming growth factor β superfamily in early differentiation and development

Transforming growth factor β (TGFβ) was first identified in media conditioned by virally transformed cells on the basis of its ability to induce anchorage independent growth reversibly in normal fibroblasts in culture (DeLarco and Todaro, 1978; Roberts *et al.*, 1981). This property is usually regarded as a characteristic of neoplastic and metastatic cells, among which EC cells may be included (Mummery *et al.*, 1987); in addition though, anchorage independent growth is also observed in ES cells growing in aggregates as embryoid bodies (e.g. Slager *et al.*, 1993a). TGFβs were shown later to inhibit the growth of many cell types, particularly of epithelial origin, and also shown to be part of a large, and still increasing superfamily of structurally related proteins. TGFβ1 is the prototype of this superfamily, which also includes the bone morphogenetic proteins (BMPs) as a large subfamily, and the activins. The discovery by Smith *et al.* (1990) and members of the Hubrecht Laboratory (van den Eijnden-van Raaij *et al.*, 1990) that activin was the mesoderm inducing activity for *Xenopus* animal caps detectable in medium conditioned by a somatic cell line from *Xenopus* (XTC) (Snoek *et al.*, 1990) was one of the exciting breakthroughs in this field in the early 1990´s. Together the data show that these peptide growth factors clearly have important functions in embryonic development and, in the adult, regulate processes that include cell growth, differentiation, matrix production and apoptosis.

We demonstrated that both mouse and human EC cells (van Zoelen *et al.*, 1989; Weima *et al.*, 1989) as well as mouse ES cells expressed mRNA encoding TGFβs and secreted protein (Mummery *et al.*, 1990a,b; Slager *et al.*, 1993b), although which of the three known mammalian isoforms this was and whether it was biologically active depended on the state of differentiation of the cells. Later, we also showed that mouse EC and ES cells express activins in a differentiation dependent manner (van den Eijnden-van Raaij *et al.*, 1992; Mummery and van den Eijnden-van Raaij, 1993) while BMPs are also differentially regulated during differentiation (Rogers *et al.*, 1992).

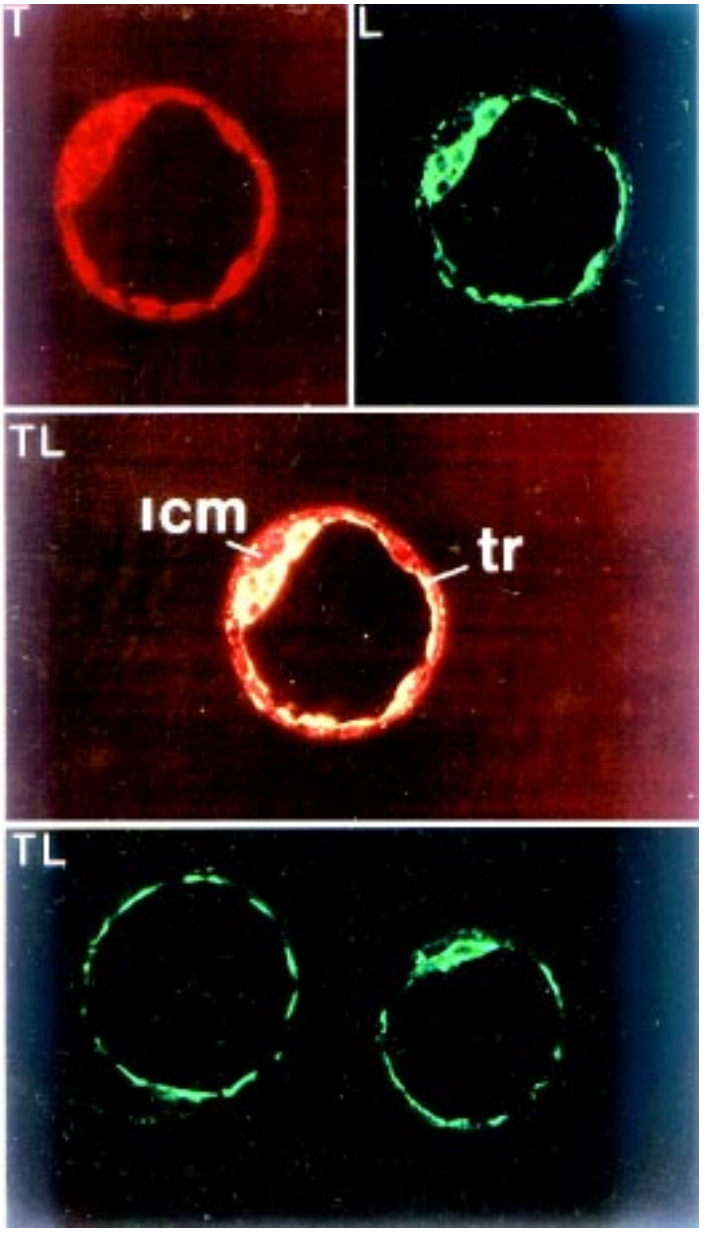

Fig. 2. Ectopic expression of TβRII in blastocysts upregulates laminin deposition. *Zygotes injected with TβRII driven by the PGK promoter, grown to the blastocyst in culture then double stained for laminin* **(L)**, *in green, and a KT3 tag* **(T)** *in the expression construct, in red. Optical section in CLSM shows colocalised staining in yellow.* **(Lower TL)** *shows normal staining for laminin in blastocysts that had not integrated the construct;* **(middle TL)** *shows a blastocyst in which the construct has been integrated; note the increase in laminin deposition, particularly around the ICM.*

These striking differential expression patterns in early embryonic cells in culture led rapidly to questions on whether differentiation of cells in the embryo was also accompanied by such changes, what the

target cells of the ligands were *in vivo* and what their functions were at different stages of development. These questions not only fascinated our group but also many others worldwide so that a wealth of data on expression patterns of ligands, receptors and downstream effector molecules are now available. In addition, much is now known about the effects of their absence or overexpression; in the sections that follow, we summarise much of these data and illustrate it in the light of some of our own contributions.

TABLE 2

EXPRESSION OF TGFβ AND ITS RECEPTORS DURING POSTIMPLANTATION DEVELOPMENT (E6.5-E10.5) (FROM GOUMANS, 1999)

	TGFβ1	TGFβ2	TGFβ3	TβRI	TβRII
E6.5					
endoderm	+[a]	+[a]			
ectoderm					
E7.5					
endoderm	+[a]	+[a], +[b]			
mesoderm	+[a]	+[a], -[b]			
ectoderm	+[a]	+[a], -[b]			
node/primitive streak				+[i]	
extraembryonic ectoderm					nd,+[a]
vasculogenesis and haematopoiesis					
visceral endoderm (E7.5)		nd, +[b]			
blood islands (E7.0)	+[g]				
allantois (E8.5)	+[g]	+[d]			+[c]
endothelial cells (E8.5)	+[e,g]				
dorsal aorta (E8.5)	+[g]				
extraembryonic mesoderm (E8.5)				+[l]	+[l]
cardiac development					
cardiac mesoderm (E7.0)	+[g]				
endocardium (E8.0)	+[e,g]				+[c]
myocytes (E8.5)	+[j]	+[d,e]	+[d]	+[m]	+[m]
outflow tract (E9.0)		+[d,e]			
AV junction (E9.0)		+[d,e]			
pericardial cells (E9.0)			+[d]		
endocardial cushion tissue (E9.0)	+[g,k]	+[d,e,h]			+[c]
neural tissue					
ventral spinal cord		+[e,h]			
neural tube				+[m]	+[m]
floor plate		+[e,h]			+[c], +[m]
neural crest (E8.5)	+[j]				+[c]
choroid plexus				+[m]	+[a,c], +[m]
skeletal					
somites		+[d,j]		+[m]	+[m]
dermamyotome (E9.5)		+[d]			
myotome (E10.5)		+[d], +/-[d]			
dermatome (E10.5)		+[d], +/-[d]			
intervertebral disc		+[e]			+[a,c]
epithelial-mesenchymal interactions					
lung mesenchyme	+[k]	+[e,h]	+[e]	+[m] (e,m)	+[c],+[m](e,m)
cephalic mesenchyme	+[j]	+[j]			+[c]
1st branchial arch mes.	+[j]	+[h,j]			+[c]
kidney mesenchyme	+[k]				+[a,c]
gut	+[k]	+[d](e), +[d]		+[m]	+[c](m), +[m]
limb bud		+[e]	+[e] (m)		

a: Roelen *et al.,* 1994 (mRNA); b: Slager *et al.,* 1991 (protein); c: Wang *et al.,* 1995 (mRNA); d: Dickson *et al.,* 1993 (both); e: Millan *et al.,* 1991 (mRNA); g: Akhurst *et al.,* 1990 (mRNA); h: Manova *et al.,* 1992 (mRNA); i: Chapter 5 (mRNA); j: Mahmood *et al.,* 1992 (protein); k: Lehnert and Akhurst, 1988 (mRNA); l: Goumans *et al.,* 1999 (mRNA); m: Mariano *et al.,* 1998 (protein). If both mRNA and protein are described then the order is mRNA, protein. e: epithelium; m: mesenchyme; e,m: expressed in both tissues. Note the difference in TβRII mRNA and protein present at sites of epithelial-mesenchyme interactions.

Model of TGFβ superfamily signal transduction

Receptors

The action of TGFβ superfamily members is mediated by a family of transmembrane serine/threonine kinase receptors that fall into two classes, type I and type II receptors (for a review see Heldin *et al.*, 1997 and Padgett *et al.*, 1998). Each member of the TGFβ family binds to characteristic combinations of type I and type II receptors, both of which are needed for signalling. The general model for receptor activation, which is based on studies of the type I and type II receptors for TGFβ as prototypes, involves ligand binding to the type II receptor followed by recruitment of the type I receptor into the complex. This results in transphosphorylation of the type I receptor in the cytoplasmic glycine-serine (GS) domain by the constitutively active type II receptor, which then triggers the downstream signalling cascade. For TGFβ only one type II receptor (TβRII) has been characterised, while two receptors exist for activin [ActRIIA and four splice variants of ActRIIB (IIB1-IIB4)]. BMPs bind to a specific BMP type II receptor (BMPRII), although BMP-7 also recognises the activin type II receptors. At least seven different type I receptors have been identified in vertebrate cells. These receptors, called ALKs (activin receptor-like kinases; ALK-1-7), determine the specificity of the intracellular signals. Originally ALK-5 (or TβRI) was thought to be the only signalling TGFβ receptor. However, recently it has been shown that TGFβ signals can also be transduced by ALK-1 (Oh *et al.*, unpublished results).

Both ALK-2 (ActRI) and ALK-4 (ActRIB) can form activin-induced heteromeric signalling complexes with either of the type II activin receptors. ALK-4 specifically mediates activin signalling. ALK-2 on the other hand also binds BMPs in the presence of activin type II receptors or the BMP type II receptor and plays a role in coordinating BMP signals. In addition, two other BMP signalling receptors, ALK-3 (BMPRIA) and ALK-6 (BMPRIB), have been characterised, which form a complex with the BMP type II receptor or activin type II receptors in the presence of BMP. We have shown that truncated activin type II receptors, lacking the entire kinase domain, not only interact with ALK-2, ALK-4 and ALK-6 in the presence of activin, but also with ALK-1, although not with ALK-3 and ALK-5 (de Winter *et al.*, 1996a). These results indicate that truncated activin type II receptors might inhibit signal transduction by various type I receptors. In addition, truncated activin type IIB2 receptors completely block activin-induced responses in P19EC cells, suggesting functional specificity for this particular splice variant (de Winter *et al.*, 1996a). ALK-7 interacts with type II receptors for TGFβ and activin in a ligand-dependent way, but so far direct binding of ALK-7 to ligand in these complexes has not been demonstrated (Ryden *et al.*, 1996).

In addition to binding to type I and type II receptors , TGFβs also bind to two structurally related forms of a TGFβ type III receptor (TβRIII), betaglycan (López-Casillas *et al.*, 1993) and endoglin (Yamashita *et al.*, 1994), large proteoglycans with a very short intracellular domain. Both receptors modulate ligand access to the TGFβ type I/type II receptor signalling complexes. For activin and BMP receptors ligand binding is controlled by follistatin, a protein that can interfere with binding of activin and BMP to type II receptors by direct interaction with these ligands (de Winter *et al.*, 1996b).

Smads

Downstream of the receptor complexes are a set of proteins related to the mediator of decapentaplegic (dpp) signalling, called mothers against dpp (MAD) in *Drosophila,* and to the Sma genes from *Caenorhabditis elegans*. These evolutionarily conserved proteins, called Smads in vertebrates, can be divided into three categories: receptor-specific Smads, common-mediator Smads and inhibitory Smads. To date, ten different Smads have been identified (Smad 1-10; for a review see Heldin *et al.*, 1997 and Padgett *et al.*, 1998; LeSueur and Graff, 1999). Receptor-specific Smads include members that interact with and become phosphorylated by specific activated type I serine/threonine kinase receptors and act in a pathway-restricted fashion. Most *in vitro* studies have indicated that Smad2 and Smad3 mediate the signals for TGFβ and activin, while Smad1, Smad5 and Smad8 are involved in the BMP signalling pathway. However, recent studies have shown that TGFβ signalling can also be mediated by Smad1 and Smad5 (Oh *et al.*, unpublished results; Bruno *et al.*, 1998; Liu *et al.*, 1998; Maciassilva *et al.*, 1998). Phosphorylation of this first group of Smads results in an interaction with the common-mediator Smad4, a member of the second group to which Smad10 also probably belongs (LeSueur and Graff, 1999), followed by translocation of the complex into the nucleus and activation of specific target genes. Work by our group and by others has demonstrated that Smad4 (originally called DPC4) is involved in TGFβ-induced growth inhibition and transcriptional response (Lagna *et al.*, 1996; de Winter *et al.*, 1997). Although the DNA binding property of Smad complexes has been established, the molecular basis for transcriptional activation by these complexes is largely unknown. Recently it has been shown that interactions of Smads with coactivators are functionally important for transactivation. These coactivators themselves bind to DNA (FAST1, FAST2) or function as a bridge between Smads and other DNA-binding proteins (p300/CBP) (Labbé *et al.*, 1998; Pouponnot *et al.*, 1998; Zhou *et al.*, 1998). Depending on which coactivators and Smads are present in the complex, transcriptional activation of a target gene will be either promoted or blocked. Increasingly, Smad binding elements are being identified in the promoters of genes induced by members of the TGFβ superfamily; among the more recent additions is a CAGACA repeat identified in the promoter of the *JunB* gene (Jonk *et al.*, 1998), an immediate early gene potently induced by TGFβ , activin and BMPs.

Another level of regulation of signalling by TGFβ superfamily members is interference with the phosphorylation of the pathway-restricted Smads. This is achieved by the third group of Smads, the inhibitory Smads (Smad6 and Smad7). These Smads stably interact with activated type I receptors thereby preventing the formation of active heteromeric Smad complexes. Smad7 can inhibit TGFβ and BMP signalling, but Smad6 preferentially inhibits signalling by BMP, not only by binding to the BMP type I receptor, but also by forming a complex with Smad1, thereby preventing Smad4 binding (for a review see Whitman, 1998). Smad6 and 7 are also autoinduced by TGFβ ligands thus creating autoregulatory, negative-feedback loops.

Current research

TGFβ: prototype of the superfamily, expression pattern, target cells and function in early mouse development

Preimplantation development.

Fertilised mouse eggs develop to blastocysts *in vitro* in relatively simple culture media without complex protein supplements (Spindle, 1990; Lawitts and Biggers, 1991). However, the rate of development and the number of cells present at the blastocyst stage is greatly enhanced when embryos, at least from outbred strains, are cultured together in groups of 10-20 in small volumes (20 μl) of chemically-defined culture medium (Paria and Dey, 1990); this suggested that factors secreted by embryos mediated growth cooperativity. All three mammalian TGFβ isoforms are among the growth factors detectable as mRNA and/or protein in preimplantation embryos, at least from the 4-cell stage onwards (Rappolee *et al.*, 1988; Slager *et al.*, 1991; Paria *et al.*, 1992; Croteau *et al.*, 1995) and, indeed, it was demonstrated that embryos cultured singly in the presence of TGFβ1 developed at a rate that matched that of those grown in groups and that the resulting blastocysts had a higher mitotic index and total cell number (Paria and Dey, 1990; Lim *et al.*, 1993). Target cells in preimplantation embryos should be

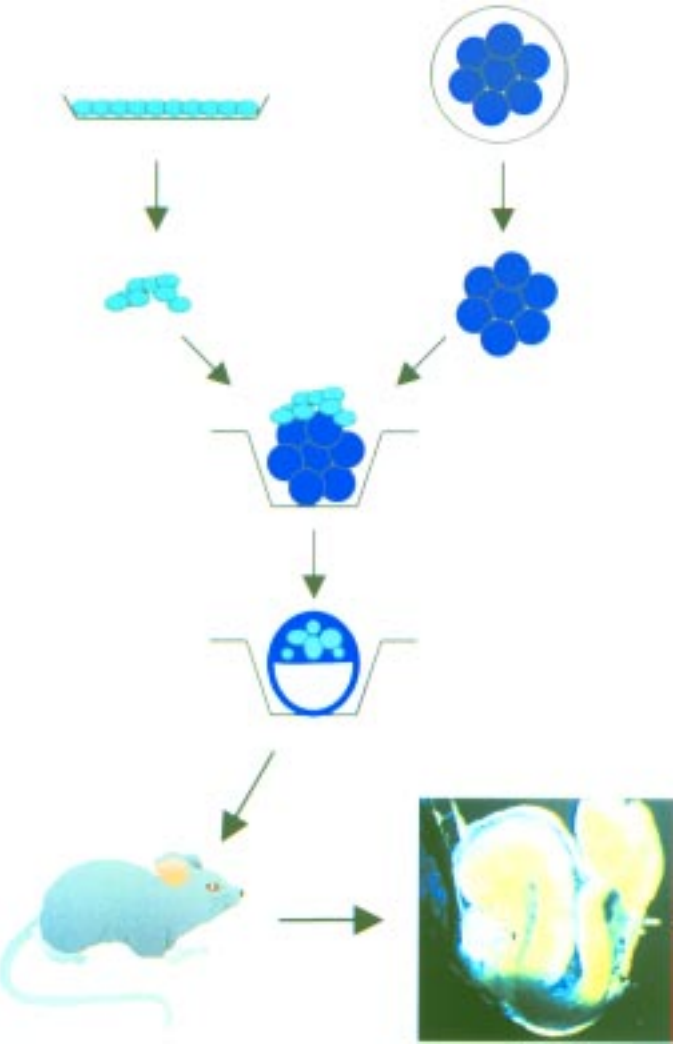

Fig. 3. Chimaeras by morula aggregation with ES cells. *Preaggregation of ES cells (green) before allowing them to attach to morula stage embryos from the ROSA26 transgenic strain (ubiquitous expression of lacZ) results in a high degree of chimaerism at least up to midgestation. Embryo shown was isolated at nominal E8.5 and is entirely ES derived with the exception of a few (blue) cells in the fore- and hindgut. The yolk sac has been peeled back but shows that its visceral endoderm component is host (blue) derived.*

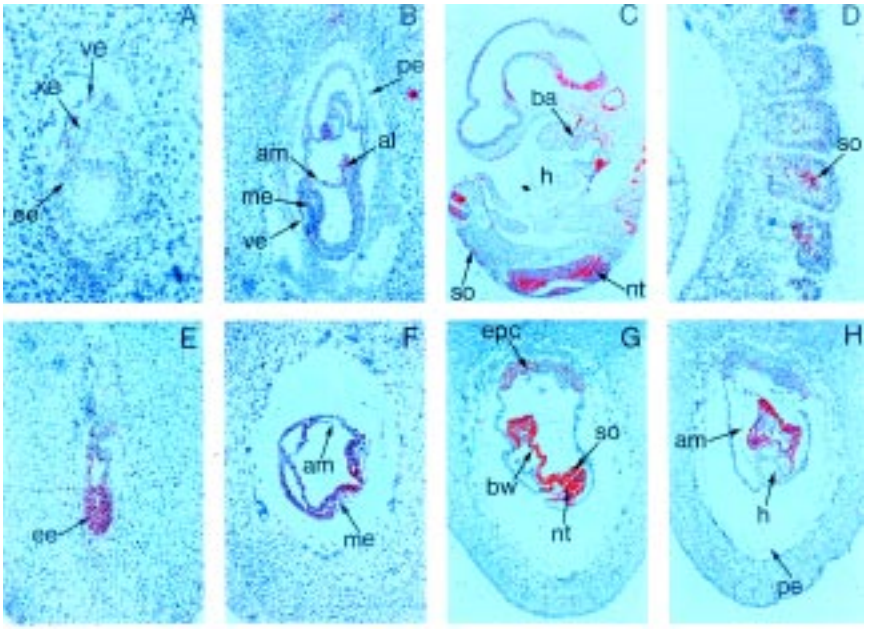

Fig. 4. Activin receptor type IIA (A-D) and IIB (E-H) receptor expression in early postimplantation mouse embryos. *In situ hybridization on sections of mouse embryos from E6.0-E9.5. Sagittal sections of E6.0* **(E)**, *E6.5* **(A)**, *E7.5* **(B)** *and E9.5* **(C-D)** *embryos.* **(F)** *Transverse section of an E7.5 embryo.* **(G-H)** *Transverse sections of an E8.5 embryo. ve, visceral endoderm; pe, parietal endoderm; ee, embryonic ectoderm; xe, extraembryonic ectoderm; me, mesoderm; am, amnion; so, somite; h, heart; epc, ectoplacental cone; nt, neural tube; ba, branchial arch; al, allantois; bw, body wall.*

identifiable as those with cell surface receptors. Binding studies with iodinated TGFβ ligands suggested that TβRI, TβRII and TβRIII were all present on the trophectoderm but not on the ICM (Paria *et al*., 1992). We later confirmed and extended these findings to show that the mRNA for all three receptors is expressed in the zygote, for TβRI only at the 2-cell stage and that no expression at all is detectable at later stages until the blastocyst forms, when TβRI and TβRII are re-expressed (Table 1); indirect immunofluorescence confirmed that TβRII is expressed exclusively on trophectoderm cells (Fig. 2) (Roelen *et al*., 1998).

These expression data contributed later to establishing a strategy for functional analysis of TGFβ in development and, in particular, to answering two specific questions in preimplantation embryos, namely (1) does TGFβ signalling have a function in zygotes, and (2) is TGFβ involved in regulating the very characteristic deposition of extracellular matrix (ECM) proteins under the trophectoderm (Thorsteinsdóttir, 1992; Hierck *et al*., 1993)?

In addressing both questions we made use of the fact that, at least to date, only one binding receptor, TβRII, has been identified for TGFβ; this receptor mediates all TGFβ responses. We therefore generated cDNA constructs which would encode TβRII lacking a cytoplasmic tail; when driven by a strong promoter (PGK) and (over)expressed ectopically, this would compete with endogenous TβRII and act as a dominant negative, blocking TGFβ signalling (Goumans *et al*., 1998). This construct was microinjected into zygotes and the embryos were cultured for 3 days; more than 60% of non injected control embryos and controls injected with empty vector had then reached the blastocyst stage. By contrast, less than 30% of those injected with the dnTβRII construct had developed to blastocysts, the majority being blocked at the 2-cell stage (Roelen *et al*., 1998). This effect could be rescued by i) co-injection of TβRII, ii) co-injection of a constitutively active (phosphorylated) TβRI (Roelen *et al*., 1998), and mimicked by injection of the inhibitory Smad7 (Goumans, 1999). The dnTβRII did not affect BMP signalling (Roelen *et al*., 1998), important for interpretation since BMPRII is expressed in zygotes and at the 2-cell stage (Roelen *et al*., 1997a). These results suggest that an intact TGFβ signal cascade is necessary for development beyond the 2-cell stage; the failure to observe a 2-cell block in embryos lacking TβRII (Oshima *et al*., 1996) may have been the result of rescue by the maternal allele initially expressed in the zygotes of heterozygous (TβRII+/-) crosses. Similarly, ActRIIB is expressed zygotically and dn actRIIB blocks development beyond the 2-cell stage (Goumans, 1999) although ActRIIB-/- embryos only die much later (Oh and Li, 1997). This transient transgenic approach can thus reveal early requirements for gene expression that may be masked in conventional knockout experiments. Of note is also the finding that the ras/raf signalling pathway is apparently essential for progression through the 2-cell stage; microinjection of a cDNA construct encoding dn ras blocked development and this was rescued by co-injection of cDNA encoding active raf, a downstream signalling molecule for ras (Yamauchi *et al*., 1994). Since TGFβ-induced ras activation has been demonstrated in epithelial cells (Mulder and Morris, 1992) and TGFβ-induced mesoderm formation is blocked by dn ras in *Xenopus* animal caps injected with TβRII (Bhushan *et al*., 1994), we examined the ability of active ras to rescue the dn TβRII 2-cell block. Rescue did not occur, indicating that the two pathways are required independently for progression through the second cell division (Goumans, 1999). Although it could be argued that the 2-cell stage in mouse development is particularly sensitive to expression of almost any dn construct, Chai *et al.* (1998) demonstrated that development of zygotes injected with a dn FGF receptor progressed beyond the 2-cell stage , the ultimate effect being on the number of cells present at the blastocyst stage.

After the 2-cell stage, the next site of TGFβ receptor expression is the trophectoderm of the blastocyst; this coincides with a gradual accumulation of the ECM protein laminin on its basal side and a redistribution of the laminin receptor α6β1 integrin also to the basal side (Hierck *et al*., 1993). Both laminin and several integrins are regulated by TGFβ. Work is in progress to assess the effect of driving dn TβRII with trophectoderm specific promoters, such as those for cytokeratins, to circumvent the 2-cell block resulting from a ubiquitous promoter. However, we have overexpressed TβRII at the blastocyst stage using the PGK promoter and zygote injection. The construct used contained a KT3 tag, recognised by a monoclonal antibody, so that staining at the blastocyst stage indicated which embryos had integrated the construct (Fig. 2T). Double staining with an antibody recognising laminin at the same stage demonstrated a significant increase in the amount of laminin under the trophectoderm of blastocysts in which the tag was detectable

compared with non-expressing controls (Fig. 2L and TL) (Zwijsen, Goumans , Mummery, unpublished). We can conclude that all of the components of the signal transduction pathway necessary to regulate laminin production (active ligand, type I receptors, Smads) are present in all cells of the trophectoderm and at least some cells (possibly primitive endoderm) of the ICM. Whether the reverse is the case i.e. blocking the TGFβ signal prevents laminin accumulation under the trophectoderm, remains to be established; the lack of laminin accumulation in the ICM during normal development may be due to the absence of endogenous TβRII in these cells. Likewise, the growth stimulatory effect of exogenous TGFβ on both trophectoderm and ICM of cultured embryos (Paria and Dey, 1990; Lim *et al.*, 1993) can only be caused (indirectly) via the trophectoderm, possibly by upregulation of mitogens like FGF or PDGF (Battegay *et al.*, 1990; Kay *et al.*, 1998), since the ICM cells lack TβRII.

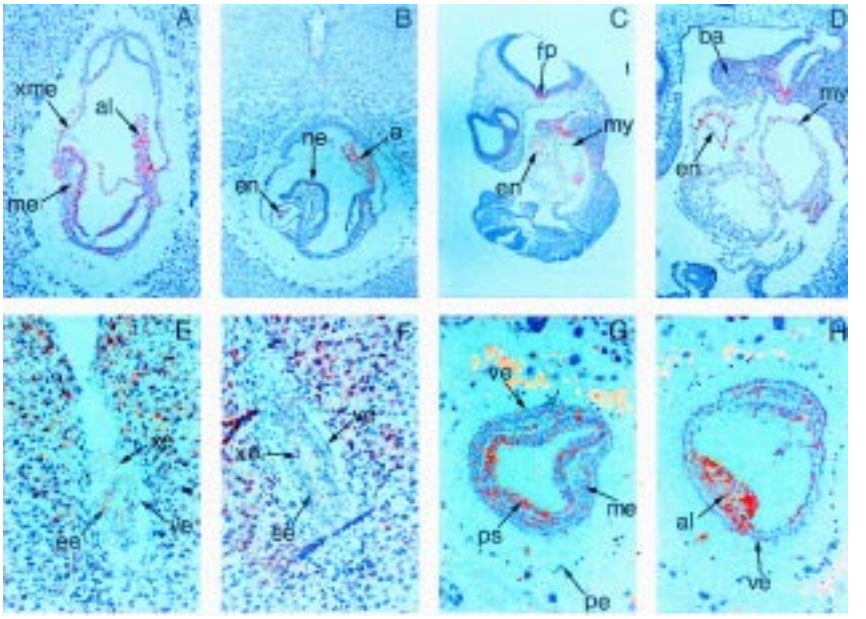

Fig. 5. ALK-2 (A-D) and ALK-4 (E-H) expression in early postimplantation mouse embryos. *In situ hybridization on sections of mouse embryos from E5.5-E9.5.* **(A-F)** *Sagittal sections of E5.5* **(E)**, *E6.0* **(F)**, *E7.5* **(A)**, *E8.5* **(B)**, *E9.5* **(C,D)** *embryos.* **(F,G)** *Transverse sections of an E7.5 mouse embryo. ve, visceral endoderm; ee, embryonic ectoderm; xe, extraembryonic ectoderm; me, mesoderm; al, allantois; pe, parietal endoderm; ne, neurectoderm; am, amnion; xme, extraembryonic mesoderm; en, endocardial cells; my, myocardium; fp, floor plate; ba, branchial arch.*

Postimplantation development

Parietal endoderm

After "hatching" from the zona pellucida, a single layer of primitive endoderm cells differentiates from ICM cells facing the blastocoelic cavity. Some of these remain in contact with the remaining ICM while others migrate over the basal surface of the trophectoderm where, probably under influence of hormones such as PTHrP (van de Stolpe *et al.*, 1993; see also Verheijen and Defize, this issue), they differentiate into parietal endoderm (PE). They eventually line the entire inner side of the blastocyst and produce large amounts of ECM, including laminin and fibronectin , to form a thick proteinous layer known as Reichert's membrane. This membrane is unique to rodents and bats and acts as a selective filter between the embryo and maternal circulation. TGFβ is known to inhibit the proliferation of PE derivatives of F9 EC cells (Rizzino, 1987; Kelly and Rizzino, 1989). We addressed the question of whether this was likely to occur *in vivo* by isolating ICMs from blastocysts by immunosurgery and plating these on to fibronectin –coated substrates or in the presence of PTHrP, conditions that favour the outgrowth of PE as solitary, migratory cells (van de Stolpe *et al.*, 1993; Behrendtsen *et al.*, 1995). TGFβ had little effect on the outgrowth of primitive- and visceral endoderm-like cells from ICMs but reduced the outgrowth of PE cells virtually to zero (Roelen *et al.*, 1998). Expression of dn TβRII under control of PE specific promoter will eventually confirm whether both ECM production and growth/migration of PE cells are controlled by TGFβ *in vivo.*

Vasculogenesis and hematopoiesis of the yolk sac

In order to analyse the function of TGFβ after implantation and to circumvent possible trophectoderm/parietal endoderm-mediated effects, we developed a novel variant of the conventional approach to generating chimeric embryos by morula aggregation (Goumans *et al.*, 1998; Zwijsen *et al.*, 1999). By pre-aggregating (mutant) ES cells in suspension culture for one hour before allowing them to attach to morula stage embryos, we forced consistently high levels of chimaerism so that the entire embryo (with the exception of some endodermal cells of the fore- and hindgut) together with the extraembryonic mesoderm component of the yolk sac were entirely ES derived (Fig. 3). The chimaeras were indistinguishable from those generated using tetraploid host embryos (Nagy *et al.*, 1990) and differed from conventional (knockout) mice in that the visceral endoderm component of the yolk sac was derived from normal, rather than mutant, cells.

Although the first descriptions of mice lacking TGFβ1 suggested that the ligand was only required post-natally (Shull *et al.*, 1992; Kulkarni *et al.*, 1993), it later became evident that sufficient maternal ligand was available to rescue the developmental requirement and most of the null mice only died after weaning (Letterio *et al.*, 1994), at least on some genetic backgrounds. On a C57Bl6/6J/Ola background, however, none of the conceptuses survived to birth (Bonyadi *et al.*, 1997) most exhibiting the defects in yolk sac vasculature that had been observed in the embryos that did not survive to birth on an NIH/Ola background (Dickson *et al.*, 1995). A modifier element was identified near the TGFβ1 gene that determined the relative dependence of embryos from different genetic backgrounds on TGFβ1 for development i.e. C57Bl6/6J/Ola mice were highly dependent on TGFβ1, NIH/Ola much less and C57Bl6 hardly at all (Bonyadi *et al.*, 1997). The first description of TGFβRII-/- embryos (Oshima *et al.*, 1996) confirmed the importance of TGFβ signalling for development, although it is of note that the construct used to generate these mice probably encoded a truncated protein lacking the cytoplasmic domain rather than causing complete ablation of the protein.

We have also confirmed and extended these findings in chimeric embryos generated as described above using ES cells expressing dn TβRII (Goumans *et al.*, 1998, 1999); we demonstrated that

defective TGFβ signalling in the extraembryonic mesodermal compartment of the yolk sac *only*, is sufficient to disrupt vasculogenesis and hematopoiesis and to account entirely for the yolk sac phenotypes in the TGFβ1 and TβRII null embryos. We believe that the phenotype is primarily caused by reduced fibronectin (FN) synthesis in mesodermal derivatives of ES cells in which TGFβ signalling is blocked, since 1) embryoid bodies from ES cells expressing dn TβRII express reduced levels of FN mRNA and protein; 2) TGFβ regulates expression of FN in many cell types in culture; 3) most importantly, in chimeric yolk sacs the deposition of FN between the visceral endoderm and mesoderm layers is greatly reduced compared with control chimaeras, possibly accounting for their abnormal separation; 4) the yolk sac phenotype in the dn TβRII chimaeras resembles that in FN and α5 (FN receptor) null embryos (George *et al.*, 1993; Yang *et al.*, 1993; Georges-Labouesse *et al.*, 1996). The normal function of TGFβ in the yolk sac may therefore be to ensure appropriate upregulation and deposition of FN by mesoderm; this, in turn, mediates interaction between the cells layers , essential for the *organisation* of endothelial cells, that do in fact differentiate from the mesoderm quite normally in the dn TβRII chimaeras, into robust vessels (Palis *et al.*, 1995).

Of interest in this context is also the observation that ALK-1, also a type I serine/threonine kinase receptor for which the endogenous ligand is not known, is highly expressed in endothelial cells; in early mouse development its expression coincides with sites of vasculogenesis and angiogenesis (Roelen *et al.*, 1997b) and in the yolk sac it is co-expressed with ALK-5 and TβRII in the mesoderm compartment whereas none of these receptors are detectable in the endoderm compartment by RT-PCR (Goumans *et al.*, 1999). *In vitro* ALK-1 can bind both activin and TGFβ in the presence of the appropriate type II receptors, but in the light of genetic evidence in humans that an autosomal dominant vascular disease known as hereditary haemorrhagic telangiectasia, is associated with mutations in ALK-1 (Johnson *et al.*, 1996), it would seem likely that TGFβ may be the endogenous ligand, at least in endothelial cells, and that the TGFβ/ALK-1/TβRII complex may be important in blood vessel maintenance. Comparison of ALK-1 and ALK-5 null mutations in yolk sac mesoderm should shed light on this question. In particular, in view of recent data demonstrating co-expression of ALK-1 and ALK-5 in yolk sac mesoderm (Goumans *et al.*, 1999), it should establish whether these type I receptors are functionally redundant in vasculogenesis *in vivo*.

TABLE 3

MAJOR DEFECTS IN TGFβ KNOCKOUT AND DOMINANT NEGATIVE RECEPTOR TRANSGENICS (FROM GOUMANS, 1999)

Model	Phenotype	Refs
TGFβ1 null mutation	Defective yolk sac vasculogenesis and haematopoiesis. Embryonic lethal (E9.5-11.5)	Dickson *et al.,* 1995
	Inflammation and autoimmunity	Kulkarni *et al.,* 1993; Shull *et al.,* 1992; Letterio *et al.,* 1994
TGFβ2 null mutation	Cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, urogenital defects Perinatal lethality	Sanford *et al.,* 1997
TGFβ3 null mutation	Cleft palate, delayed lung maturation. Mutants die shortly after birth	Proetzel *et al.,* 1995 Kaartinen *et al.,* 1995
TβRII null mutation	Defective yolk sac vasculogenesis Embryonic lethal (E10)	Oshima *et al.,* 1996
Smad2 null mutation	Embryonic lethal (E7.5-E8.5)	
	Failure in egg cylinder elongation, gastrulation and mesoderm formation	Weinstein *et al.,* 1998 Nomura and Li, 1998
	Difference in Smad2-/- in extraembryonic tissue differentiation and gastrulation defect in heterozygotes	Waldrip *et al.,* 1998
Smad3 null mutation	Metastatic colorectal cancer (4-6 months of age)	Zhu *et al.,* 1998
Smad4 null mutation	Growth retardation, no mesoderm formation, abnormal visceral endoderm. Embryonic lethal (E7.5-E8.5)	Sirard *et al.,* 1998 Yang *et al.,* 1998
ΔTβRII in epidermis (mouse loricrin promoter)	Epidermal hyperproliferation, hyperplasia and hyperkeratosis.	Wang *et al.,* 1997
ΔTβRII in skeletal tissue (metallothionein II prom)	Progressive skeletal degeneration (osteoarthritis). Terminal differentiation of chondrocytes	Serra *et al.,* 1997
ΔTβRII in osteoblasts (Osteocalcin promoter)	Decrease in osteocyte density Decrease in rate of osteoblastic differentiation	Erlebacher *et al.,* 1998
ΔTβRII in pancreatic acini (metallothionein I prom)	Acinar hyperproliferation and altered differentiation. Fibrosis, macrophage infiltration and neoangiogenesis.	Böttinger *et al.,* 1997
ΔTβRII in mammary epithelium (mmtv-ltr).	Inappropriate alveolar development and differentiation. Expression of milk proteins in virgin mice.	Gorska *et al.,* 1998
ΔTβRII in early mouse embryos (PGK)	Developmental arrest at the 2-cell stage	Roelen *et al.,* 1998

It is of note that yolk sac defects similar to those described here have also been reported for null mutations either in other ligands of the superfamily, such as BMP4 (Winnier *et al.*, 1995), or in the Smad genes downstream of the receptors, such as Smad 5 (Chang *et al.*, 1999). It remains to be established exactly how these pathways might be linked and whether there are common target genes that are transcriptionally regulated *in vivo*.

Neurogenesis and somitogenesis

Although expression of TGFβ receptors is largely restricted to extraembryonic tissues during very early postimplantation development, at 8.5 dpc both TβRI/ALK-5 and TβRII are detectable in the heart (Roelen *et al.*, 1994, 1998; Wang *et al.*, 1995; Mariano *et al.*, 1998). In the central nervous system slightly later, both receptors have been detected in the neural tube and in migrating neural crest cells, while at 12.5 dpc they are expressed in a variety of organs and tissues in which epithelial-mesenchyme interactions take place (see Table 2 for summary).

We addressed the possible function of TGFβ signalling in these tissues by analysing high ES contribution chimeric embryos, generated as described earlier. Using the PGK promoter to drive expression of dn TβRII resulted in embryonic lethality at nominal days 9.5-10.5 dpc (Goumans *et al.*, 1999). In addition to pleiotropic (non-specific) effects secondary to poor yolk sac circulation, dn TβRII chimaeras have specific defects in neural tube formation and in somite maturation. The neural tube failed to close in the hindbrain of many chimaeras, possibly the result of overproliferation of neurectoderm, and in about 30% of chimaeras was undulated, reminiscent of FN-/- embryos; however; we failed to detect alterations in FN distribution in the neural tube of the mutant chimaeras (Goumans, 1999). Somite development was also defective and instead of forming characteristic “blocks”, in some chimaeric embryos they fail to epithelialise and remain distinctly rounded. Pericardial effusion characterised heart development but this probably secondary to circulation defects; future studies will establish whether defective TGFβ signalling causes specific defects in heart development.

Although there have been several descriptions of tissue specific (ectopic) expression of dn TβRII, these have generally concerned later development (e.g. Filvaroff *et al.*, 1994; Serra *et al.*, 1997; Erlebacher *et al.*, 1998; Gorska *et al.*, 1998; see Table 3) and may have been selected in part to circumvent the 2-cell block resulting from use of ubiquitous promoters, described above. To our knowledge, the results described above are the first details of the function of TGFβ signalling in early post implantation development. Which of the downstream Smads couples to the TGFβ signalling pathway in each specific tissue remains to be established, although comparison of the null phenotypes of these genes with the mutant chimaeras may provide some vital clues.

Activins and activin-binding proteins in early mouse development

Expression in vitro and effects on differentiation

From the discovery of activin as a potent mesoderm-inducing factor in *Xenopus* blastula explants (Asashima *et al.*, 1990; Smith *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1990) this factor became the subject of extensive research in the field of developmental biology. Activin is a homo- or heterodimer of 2 subunits known as βA and βB; each of these subunits may also be found in combination with an (inhibin) α subunit, giving rise to (gonadal) proteins known as inhibin A and inhibin B. We first investigated the expression patterns of the different activin/inhibin subunits, follistatin and the activin type II receptors in P19EC and ES cells, and in the embryo itself. Expression of activin βA subunit transcripts and the inhibin α transcript is restricted to certain differentiated cell types, while βB subunits are expressed in both differentiated and undifferentiated cells (van den Eijnden-van Raaij *et al.*, 1992). In MES-1 cells, a mesodermal derivative of P19 EC, β subunit expression is modulated by TGFβ, indicating a possible role for type beta transforming growth factors as regulators of activin expression during early embryogenesis (van der Kruijssen *et al.*, 1993). The activin-binding protein follistatin and the type IIA activin receptor both are regulated during differentiation of P19 EC and ES cells, while mRNA levels of the activin type IIB and type I receptors (ALK-2 and ALK-4) are not affected by various differentiation stimuli (van den Eijnden-van Raaij *et al.*, 1992; van der Kruijssen *et al.*, 1995). The apparent increase in type IIA mRNA expression during neural differentiation of P19 EC cells appeared to be due to transcriptional induction of the activin type IIA-N receptor, an alternative splicing product of the type IIA receptor (Shoji *et al.*, 1998).

The functionality of activin type I and type II receptors in EC and ES cells was shown by the effects of activin on their differentiation. Activin has no mesoderm-inducing activity in P19 EC cells, but it inhibits their differentiation to derivatives of all three germ layers (van den Eijnden-van Raaij *et al.*, 1991). In P19 EC cells which constitutively express receptor protein tyrosine phosphatase-α (RPTPα) (den Hertog *et al.*, 1993) activin suppresses the RA-induced neuronal differentiation (Ameerun *et al.*, 1996). A similar effect of activin on neuronal differentiation has been observed for ES cells aggregated in chemically defined medium (van den Eijnden-van Raaij *et al.*, unpublished results).

Together these expression data in differentiating EC and ES cells and the effects of activin on their differentiation pointed to a potential function of activins and/or their binding proteins in the mouse embryo.

Expression in mouse development

The expression of activins and activin-binding proteins was examined in pre- and postimplantation mouse embryos. Using polyclonal antibodies that are specific for each of the activin β subunits we have shown that both the βA and βB subunits are

TABLE 4

EXPRESSION OF ACTIVIN/INHIBIN SUBUNITS, ACTIVIN RECEPTORS AND FOLLISTATIN DURING PREIMPLANTATION DEVELOPMENT

	egg	zygote	2-cell	4-cell	8-cell	morula	blastocyst
a	-[a]	-[a]	-[a]		-[a]		+[b]
βA	+[a]	+[a]	+[a]		+[a]		-[b], +[e]
βB	+[a]	+[a]	+[a]		+[a]		+[b], +[e]
ActRIIA							+[b]
ActRIIB	+[a]	+[a]	-[a]		+[a]		
ALK-2		+[c]	+[c]	+[c]			+[c]
Follistatin	+[d]	-	+[d]			+[d]	+[d], -[b]*

a: Lu *et al.,* 1993; b: van den Eijnden-van Raaij *et al.,* 1992; c: Roelen *et al.,* 1994; d: Albano and Smith, 1994; e: Paulusma *et al.,* 1993.
*Positive in plated blastocysts (b).

present in blastocysts (Paulusma *et al.*, 1994). As shown in Table 4, RT-PCR analysis has also shown that βA and βB transcripts, but not α transcripts are present from the oocyte to 8-cell stage (Lu *et al.*, 1993). Transcripts for follistatin and activin type II receptors were also detected at the various stages of preimplantation development (van den Eijnden-van Raaij *et al.*, 1992; Lu *et al.*, 1993; Albano and Smith, 1994).

In postimplantation embryos expression has been studied by *in situ* hybridization. From E6.0 to E9.5 activin βA and βB subunit mRNAs are not detected in the embryo itself, but are expressed in a specific subset of decidual cells (Manova *et al.*, 1992; Albano *et al.*, 1994; Feijen *et al.*, 1994). At E10.5 and E12.5 βA subunit transcripts are expressed predominantly in mesenchymal structures, including those surrounding the epithelia of several internal organs (Feijen *et al.*, 1994). In contrast, βB transcripts are present in the central nervous system (Feijen *et al.*, 1994). However, several sites of coexpression or adjacent expression of βA and βB subunits have been observed, but the developing gonads are the only sites where α and β subunits are colocalised.

Follistatin has a very characteristic expression pattern in early postimplantation mouse embryos (Feijen *et al.*, 1994). Its transcripts are present in both the decidua and the embryo proper. In particular, the primitive streak region, the even rhombomeres in the developing hindbrain, somites, paraxial mesoderm and parietal endoderm cells attached to Reichert's membrane show strong expression of follistatin (Table 5). Follistatin is not expressed in the visceral yolk sac (endoderm and mesoderm) of E8.5 mouse embryos (Fig. 6). During organogenesis follistatin is expressed in various mesoderm- and ectoderm-derived tissues (Feijen *et al.*, 1994).

Expression of the activin type I and type II receptors in mouse development has been well-studied for the period of organogenesis (Feijen *et al.*, 1994; Verschueren *et al.*, 1995). However, as yet there has been no comparative description of their expression patterns in earlier postimplantation development; this is now included here, with examples of activin RIIA, RIIB, ALK-2 and ALK-4 expression at E5.5 to E9.5 in Figures 4 and 5. The results, summarised in Table 5, demonstrate clear differences in the expression patterns of the different receptor types, but also overlapping sites of expression. As shown in Figure 4, activin type IIA receptor is weakly expressed from E6.5 in both the embryonic and extraembryonic ectoderm, and in the visceral endoderm (Fig. 4A). No signal above background was observed in the parietal endoderm or in the decidua surrounding the conceptus. Manova *et al.* (1995) reported expression of ActRIIA earlier in E5.5 mouse embryos, but it is questionable whether the signal was above background. In E7.5 mouse embryos the signal is more pronounced. All three germ layers express the receptor (Fig. 4B). Relatively strong expression was detected in the extraembryonic mesoderm, including the allantois and the mesodermal component of the amnion and chorion. RT-PCR analysis has shown that ActRIIA is present in the endoderm, but not in the mesoderm of the visceral yolk sac (Fig. 6). In E9.5 embryos the expression of ActRIIA becomes more restricted to neural tissues, including the brain, neural tube and ganglia (Fig. 4C). In addition, a signal was observed in the branchial arches, the branchial arch arteries and in the myotome cells of the somites (Fig. 4D). The heart was negative at this stage.

Activin type IIB receptor is strongly expressed from E5.5 (Manova *et al.*, 1995). In E6.0 mouse embryos there is ubiquitous expression in the extraembryonic and embryonic ectoderm, although expression levels are higher in the embryonic part (Fig. 4E). During development from E6.5 to E7.5 the receptor also becomes expressed in the developing mesoderm, while the visceral endoderm, the parietal endoderm and the amnion are negative (Fig. 4F). There was also no signal in the decidua. In E8.5 embryos activin type IIB receptor is expressed in the neural tube, somites and the body wall (Fig. 4G,H). The heart, amnion and parietal endoderm are negative. Weak expression could be observed in the ectoplacental cone (Fig. 4G,H). Using RT-PCR a weak signal for activin type IIB receptor was observed in the endoderm, but not in the

TABLE 5

EXPRESSION OF ACTIVIN RECEPTORS AND FOLLISTATIN DURING POSTIMPLANTATION DEVELOPMENT (E5.5-E9.5)

		ActRIIA	ActRIIB	ALK-2	ALK-4	Follistatin
E5.5	visceral endoderm				-[a]	
	embryonic ectoderm	+[b]	+[b]		+[a]	
	extraembryonic ectoderm	+[b]	+[b]		+[a]	
	parietal endoderm				-[a]	
E6.0	visceral endoderm		-[b,c]		-[a]	-[d]
	embryonic ectoderm	+[b]	+[b,c]		+[a]	+[d,1]
	extraembryonic ectoderm	+[b]	+[b,c]		+[a]	-[d]
	parietal endoderm	-[b]	-[b,c]		-[a]	+[d]
E6.5	visceral endoderm	+[b,c]	-[b,c]	+[e]	-[a]	-[d,f]
	mesoderm	+[b,c]	+[b,c]		+[a]	+[d,f,1]
	ectoderm	+[b,c]	+[b,c]	-[e]	+[a]	+[d,f,1]
	parietal endoderm	-[b,c]	-[b,c]		-a	+[d,f]
	extraembryonic ectoderm	+[b,c]	+[b,c]		+[a]	-d,f
E7.5	visceral endoderm	+[b,c]	-[b,c]	+[e]	-[a]	-[d,f]
	primitive streak	+[b,c]	+[b,c]	-[c]	+[a]	+[d,f]
	allantois	+[c]	+[b,c]	+[c]	+[a]	-[d,f]
	parietal endoderm	-[b,c]	-[b,c]		-[a]	+[d,f]
	embryonic mesoderm, ant.	+[b,c]	+[b,c]	+[c]	+[a]	-[d,f]
	extraembryonic mesoderm	+[b,c]	+[b,c]	+[c]		-[d,f]
E8.5	neural tube		+[c]			+[d,f]
	developing brain		+[c]			+[d,f,2]
	somites	+[c]	+[c]		+[c]	+[d,f]
	endocardial cells			+[d]		
	allantois			+[c]		-[d,f]
	head mesenchyme			+[c]		
	parietal endoderm					+[d,f]
	vys endoderm	+[c]	+[c]	+[c]	+[c]	-[c]
	vys mesoderm	-[c]	-[c]	+[c]	+[c]	-[c]
E9.5	branchial arches/arteries	+[c]		+[c]		
	neural tube/ganglia	+[c]	+[c]			+[f]
	somites	+[c]	+[b,c]			+[f]
	floor plate			+[c]		
	brain	+[c]	+[b,c]			+[f]
	endocardium/myocardium/ cushion tissue of the heart	-[c]	-[c]	+[c]		
	otic pit			+[c]		

1: in putative primitive streak; 2: in developing hindbrain, rhombomeres 2, 4 and 6.
NB: In E6.0-9.5 mouse embryos expression of activin/inhibin βA, βB and α subunits is absent (Feijen *et al.,* 1994; Albano *et al.,* 1994; Manova *et al.,* 1992).

a: Gu *et al.,* 1998; b: Manova *et al.,* 1995: c: Van den Eijnden-van Raaij, A.J.M., Goumans, M.J., Loonstra, A., Rouws, C. and Feijen, A. (unpublished results): d: Feijen *et al.,* 1994; e: Roelen *et al.,* 1994; f: Albano *et al.,* 1994.

mesoderm of the visceral yolk sac of an E8.5 embryo (Fig. 6).

The expression pattern of the two type I receptors, ALK-2 and ALK-4, is shown in Figure 5. In early postimplantation mouse embryos (E6.0-E6.5) the ALK-2 signal is too weak for detection by *in situ* hybridization. RT-PCR studies have shown that ALK-2 is expressed in E6.0 embryos, and in the visceral endoderm, but not the embryonic ectoderm of E6.5 embryos (Roelen *et al.*, 1994). *In situ* hybridization on sections of E7.5 embryos have indicated a signal for ALK-2 in the extraembryonic mesoderm, including allantois, as well as in the embryonic mesoderm in the headfold region (Fig. 5A). In addition, the mesodermal component of amnion and chorion is positive, while the embryonic and extraembryonic ectoderm is negative. Expression in the visceral and parietal endoderm was difficult to observe, but RT-PCR studies have shown that the visceral endoderm and mesoderm, but not the ectoderm of E7.5 embryos is positive for ALK-2 (Roelen *et al.*, 1994). In E8.5 embryos the endothelial cells lining the endocardial cavity are positive, as well as the head mesenchyme and the allantois (Fig. 5B; Gu *et al.*, unpublished results). ALK-2 is also expressed in both the endodermal and mesodermal layer of the visceral yolk sac (Fig. 6). In E9.5 embryos the most pronounced site of ALK-2 expression is the developing heart. Expression is strongest in the endocardial cells lining the outflow tract, in cells lining the branchial arch arteries (Fig. 5C,D) and in the otic pit (not shown). ALK-2 is also strongly expressed in the floor plate (Fig. 5C,D). Weaker expression of ALK-2 could be observed in the myocardial wall of the heart chambers, the cushion tissue and in the branchial arches.

ALK-4 transcripts were detected uniformly at low levels in the extraembryonic ectoderm and the epiblast of E5.5 and E6.0 embryos. The signal in the visceral endoderm of these embryos was not above background (Fig. 5E,F; Gu *et al.*, 1998). During gastrulation (E6.5-E7.0) ALK-4 expression continued to be detectable in the epiblast and extraembryonic ectoderm, whereas expression in the ectoplacental cone and proximal visceral endoderm was not significant (Gu *et al.*, 1998). The distal endoderm cells, however, expressed ALK-4. At E7.5 all three germ layers in the embryonic region expressed ALK-4 (Fig. 5G) and the signal in the ectoderm appeared to be stronger in the primitive streak. In the extraembryonic region ALK-4 is strongly expressed in the allantois (Fig. 5H) and chorion, while the cuboidal proximal visceral endoderm cells still lack ALK-4. ALK-4 expression was not detected in the parietal endoderm at any stage of early development. In E8.5 embryos ALK-4 is expressed in the endoderm and mesoderm of the visceral yolk sac (Fig. 6).

Very recently the expression patterns of several Smads have also been described in the mouse embryo. Smad1 is expressed first in the early embryonic mesoderm and later ubiquitously, whereas Smad2 and Smad4 are ubiquitously expressed in both extraembryonic and embryonic tissues from E8.5 (Waldrip *et al.*, 1998; Yang *et al.*, 1998). During organogenesis Smad1 and Smad2 expression is observed in a variety of developing organs at sites of epithelial-mesenchymal interactions (Dick *et al.*, 1998).

From these expression studies it can be concluded that a suitable combination of two activin receptors, (type I and type II), and of two signal transduction components, Smad 2 and Smad 4, necessary for activin signalling, is present in cells of several tissues during the early stages of postimplantation development. The difference in expression pattern between ALK-2 and ALK-4 suggests that they have different roles in postimplantation development. Results of functional analyses of the type I and type II activin receptors in early mouse development, in as far as they are complete, are described below. The nature of the possible ligand(s) for these receptors *in vivo*, of follistatin as a ligand modifier and the involvement of different Smads in ligand-specific signalling pathways are also discussed.

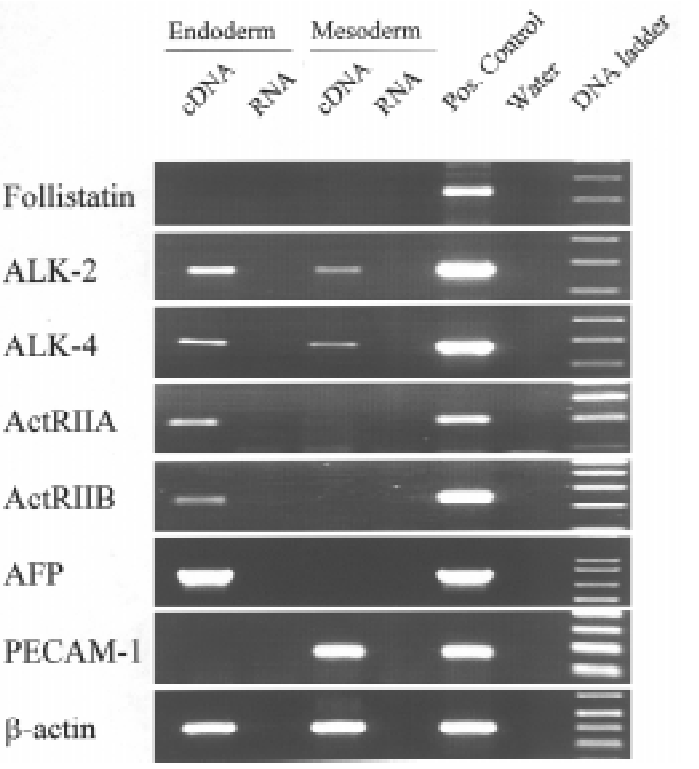

Fig. 6. Detection of activin receptors and follistatin in the endodermal and mesodermal layer of the visceral yolk sac at E8.5 by RT-PCR. *Amplified products of the type IIA and IIB activin receptor, type I receptors (ALK-2 and ALK-4), follistatin, endothelial cell marker PECAM-1, visceral endoderm marker α-fetoprotein (AFP), and β-actin are indicated.*

Functional analysis

In order to elucidate the function of activins and activin-binding proteins in early mouse development their coding genes have been disrupted by gene targeting, and the phenotypes of the resulting mice, which are deficient in one or more genes, have been analysed. Disrupting one of the activin type II receptor genes (type IIA or IIB) has no effect on preimplantation and early postimplantation development. Some of the mice deficient in the activin type IIA receptor have skeletal and facial abnormalities, which corresponds with the expression of this receptor in the first branchial arch. However, most activin receptor type IIA-deficient mice lack these defects and develop to adulthood. Their follicle-stimulating hormone was suppressed and their reproductive performance was defective (Matzuk *et al.*, 1995a). The phenotype of activin type IIB receptor-deficient mice is quite different. The mice die after birth from complicated cardiac defects that include randomised heart position, malposition of the great arteries, and ventricular and atrial septal defects. The heart anomalies are associated with right pulmonary isomerism and splenic abnormalities. In addition, the

mice undergo homeotic transformation of the axial skeleton (Oh and Li, 1997). These results indicate that the absence of one of the activin type II receptors does not disturb normal preimplantation development and gastrulation, but has an effect on organogenesis and fertility. However, recent studies have shown that mice deficient in both receptors have defects in primitive streak formation and gastrulation (Song *et al.*, 1999), indicating the requirement of at least one functional type II receptor for these processes.

From their expression patterns in early postimplantation embryos it was suggested that ALK-2 and ALK-4 have different functions in development. We compared the phenotype of ALK-2 and ALK-4 deficient mice with the expression pattern of the receptors. Analysis of the ALK-4-deficient embryos showed that the epiblast and the extraembryonic ectoderm were disorganised, resulting in disruption and developmental arrest of the egg cylinder before gastrulation (Gu *et al.*, 1998), which is consistent with the expression in embryonic and extraembryonic ectoderm. The trophoblast and parietal endoderm show no morphological defects, which corresponds with the lack of ALK-4 expression in these tissues. Analysis of chimaeras derived from injection of wild type ES cells into an ALK-4-deficient host or vice versa provided evidence that ALK-4 functions in both epiblast and extraembryonic cells to mediate signals that are required for egg cylinder organisation and primitive streak formation. ALK-4, however, is not essential for mesoderm formation.

Mouse embryos lacking a functional ALK-2 gene were arrested at the early gastrulation stage and displayed multiple morphological defects including abnormal morphology of the visceral endoderm, the lack of an elongated primitive streak, and delay and disruption of mesoderm formation (Gu *et al.*, 1999). Chimaera analysis has shown that ALK-2 functions in the visceral endoderm to regulate normal mesoderm formation and gastrulation in the mouse, which is consistent with the expression of ALK-2 in this tissue. Like ALK-4, ALK-2 is also not required for mesoderm formation in an autonomous manner (Gu *et al.*, 1999). From these functional studies it can be concluded that signalling via a combination of a type II activin receptor (IIA or IIB) and ALK-2 or ALK-4 by activin or activin-like molecules is important for normal early postimplantation development.

The unsolved problem is which ligand or ligands are involved. It is very unlikely that activin itself is a key ligand in early postimplantation development since the phenotype of activin-deficient mice is quite different from those of the receptor-deficient mice. Mice lacking functional activin βA subunits are viable at birth, lack whiskers and incisors, and die because of palate defects (Matzuk *et al.*, 1995b). Activin βB subunit-deficient mice suffered from failure of eyelid fusion and mutant females, but not males, manifested an impaired reproductive ability (Schrewe *et al.*, 1994; Vassalli *et al.*, 1994). Mice deficient in both activin subunits show the defects of both individual mutants (Matzuk *et al.*, 1995b). From these studies it appeared that zygotic activin A, activin B or activin AB and maternal activin B are not essential for mesoderm formation and gastrulation during mouse development. Although it cannot be excluded that other activin subunits (βC, βD and βE; Hotten *et al.*, 1995; Fang *et al.*, 1996; Oda *et al.*, 1995) are involved, a role for activin proteins is made even more unlikely by huge amounts of follistatin protein both in the decidua and in the embryonic part of the early conceptus (van den Eijnden-van Raaij *et al.*, unpublished results). This probably neutralises any trace of activin activity. A clear function for follistatin in early postimplantation development, however, has not been found since follistatin-deficient mice have only later defects, including decreased mass of the diaphragm and muscles, shiny taut skin, skeletal defects, abnormal whisker and tooth development. They fail to breathe and die within hours of birth (Matzuk *et al.*, 1995c). The lack of an early phenotype might be the result of rescue by maternal follistatin or redundancy with other BMP-binding proteins like chordin and noggin (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996).

A candidate ligand for signal transduction through an activin type II receptor in combination with ALK-4, however, is nodal, another member of the TGFβ superfamily. The phenotype of nodal-deficient mice suggests that nodal is not required for mesoderm formation, but is essential for primitive streak formation (Conlon *et al.*, 1994; Varlet *et al.*, 1997). The similarities in defects in primitive streak formation between ALK-4- and nodal-deficient mice raise the possibility that ALK-4 functions as the type I receptor for nodal during this process.

The TGFβ-like molecule signalling via ALK-2 is very likely to be a BMP. The phenotype of BMP-2-deficient mice closely resembles that of ALK-2 mutant mice, since both show abnormal development of amnion, chorion, proamniotic canal and allantois (Zhang and Bradley, 1996). The phenotype of BMP-2-deficient mice is less severe, which might be explained by partial rescue by maternal BMP-2 or BMP-4. In addition, studies in *Xenopus laevis* of ALK-2 and ALK-4 have indicated that ALK-2 behaves as a BMP receptor, and not as an activin receptor (Armes and Smith, 1997).

Another intriguing question is which Smads are involved in signalling by the different type I receptors. The answer to this question is complicated by the different phenotypes that have been reported by different groups as resulting from deletion of a single Smad. Waldrip *et al.* (1998) have reported that embryos lacking Smad2 have normal extraembryonic tissues, but fail to have a distinct proximal-distal polarity. The entire epiblast adopts a posterior mesodermal fate and differentiates as extraembryonic mesoderm, expressing Brachyury T at early gastrula stages. Nomura and Li (1998) and Weinstein *et al.* (1998), however, reported severe defects in extraembryonic tissues in Smad2 mutants and no expression of Brachyury T in the epiblast at gastrulation. In addition, and in contrast to the results of Waldrip *et al.* (1998) and Weinstein *et al.* (1998) the heterozygotes of Nomura and Li (1998) exhibit severe gastrulation defects. The reason for these differences is yet unclear. The similarity between the phenotype of Smad2 mutants and ALK-4 and nodal mutants strongly suggests that Smad2 mediates signalling through ALK-4. The hypothesis that Smad2 is involved in nodal signalling is further strengthened by the observation that mice trans-heterozygous for both Smad2 and nodal mutations had an identical gastrulation phenotype as Smad2 heterozygous mice (Nomura and Li, 1998).

Smad4-deficient mice had an unexpected phenotype. These mice are growth retarded during pregastrulation development, have abnormal visceral endoderm and have no mesoderm (Sirard *et al.*, 1998; Yang *et al.*, 1998). Chimaera analysis has shown that Smad 4 is not required in embryonic tissues for early mesoderm specification and gastrulation, while ALK-3 is required for these processes in the epiblast in an autonomous manner (Mishina *et al.*, 1995). These results suggest that either another Smad4 is involved in mesoderm specification or that signalling via Smad4, or in fact any Smad, is not required for this process.

Ablation of Smad3 on the other hand, in contrast to Smad2 which on the basis of studies in cells in culture is also considered to be involved in TGFβ and activin signalling, is compatible with

normal development although it does result in the formation of metastatic colorectal cancer in newborn mice (Zhu *et al.*, 1998).

Discussion

The current models for signal transduction by ligands of the TGFβ superfamily via serine/threonine kinase receptors and Smads that lead to transcriptional regulation of target genes and ultimately changes in cell behaviour, have been developed largely from experimental analysis of cells in culture, in particular from cells bearing mutations in genes of the signal transduction pathway. The cell lines that have been used in these studies are those most sensitive to ligand; since TGFβ1 is the prototype of the superfamily, experiments with this ligand in highly TGFβ-sensitive epithelial cell lines have essentially formed the basis of the models applicable to other ligands. However, we have seen in the sections above, that functional analysis of genes in the signal transduction cascade *in vivo* in mice either lacking some genes entirely or expressing dominant negative forms of particular proteins, inevitably lead to questions on the general applicability of the models derived from *in vitro* studies as they currently exist. For example, experiments in cell lines have suggested that ALK-5 is the only type I signalling receptor for TGFβ yet we have seen that in endothelial cells in particular ALK-1 may have the same function. It also seems likely that ALK-1 may be able to signal via Smad1, rather than Smad2, so that in some cells TGFβ signals might be mediated by Smad1. This contradicts the generally accepted view from cell lines which have led to Smad1, Smad5 and Smad8 being regarded as exclusively in the BMP pathway. Further, in cell lines in culture, Smad2 and Smad3 have often been reported to be functionally indistinguishable; the cellular response is the same whether Smad2 complexes with Smad4 or whether Smad3 complexes with Smad4 before translocation of the complex to the nucleus. The data in mice, however, suggest this is not the case *in vivo*; Smad2 is clearly essential for development beyond gastrulation while mice deficient in Smad3 develop normally to term. Either Smad3 is not necessary for development or it can be replaced by Smad2, as *in vitro,* but this is clearly not the case the other way around: Smad3 cannot replace Smad2.

Together, the data demonstrate that cell lines in culture have been exceedingly useful for developing concepts of how signals are transduced between the cell surface and the nucleus, and more recently how this leads to changes in the expression of target genes, but that only after further analyses of the consequences mutations in genes of the pathway have on development, in particular determination of which cells and tissues are affected, will it become clear exactly how this extremely important gene family works and the degree of redundancy there is in each tissue for each gene in the cascade.

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