TGFβ signaling at the summit

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Summary

Ligands belonging to the transforming growth factor (TGF) β superfamily have emerged as major regulators of a wide variety of developmental events, ranging from the earliest steps in germ layer patterning of the pre-gastrula embryo to tissue healing, regeneration and homeostasis in the adult. Recently, Caroline Hill and Bob Lechleider organized the third in a bi-annual series of FASEB meetings on TGFβ signaling and development at Snowmass (CO, USA). This meeting highlighted the ongoing interplay between advances in our understanding of the molecular biology of TGFβ family signaling and in investigations into its roles in specific developmental events.

Introduction

Since their identification in 1995, the Smad proteins have emerged as the major transducers of the TGFβ signaling pathway, which regulates transcription during embryogenesis and adulthood (Fig. 1) (Massague and Wotton, 2000). Although important responses to TGFβ signaling can occur both without Smads (Derynck and Zhang, 2003) and without transcriptional regulation (Ozdamar et al., 2005), the predominant focus of this meeting was on mechanisms that regulate Smad activity and on how these mechanisms confer specific transcriptional responses. This emphasis arose largely from the sheer number and variety of candidate regulators and targets of Smad function that have been identified over the past few years (Fig. 1, Table 1) (Derynck and Zhang, 2003; ten Dijke and Hill, 2004). A second focus of the meeting concerned how the TGFβ ligand superfamily are extracellulary regulated (Fig. 2). It has been well recognized for many years that a complex set of extracellular antagonists and co-factors modulate TGFβ ligand activity. How these regulators fit together to generate spatially and temporally complex patterns of highly specific gene activation remains a crucial area of investigation. A number of talks reported on novel mechanisms of ligand regulation, some of which were placed in specific developmental contexts.

Transcriptional regulation by Smads: targets and co-factors

Numerous efforts have identified individual examples of promoters or enhancers that are regulated by the interaction of the Smads with tissue-specific transcription factors (e.g. Chen et al., 1996, Seone et al., 2004) (reviewed by Massague and Wotton, 2000), which have laid the basis for a more systematic analysis of sets of transcriptional responses to TGFβ. Several talks explored the problem of identifying synexpression sets of transcriptional responses to TGFβ signals (synexpression sets are sets of genes that are coordinately expressed in response to a given stimulus). Joan Massague (Sloan-Kettering Institute, New York, NY, USA) built on prior work from his laboratory that established the Foxo transcription factors as being Smad3 interactors (Seoane et al., 2004). At this meeting, he reported on the transcriptional profiling of *Foxo*-null cells to identify transcriptional responses that are specifically mediated by Smad3/Foxo interactions. Analysis of Smad3/Foxo responsive elements from several target genes, such as *p21CIP1* (*Cdkn1a* – Mouse Genome Informatics) (Seoane et al., 2004), revealed considerable diversity in the spatial relationship between Smad- and Foxo-binding sites, thus indicating relatively loose constraints on how complexes of these factors recognize target promoters. Liliana Attisano (University of Toronto, Toronto, Canada) presented an in silico approach to identifying the targets of Foxh1, a transcription factor that associates with activated Smad2/Smad4 complexes during patterning of early embryos by the nodal subset of TGFβ ligands (Chen et al., 1996). By combining information on the genomic location of predicted DNA-binding sites for Foxh1 and Smad4 with cross-species comparisons, these investigators identified and validated several novel embryonic targets of Foxh1 signaling.

Aristidis Moustakas (Ludwig Institute, Uppsala, Sweden) focused on responses that either coordinate or distinguish TGFβ effects on epithelial-to-mesenchymal transitions (EMTs) and on inhibition of cell proliferation. He described the functional characterization of genes identified in a broadly targeted microarray screen that compared genes that are induced by TGFβ in NMuMg mammary epithelial cells (in which TGFβ concomitantly induces cytostasis and EMT) with those that are induced by Bmp7 (which induces neither response in these cells). Members of the Id family of basic helix-loop-helix (bHLH) factors, particularly Id2, emerged from this study as being crucial targets for the downregulation of gene expression by TGFβ during the regulation of both cytostasis and EMT. Two additional gene targets of TGFβ signaling were identified: a high-mobility group protein, which acts as a potential upstream regulator of EMT; and a homeodomain protein, which regulates the epithelial cytostatic response.

Kunxin Luo (University of California, Berkeley, CA, USA) approached the issue of TGFβ-regulated transcription and EMT versus cytostasis from a different angle by examining the role of the co-repressors Sno and Ski in these processes. Sno and Ski have previously been shown by the Luo laboratory and others to act as co-repressors that interact with Smads to suppress TGFβ-stimulated transcription (Luo, 2004). Although the functions of Sno and Ski have generally been viewed as being overlapping, Luo used RNAi analysis to show that their individual knock down produces distinctive effects on cell motility, EMT and growth arrest, suggesting that the relative endogenous levels of Sno and Ski may lead to distinctive responses to TGFβ signals.

The differential interaction of Smad complexes with co-repressors and co-activators is emerging as a major issue in mechanisms that generate specific cell-type responses to TGFβ signals. Rik Derynck (University of California, San Francisco,

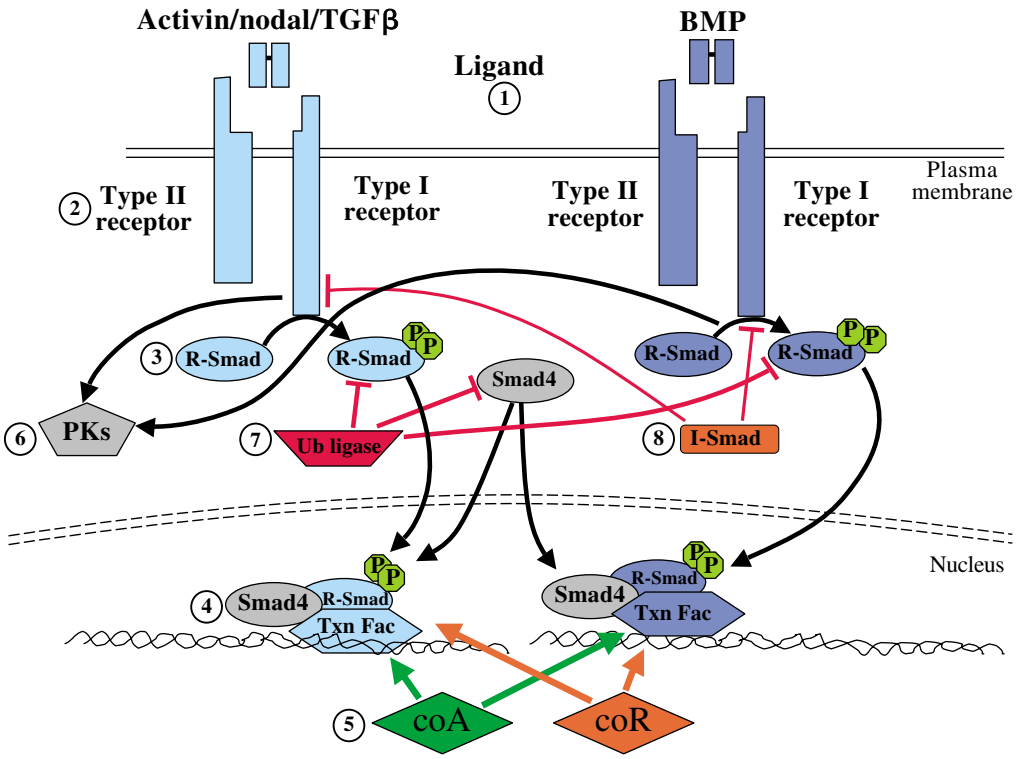

Fig. 1. A schematic overview of TGFβ signal transduction. Numbers in circles refer to specific examples given in Table 1. Abbreviations: BMP, bone morphogenetic protein; coA, co-activator; coR, co-repressor; I-Smad, inhibitory Smad; P, phosphate; Pks, protein kinases; R-Smad, receptor-regulated Smad; Txn Fac, transcription factor; Ub ligase, ubiquitin ligase. Black and green lines indicate positive interactions, red lines indicate inhibitory ones.

CA, USA) touched on this issue for Smad3/Runx2 complexes, which interact with specific histone deacetylases (HDACs) to shut off bone-differentiation genes in mesenchymal stem cells. By contrast, Runx1 and Runx3 appear to form transcriptional activation complexes with Smads in other cell types (Ito and Miyazono, 2003).

Although a wide range of transcriptional components that mediate TGFβ signals have been identified, the mechanistic basis for transcriptional activation by Smads has not been directly examined. Caroline Hill (Cancer Research UK, London, UK) addressed this gap by establishing an in vitro assay for Smad-mediated transcriptional activation. This assay is likely to provide an important tool for understanding the specifics of how Smads interact with and modulate the local activity of the transcriptional machinery.

Distinct and overlapping Smad functions

Although functional distinctions between different Smads have been established in assays in vitro, the significance of these distinctions has been awaiting in vivo tests. Smad2 and Smad3 are regulated by similar upstream pathways (Fig. 1) but differ biochemically in that Smad3 can bind directly to DNA, whereas Smad2 cannot, owing to an insert in the otherwise conserved DNA-binding region (Dennler et al., 1999). This insert is excised in an alternative splice variant of Smad2 (Smad2δ exon3), enabling it to bind DNA, but the significance of this splice variant in vivo is not known. Inactivation of the *Smad3* gene in mice does not result in disruption of early embryogenesis, indicating that Smad2 can effectively compensate for Smad3 at these stages (Datto et al., 1999; Yang et al., 1999). Liz Robertson (Oxford University, Oxford, UK) presented new evidence indicating that Smad2δ exon3 can functionally substitute for both Smad3 and Smad2 when it is expressed in the mouse epiblast. Her genetic interaction data suggest that Smad3 can partially compensate for some Smad2 functions. Thus, the DNA-binding activities of Smad2δ exon3 and Smad3 appear to be of crucial importance in the early mouse embryo. By contrast, Lalage Wakefield (National Cancer Institute, Bethesda, MD, USA) reported significant differences in how mammary epithelial cells that lack either Smad2 or Smad3 genes or proteins (?) respond to TGFβ. Thus, functional divergence between Smad2 and Smad3 seems to be a cell-context-dependent phenomenon, and defining the basis for this divergence will be an important area for further investigation. Findings from both the Robertson and Wakefield laboratories indicate that the level of Smads (as controlled by gene dose) is an important component of phenotypic outcome, consistent with work from many other laboratories demonstrating that the ubiquitin ligase-targeted degradation of Smads is an important mechanism of Smad regulation (Datto and Wang, 2005).

TGFβ signaling and other signaling pathways: integration and relays

A common theme at the meeting among studies in different developmental systems was the integration of TGFβ signaling with other extracellular signals. Xiao-Fan Wang (Duke University, Durham, NC, USA) discussed mechanisms that integrate cooperative transcriptional regulation by Wnt and TGFβ in mesenchymal progenitor cells. Peter ten Dijke (Leiden University Medical Center, Leiden, The Netherlands) reported that Wnt signaling is required for BMP-induced osteoblast differentiation. This cooperative interaction may be important in vivo because a Wnt antagonist is required for bone homeostasis in vivo. Several talks described the induction of a second signaling cascade by a TGFβ signal. For example, Laurel Raftery (Massachusetts General Hospital, Boston, MA, USA) described that stimulation of Notch signaling by BMP

signaling is important in *Drosophila* follicle cell patterning. The activation of FGF signaling was reported to be important for the TGFβ-induced proliferation of craniofacial bone development by Yang Chai (University of Southern California, Los Angeles, CA, USA). However, this TGFβ-FGF relay mechanism appears to be specific for the induction of bone formation from neural crest mesenchyme, as opposed to the induction of smooth muscle differentiation. Bob Lechleider (Georgetown University Medical Center, Washington, DC, USA) reported that it is downregulation of FGF signaling that is important for TGFβ-induced smooth muscle differentiation. The close intertwining of TGFβ signaling with other pathways appears to be an important component of cell fate determination by TGFβ family members.

Table 1. Components of vertebrate and *Drosophila* signaling

Components*	Activin/nodal/TGFβ	BMP
1 Ligands	Activin	BMP2, BMP4, BMP5, BMP6, BMP7, BMP9
	Nodal	GDF5, GDF6, GDF7
	TGFβ1, TGFβ2, TGFβ3	Dpp, Gbb, 60A
	GDF1, GDF8, GDF11	ADMP
	Cyc, Sqt, Spw	Radar
	Xnr1, Xnr2, Xnr4, Xnr5, Xnr6	AMH (MIS)
	Vg1, Derriere	
	dActivin, Myoglianin, Alp23	
2 Type I receptors	Alk1	Alk2
	Alk4	Alk3
	Alk5	Alk6
	Alk7	Alk8
	TARAM A	Tkv, Sax
	Babo	
Type II receptors	TβRII	BMPRII
	ActRIIA, ActRIIB	ActRIIA, ActRIIB
	Punt	AMHR-II
		Punt, Wit
3 R-Smads	Smad2, Smad2Δ exon3	Smad1
	Smad3	Smad5
	Smox	Smad8
		Mad
4 Transcription factors	Foxh1	OAZ
	Foxo	Nkx3.2
	Mixer	Hoxc8
	Runx2	Runx2
	AP1	Zen
	TFE3	Labial
	E2F4	Tinman
5 Co-activators	p300	p300
	CBP	CBP
	MSG1	
	Swift	
Co-repressors	HDAC1	HDACs
	Sno	Sno
	Ski	Ski
	Evi1	Evi1
	Sin4	Schnurri
	TGIF	
	p107	
6 Cytosolic protein kinases	TAK1	TAK1
	ERK	ERK
	JNK	JNK
	p38	p38
	NLK	
7 Ubiquitin ligases	Smurf2	Smurf1
	Ectodermin	Ectodermin
		dSmurf
8 Inhibitory Smads	Smad7	Smad7
		Smad6
		Dad

*Lists are not intended to be fully comprehensive; for additional examples, readers are directed to reviews cited in the text.

Abbreviations: ADMP, anti-dorsalizing morphogenetic protein; Alk, activin receptor-like kinase; AMH, anti-Müllerian hormone; CBP, CREB-binding protein; GDF, growth and differentiation factor; HDAC, histone deacetylase.

Extracellular regulation of TGFβ ligands

The extracellular regulation of TGFβ ligand activity is an expanding area of investigation, and has been intensely investigated during *Drosophila* dorsoventral patterning. In *Drosophila* blastoderm embryos, extracellular BMP-binding proteins (Sog and Tsg) and the metalloprotease Tolloid are required to localize BMP activity to a narrow spatial domain at the dorsal midline of the late blastoderm embryo (reviewed by Ashe, 2005; Raftery and Sutherland, 2003). Mike O'Connor (University of Minnesota, Minneapolis, MN, USA) presented his group's studies of the related extracellular DPP/BMP-binding proteins, Crossveinless2 (Cv2), Crossveinless (Cv) and Tolloid-related (Tlr), which are required to localize BMP activity to the narrow line of primordial wing cells in *Drosophila* where the posterior crossvein will form (Ralston and Blair, 2005; Serpe et al., 2005; Shimmi et al., 2005). Differences in kinetics between the Sog/Tsg/Tolloid system and the Cv2/Cv/Tlr system correlate with the differences in temporal constraints for patterning in these two tissues. O'Connor described a bind and release mechanism for localizing active BMP ligands that almost certainly also occurs in vertebrate tissues because all three classes of extracellular regulators are also found in vertebrates: the Sog/CV/Chordin type of BMP-binding proteins; Tolloid/Tlr/BMP1 extracellular metalloproteases; and Tsg-like proteins (reviewed by Dale, 2000).

Metalloproteases related to Tolloid are proving to be a versatile group of BMP regulatory proteins. Tolloid and its *Xenopus* homolog Xolloid have previously been shown to have similar abilities to cleave the BMP antagonists Sog and Chordin (reviewed by Mullins, 1998). Each is now reported to release active TGFβ family ligands by targeting a different class of latent complexes. Some TGFβ ligands form latent complexes when the initially synthesized propeptide is cleaved during secretion; the C-terminal ligand domain remains associated with the N-terminal pro-domain in a latent complex (reviewed by Massague, 1998). Malcolm Whitman (Harvard Dental School, Boston, MA, USA) reported that *Xenopus* GDF11, a TGFβ ligand, is secreted as a latent complex of mature ligand and pro-domain. This complex is cleaved, so that GDF11 is activated by Xolloid in the developing tail, where these factors are co-expressed. Mihaela Serpe (University of Minnesota, Minneapolis, MN, USA) reported that *Drosophila* Tolloid and Tolloid-related both can activate latent ligands in a cell culture system. Murine Bmp1, another member of this family, can also activate latent ligands (Ge et al., 2005). This class of metalloproteases has broad biological activities, as Bmp1 cleaves a number of extracellular matrix proteins in biologically important reactions (Gonzalez et al., 2005).

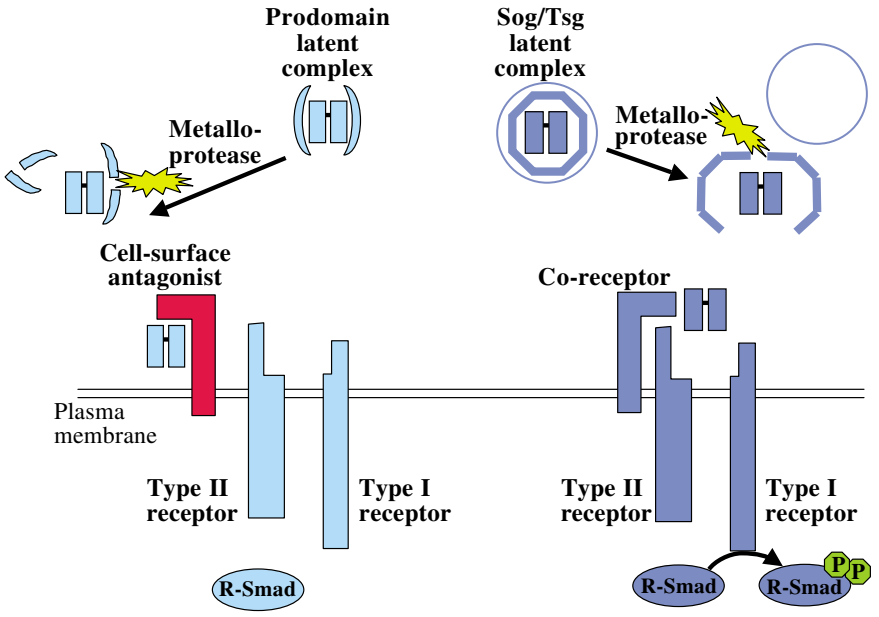

Fig. 2. Extracellular regulation of TGFβ ligand family activity. Ligand activity can be masked in latent complexes and activated by the action of extracellular metalloproteases in the BMP1/Tolloid family (Massague, 1998; Dale, 2000; Ashe, 2005). Activity can be modulated both positively and negatively by cell surface proteins, as discussed in the text. This figure provides a snapshot of systems discussed at the meeting, and is not intended to be a comprehensive review. Abbreviations: Sog, Short gastrulation, Tsg, Twisted gastrulation

The ability of pro-domains to remain associated with the ligand in a secreted latent complex was first identified for TGFβ1, TGFβ2 and TGFβ3 (reviewed by Massague, 1998). However, not all ligands in this family form such complexes; for example, there have been no reports of such complexes for Bmp2 and Bmp4. Intriguingly, Bmp9 appears to retain full biological activity, while it is associated with its prodomain (Senyon Choe, Salk Institute, San Diego, CA, USA). Perhaps the formation of pro-domain latent complexes is a characteristic of broadly expressed or circulating ligands, whereas ligands with more limited expression patterns might be regulated by other mechanisms.

Cell-surface co-receptors were highlighted at the meeting as another area of expanding significance. The importance of co-receptors for TGFβ family signaling was recognized early on, with the identification of betaglycan as a cell surface protein that binds TGFβ ligands and facilitates the activation of the signaling receptor complex (Lewis et al., 2000). More recently, it has been found that cripto is an essential co-receptor for nodal signaling (reviewed by Schier, 2003). Michael Shen (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA) discussed evidence for non-cell-autonomous functions of murine cripto (Yan et al., 2002). Rick Padgett (Rutgers University, Piscataway, NJ, USA) presented the genetic identification of candidate co-receptors of the *C. elegans sma* pathway, and Jodie Babbitt (Massachusetts General Hospital, Boston, MA, USA) reported that RGMa, like other proteins in the Dragon family, is a co-receptor for BMPs (Babitt et al., 2005).

The functional pairing of TGFβ superfamily receptors to ligands, like functional divergence among the Smads, has been established in vitro but has not been fully investigated in vivo. TBRII and Alk5 have been identified as type II and type I receptors, respectively, for TGFβ. Yang Chai (University of Southern California, Los Angeles, CA, USA) and Vesa Kaartinen (Saban Research Institute, Children's Hospital, Los Angeles, CA, USA) presented data on the loss of TβRII and Alk5, respectively, in neural crest derivatives in mice. In each case, dramatic craniofacial malformations result, consistent with the action of these receptors in a common neural crest pathway. Intriguingly, however, loss of *Alk5* results in additional defects not seen with loss of the gene encoding TβRII, raising the possibility that the Alk5 Type I receptor may act in conjunction with Type II receptors other than TβRII in the developing neural crest.

TGFβ signaling and vascular development

Studies of TGFβ signaling in mouse angiogenesis have been informative both for uncovering novel mechanisms of TGFβ signal transduction and for elucidating the etiology of vascular defects in human hereditary hemorrhagic telangiectasia (HHT) (reviewed by Marchuk et al., 2003). HHT1 is associated with mutations in *eng*, the gene for endoglin. HHT2 is associated with mutations in *ACVRL1*, the gene for Alk1, a type I receptor that can bind TGFβ. Both syndromes are marked by the formation of direct arterial-venous malformations in some tissues, in which arteries and veins are directly connected with no intervening capillary network. These syndromes suggest that a sufficient level of TGFβ signal transduction is necessary to maintain capillary networks in certain tissues. Consistent with this hypothesis, TGFβ has dose-dependent effects on endothelial cells in vitro. Doug Marchuk (Duke University, Durham, NC, USA) presented the human genetic perspective on TGFβ signaling. In addition to the two autosomal dominant HHT syndromes that have been described in humans, Marchuk reported that individuals with juvenile polyposis, which is associated with mutations in *MADH4* (Smad4), also exhibit HHT-like lesions (Gallione et al., 2004). It seems likely that these human syndromes identify rate-limiting steps for TGFβ regulation of capillary remodeling.

There was lively discussion during the vasculogenesis session, as investigators sought to resolve the complex phenotypes observed in different studies of receptor mutants in murine endothelial cells. A substantial effort has been directed towards developing mouse genetic models of HHT, and towards understanding the mechanisms of TGFβ signaling in

angiogenesis. In vitro studies of angiogenesis divide the process into two phases, both of which are stimulated by TGFβ1 (reviewed by Lebrin et al., 2005; Marchuk et al., 2003). Vascular remodeling begins with endothelial cell migration, proliferation and lumen formation, which are aspects of the activation phase of angiogenesis. Remodeling is completed with the cessation of endothelial cell proliferation and migration, the production of new basement membrane, and the recruitment and differentiation of smooth muscle cells, i.e. the maturation, or resolution, phase of angiogenesis. Mice homozygous null for either *Eng* or *Alk1* (*Acvrl1* – Mouse Genome Informatics) have defects in the embryonic vasculature; initial vasculature formation occurs, but subsequent remodeling is defective (Li et al., 1999; Oh et al., 2000). The effects of these mutations on angiogenesis in vivo and in embryonic endothelial cells are under intensive investigation.

TGFβ can stimulate two Smad pathways in endothelial cells: the canonical Smad2/Smad3/Smad4 pathway via its predominant type I receptor, Alk5; and the Smad1/Smad5/Smad4 pathway via Alk1 (Fig. 3) (reviewed by Lebrin et al., 2005; Marchuk et al., 2003). ten Dijke presented data that support an attractive model in which Alk1/Smad1 signaling promotes the activation phase of angiogenesis and Alk5/Smad2/Smad3 signaling promotes the resolution phase (Goumans et al., 2002). However, this model may be incomplete, as Paul Oh (University of Florida, Orlando, FL, USA) and Sabine Bailly (INSERM, Grenoble, France) reported results indicating that Alk1 promotes maturation (Lamouille et al., 2002; Seki et al., 2003). At present, it is not clear whether these conflicting results arise from the use of different endothelial cell types, or whether some of the effects observed arise from compensatory changes in constitutively mutant tissues. O'Connor reported embryonic vascular defects in *Tak1* knockout homozygotes, raising the possibility that angiogenesis may also involve non-Smad signal transduction via this MEKK kinase.

Endoglin was identified soon after betaglycan as a potential endothelial component of TGFβ receptor complexes; initial data suggested that it antagonizes TGFβ signaling (Barbara et al., 1999). ten Dijke reported that endoglin can promote signaling through Alk1 and antagonize signaling through Alk5; Jeff Wrana (Samuel Lunenfeld Institute, Toronto, Canada) touched on results that indicate that endoglin antagonizes signaling by regulating Alk5 levels (Lebrin et al., 2004; Pece-Barbara et al., 2005). The molecular mechanisms by which endoglin can bias receptor choice by TGFβ ligands are still unclear. Understanding the mechanisms for receptor choice

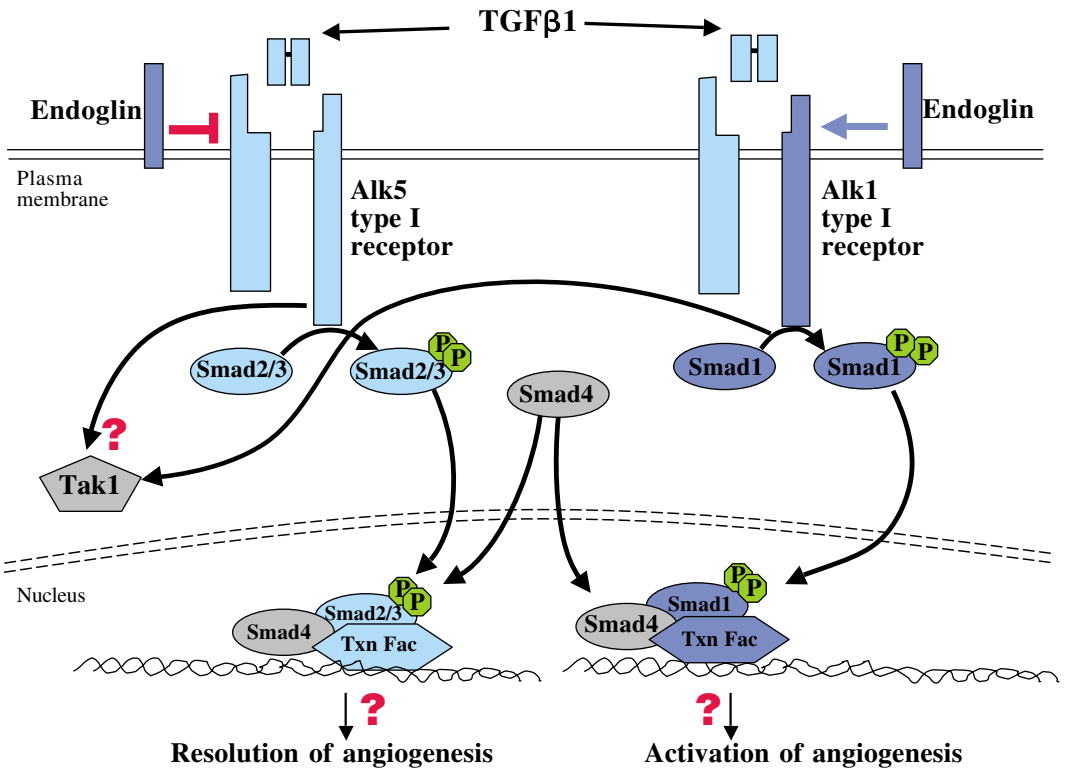

Fig. 3. A model for TGFβ1 signal transduction during mouse angiogenesis. TGFβ1 can activate two type I receptors in cultured endothelial cells (reviewed by Byfield and Roberts, 2004). As expected, it activates Alk5 (also called TβRI), the canonical TGFβ type 1 receptor, and surprisingly also Alk1, an orphan type 1 receptor that stimulates Smad1 phosphorylation, which is normally associated with BMP signaling (see Table 1). Different levels of TGFβ promote different endothelial cell responses, supporting a model in which low signaling activity promotes activation responses and high signaling activity promotes resolution responses. The specific phase of angiogenesis regulated by Alk1 is controversial ('?'). The cell-surface protein endoglin reportedly stimulates Alk1 signaling and antagonizes Alk5 signaling (Lebrin et al., 2004; Pece-Barbara et al., 2005) through unclear mechanisms. *Tak1* knockout mice have embryonic vasculature defects, suggesting that this non-Smad pathway is involved in TGFβ signaling during angiogenesis. [For comprehensive discussions of this model, see Marchuk (Marchuk, 2003) and Lebrin et al. (Lebrin et al., 2005).] Alk, activin receptor-like kinase; P, phosphate; Tak1, TGFβ activated kinase 1; Txn Fac, transcription factor.

and the specific cell types that are most sensitive to each signal transduction pathway remain crucial issues for this field. These studies of TGFβ signaling in mouse angiogenesis, like studies of BMP signaling in *Drosophila* patterning, underscore the importance of developmental genetics for uncovering the delicate balance of mechanisms that mediate dose-dependent responses to TGFβ family ligands in normal development.

Conclusions

The TGFβ field has made dramatic progress in the identification of components of the pathways that regulate TGFβ signaling both extracellularly and intracellularly. The importance of in vivo developmental analyses to test these molecular mechanisms is highlighted by the evolving story on TGFβ1 signaling in angiogenesis. Similar studies of other in vivo mechanisms will be fertile ground for presentations in 2 years at the next meeting.

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