REVIEW

Smads and early developmental signaling by the TGF β superfamily

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Members of the transforming growth factor β (TGF β) superfamily of peptide growth factors regulate a broad range of cellular functions, including proliferation, apoptosis, extracellular matrix secretion and adhesion, terminal differentiation, and specification of developmental fate (Roberts and Sporn 1993; Wall and Hogan 1994; Moses and Serra 1996). Although regulation of each of these functions by TGF β superfamily factors is important throughout embryonic development, it is the potency of these factors in regulating developmental fate that has been the focus of recent excitement among embryologists. This has been primarily because of indications in both vertebrate and invertebrate model systems that TGF β s can serve as morphogens, acting across developing tissues in a graded fashion to specify a patterned array of cell fates (Gurdon et al. 1994; Nellen et al. 1996; Neumann and Cohen 1997). A defining feature of morphogens is their ability to specify multiple cell types over a range of concentrations. This capacity has been demonstrated for several members of the TGF β superfamily in early *Xenopus* embryos (Green and Smith 1990; Dosch et al. 1997; Wilson et al. 1997) and for the bone morphogenetic protein (BMP) homolog *decapentaplegic* (*dpp*) in the early *Drosophila* embryo and wing disc (Gelbart 1989; Ferguson and Anderson 1992; Wharton et al. 1993; Lecuit et al. 1996; Nellen et al. 1996). The intracellular mechanisms by which ligand dose can be transduced into multiple developmental fates is therefore a current focus of interest for understanding TGF β s as morphogens.

A second striking feature of TGF β superfamily signals is the variety of effects they can evoke, contingent on the developmental history of the responding cell. For example, at the gastrula stage, BMP2 can specify frog blastomeres as epidermal rather than neural progenitors (Wilson et al. 1997); at later stages it can specify dorso-ventral pattern in the neural tube (for review, see Tanabe and Jessell 1997), neural crest (NC) cells to form neuronal rather than Schwann cells (for review, see Mehler et al. 1997), and apoptotic patterning of the NC during rhombomere segmentation (Graham and Lumsden 1996). How the developmental history of a particular cell intersects with an instructive signal from a TGF β super-

family ligand to generate a cell type-specific developmental decision is therefore a second critical question in understanding how these ligands function in developmental patterning. A third central issue, closely tied to the first two, is how other signaling pathways that participate in developmental patterning interact with TGF β superfamily signals. Over the past 10 years considerable progress has been made in understanding where and when different TGF β superfamily ligands signal in embryos and defining characteristic transcriptional responses that mark their effects. Understanding the molecular basis for patterning, however, will also require a knowledge of how the intracellular signal transduction of TGF β s integrates ligand dose, developmental history, and signals from other pathways to generate the complex set of responses observed during embryogenesis.

Until recently, very little has been known about how TGF β superfamily signals are transduced intracellularly. This situation has changed rapidly over the past 3 years, however, with the discovery and characterization of the Smad family of gene products as transducers of TGF β superfamily signals (for review, see Derynck and Feng 1997; Heldin et al. 1997; Attisano and Wrana 1998; Massagué 1998). Although our understanding of how Smads mediate cell type-specific transcriptional regulation is still incomplete, characterization of these signal transducers has begun to provide a molecular framework within which to investigate in detail how the TGF β superfamily of factors regulates complex patterns of cell specification. In addition to providing our first picture of how TGF β signals directly regulate transcription, studies of Smad regulation have begun to provide clues as to how cells integrate multiple signals from both TGF β and non-TGF β signaling pathways. Recent work on the extracellular regulation of BMPs during embryogenesis has provided a dramatic advance in the understanding of BMP-dependent patterning mechanisms conserved between vertebrates and invertebrates (Holley et al. 1995; Piccolo et al. 1996; Zimmerman et al. 1996); recent advances in understanding intracellular regulation by TGF β s provides new opportunities for examining their function in patterning at multiple steps in embryogenesis.

TGF β superfamily signal transduction has been the subject of numerous reviews over the past 2 years (see above). This review will provide a relatively cursory

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summary of the work reviewed previously to concentrate on more recent work on Smads as a focal point for thinking about possible mechanisms both for TGF β signaling specificity and for integration of multiple signaling pathways during embryogenesis.

Transduction background: the receptors

The first step in signaling by TGF β superfamily factors is the binding of ligand to transmembrane receptors. In general, TGF β superfamily ligands bind and thus bring together two distinct types of receptors, designated type I and type II (see Derynck and Feng 1997; Massagué 1998). Both receptor types are serine–threonine-specific protein kinases; upon ligand binding, the type II receptor phosphorylates the type I receptor, which is activated and, in turn, is responsible for intracellular signaling. Although type I and type II receptors have been named according to the ligands they can bind, the full set of physiologically significant combinations of ligands and type I/II receptors that interact *in vivo* has not been fully described (Derynck and Feng 1997). Some ligands (e.g., TGF β and possibly activin) also have a non-serine–threonine kinase type III receptor that facilitates or modulates binding to type I/II receptors (Lopez-Casillas et al. 1993), and different I/II pairs may vary significantly in the relative contributions of the type I and type II receptors to the affinity and specificity of ligand binding (for review, see Derynck and Feng 1997; Massagué 1998). Because the type I receptors appear to be the primary determinants of intracellular signaling specificity (Carcamo et al. 1994), the range of cellular responses a given ligand can elicit may depend significantly on exactly which combinations of type I/II receptors can bind ligand in a given target cell.

The discovery of Smads in *Drosophila* and *Caenorhabditis elegans*

The characterization of type I receptors as signal transducing serine–threonine-specific protein kinases immediately raised the question of what are the downstream targets for these kinases. A potential answer came from a *Drosophila* genetic screen for maternal effect mutations that enhance the phenotype of weak alleles of *dpp*, a *Drosophila* BMP homolog (Raftery et al. 1995). Multiple enhancing mutations were found in a gene named *Mothers against dpp* (*Mad*) (Raftery et al. 1995; Sekelsky et al. 1995). Genetic and sequencing analysis showed that *Mad* encodes a novel protein that acts cell autonomously downstream of Dpp and the Dpp type I receptor *thick veins* (*Tkv*) (Sekelsky et al. 1995; Hoodless et al. 1996; Newfeld et al. 1996; Wiersdorff et al. 1996). A second gene from the same screen, *Medea*, has subsequently been shown to share significant homology with *Mad* and to also act downstream of Dpp (Das et al. 1998; Hudson et al. 1998; Wisotzkey et al. 1998). In the nematode *Caenorhabditis elegans*, cloning and sequencing of several genes implicated in a pathway regulated by a TGF β receptor homolog (*daf-4*) (Estevez et al. 1993) identified a

family of genes (*sma-2*, *sma-3*, and *sma-4*) with clear homology to *Mad*, suggesting that the *Mad/sma* genes might represent a broadly conserved family encoding proteins involved in TGF β superfamily signaling (Savage et al. 1996). Because of the dual origin of this family of genes, and the pre-existence of unrelated proteins abbreviated as MAD, the family defined by the homology between MAD and *sma-2,3,4* was renamed Smad (Derynck et al. 1996).

The vertebrate Smads

Following closely on the heels of the identification of *Drosophila* and nematode Smads, vertebrate Smad homologs were identified in EST database searches, by degenerate oligonucleotide PCR and by independent functional expression screens in *Xenopus*. A total of nine vertebrate Smads have been identified (a number that is still rapidly changing), as well as two in *Drosophila*, and five in nematodes. Most of the invertebrate Smads have been identified as homologs of specific vertebrate Smads on the basis of both primary sequence and functional criteria; for example, *Mad* is functionally similar to Smad1 (Newfeld et al. 1996) and *Medea* and *Sma-4* are similar to Smad4 (Savage et al. 1996; Das et al. 1998; Wisotzkey et al. 1998). However, some invertebrate Smads (e.g., *daf-3*) (Patterson et al. 1997) have no clear homolog yet identified in vertebrates.

An indication that Smads might be able to mimic TGF β superfamily signals came from overexpression studies in *Xenopus*. Expression of either full-length Smad2 or its carboxy-terminal half mimics the mesoderm-inducing effects of activin on animal pole blastomeres (Baker and Harland 1996; Graff et al. 1996). Overexpression of Smad1 or Smad5, in contrast, mimics the ventralizing effects of BMP4 (Liu et al. 1996; Thomsen 1996; Suzuki et al. 1997a). These observations not only demonstrated that Smads can mimic TGF β superfamily signals but also suggested that different Smads might transduce distinct TGF β superfamily signals. Concomitant observations that BMP4 stimulates Smad1 phosphorylation and translocation to the nucleus (Hoodless et al. 1996; Liu et al. 1996), whereas activin or TGF β stimulates Smad2 phosphorylation and nuclear translocation (Baker and Harland 1996; Eppert et al. 1996; Yingling et al. 1996), confirmed that these Smads participate in distinct TGF β superfamily signaling pathways. Identification and characterization of additional Smads has led to the designation of three distinct categories of Smads: (1) those that are directly phosphorylated by type I receptors and when overexpressed mimic specific signaling pathways (“pathway-specific” or “receptor-regulated” Smads, e.g., vertebrate Smad1, Smad2, Smad3, Smad5, and Smad8, *Drosophila* *Mad*); (2) those that appear to be shared among multiple signaling pathways (e.g., vertebrate Smad4 and *Drosophila* *Medea*); and (3) those that when overexpressed inhibit TGF β superfamily signaling [“inhibitory Smads,” e.g., vertebrate Smad6, Smad7, and *Drosophila* Daughters against Dpp (*Dad*)]. There remain some Smads that have not been catego-

alized definitively in this framework (e.g., *daf-3*), and as there are still many more TGF β superfamily ligands than there are receptor-regulated Smads, it seems likely that many ligands, and possibly some type I receptors, may share common receptor-regulated Smads for their signaling. Although the three categories listed above provide a general framework for how Smads work, the definition and number of these categories may change as our understanding of Smads progresses. Table 1 provides a summary of some of the best understood sets of components of TGF β superfamily signaling pathways.

Domain structure of Smads

Examination of the primary sequence of Smads revealed extensive sequence homology among Smads in two distinct regions: an amino-terminal domain designated the N or Mad homology 1 (MH1) domain, and a carboxy-terminal domain designated the C or MH2 domain. All Smads identified to date contain a recognizable MH2 domain; however, the inhibitory Smads have poorly conserved MH1 domains. The region between the MH1 and MH2 domains is referred to as the linker region and is poorly conserved among Smads. Functional analysis of Smad point and deletion mutations (Baker and Harland 1996; Hata et al. 1997) suggests that in the inactive state, the MH1 and MH2 domains associate with one another. Receptor activation then opens up this structure to activate the effector functions of each domain (discussed below).

Smad heterodimerization—the necessity of Smad4

Genetic analysis of the *C. elegans* Smads (*sma-1,2,3*) indicated that multiple Smads are required in the same tissue to transmit upstream signals (Savage et al. 1996). A specific mechanism for this cooperation was provided

by biochemical analysis of the vertebrate Smads: The pathway-specific Smads heterodimerize with Smad4 in response to ligand stimulation (Lagna et al. 1996; Zhang et al. 1996, 1997). This heterodimerization appears to be necessary for signaling, as cell lines that lack Smad4 do not respond to TGF β signals, and signaling can be restored by transfection of wild-type Smad4 (Lagna et al. 1996; de Caestecker et al. 1997). Recent genetic data from *Drosophila* reveal a requirement for the Smad4 homolog Medea in Dpp/Mad signaling, indicating that this heterodimerization mechanism is conserved between vertebrates and invertebrates (Das et al. 1998; Hudson et al. 1998; Wisotzkey et al. 1998). Crystallization of the MH2 domain of Smad4 revealed that it forms a trimer (Shi et al. 1997), and conservation of primary amino acid sequence at the crystal trimer interfaces suggests that all the Smads may have the capacity to trimerize. In the case of Smad2 and Smad3, which have highly homologous MH2 domains and interact with one another in a TGF β -regulated manner (Nakao et al. 1997b), receptor-regulated trimerization may also occur. In light of the extensive primary sequence homology between MH2 domains of different Smads, whether the Smad4–MH2 homotrimers observed in vitro reflect a propensity solely for homotrimerization in vivo, or whether heterotrimerization may be mediated by homologous interfaces in other Smads, is not understood. Mutations that disrupt trimerization disrupt Smad4 function, indicating that trimeric MH2–MH2 interactions are functionally important (Shi et al. 1997), but to what extent Smad trimerization occurs among full-length Smads in vivo, whether this trimerization is constitutive or regulated, and to what extent it is homotrimerization or heterotrimerization all remain to be established.

Activation of Smads

Smad1, Smad2, and Smad3 and, by inference, Smad5 and Smad8, are directly phosphorylated at their carboxyl termini by type I TGF β superfamily receptors (Macias-Silva et al. 1996; Zhang et al. 1996; Kretzschmar et al. 1997b) and therefore are often referred to as receptor-regulated Smads. Smad4 lacks this carboxy-terminal phosphorylation sequence and does not appear to be a substrate for the type I receptors. The specificity of this phosphorylation by upstream receptors generally consistent with the observed downstream effects of ectopic Smad activation—that is, Smad1, which when overexpressed mimics BMP signaling, is phosphorylated by the type I BMP receptor (Kretzschmar et al. 1997b), and Smad2, which mimics the effects of activin, is phosphorylated by the ActRIB or T β R1 receptors (Macias-Silva et al. 1996). Phosphorylation occurs at two serines in a SSXS motif conserved among the pathway-specific Smads (Abdollah et al. 1997; Souchelnytski et al. 1997), and TBR1 the specificity of receptor interaction–phosphorylation is determined by a conserved 17-amino-acid region in the MH2 domain referred to as the L3 loop (Lo et al. 1998). Phosphorylation is mediated by direct association between the Smad and the type I receptor, occurs within

Table 1. Molecular components of TGF β superfamily signal transduction

Ligands	Receptor		Receptor-regulated Smad	Common Smad
	type II	type I		
Activin	→ ActRII ActRIIB	→ ActRIB	→ Smad2 Smad3	→ Smad4
TGF β	→ T β RII	→ T β RI	→ Smad2 Smad3	→ Smad4
BMP2/4	→ BMPRII ActRII	→ BMPRI BMPRIIB	→ Smad1 Smad5 Smad8	→ Smad4
Dpp	→ Punt	→ Tkv Sax	→ MAD	→ Medea

Listed are examples of components of relatively well-characterized TGF β superfamily transduction pathways discussed in this review. The list is not intended to be comprehensive either with respect to ligands in the superfamily or transducers that may potentially act downstream of a ligand shown. More comprehensive discussions of TGF β superfamily ligands and their receptors may be found in Derynck and Feng (1997) and Massagué (1998).

minutes of receptor stimulation, and is required both for signaling and association of receptor-regulated Smads with Smad4 (Macias-Silva et al. 1996; Kretzschmar et al. 1997b).

Upon ligand stimulation, Smads translocate from the cytosol to the nucleus, and this translocation also appears to be regulated by phosphorylation at the carboxyl terminus (Liu et al. 1996). Ligand-stimulated nuclear translocation of the receptor-regulated Smads does not appear to require association with Smad4 (Liu et al. 1997; Wisotzkey et al. 1998). Smad4, in contrast, does require a phosphorylated pathway-specific Smad to accumulate in the nucleus, indicating that heterodimerization with receptor-regulated Smads drives Smad4 nuclear accumulation (Liu et al. 1997; Wisotzkey et al. 1998). Isolated MH2 domains of receptor-regulated Smads accumulate in the nucleus constitutively (Baker and Harland 1996), suggesting that the MH1/linker domains may either occlude sequences in the MH2 domain that are responsible for nuclear localization or contain a sequence that directs cytoplasmic retention of the protein in the absence of carboxy-terminal phosphorylation. Smads do not contain a recognizable nuclear localization signal, and so the mechanism by which they accumulate in the nucleus remains unknown.

How Smads signal

The phosphorylation of pathway-specific Smads by type I TGF β superfamily receptors and their subsequent heterodimerization with Smad4 and translocation to the nucleus form the basis for a simple model for how Smads transduce signals from the plasma membrane to the nucleus (Fig. 1). The next question is: What do Smads do there? Three types of activities have been demonstrated for Smads that point to mechanisms by which they

might direct specific transcriptional responses: (1) a transcriptional transactivation function intrinsic to the MH2 domain; (2) specific association with a nuclear transcription factor; or (3) DNA-binding activity intrinsic to the MH1 domain.

The MH2 domain is a transcriptional activator

Intrinsic transcriptional transactivation activity has been demonstrated for MH2 domains of both receptor-regulated Smads and Smad4 by fusion of the MH2 domain to a Gal4 DNA-binding domain (Liu et al. 1996). Full-length Smad4 fused to the Gal4 DNA-binding domain lacks this activity, and full-length Smad1 fused to Gal4 requires activation by BMP receptor for activity, supporting the idea that the MH1 domain may negatively regulate the activity of the MH2 domain. In addition, transcriptional activation by Gal4-Smad1 or Gal4-Smad2 in vertebrate cells requires the coexpression of Smad4 or, at a minimum, expression of the Smad4 MH2 domain (Liu et al. 1997). Heterodimerization of the receptor-regulated Smads with Smad4 may therefore be essential for the localization of the transcriptional activation function of the Smad4 MH2 domain to the nucleus.

Smad association with a nuclear transcription factor

A mechanism for targeting the Smad transcriptional activation function to specific responsive promoters has been provided by the demonstration of a regulated association between Smads and a nuclear transcription factor, FAST-1, in early frog embryos (Chen et al. 1996, 1997; Liu et al. 1997). In this system, stimulation of cells with activin leads to the association of Smad2 and Smad4 with FAST-1, a winged helix transcription factor. This complex can bind to a regulatory element, the ac-

Figure 1. A general scheme of Smads as TGF β superfamily signal transducers. (See text for a discussion).

activin responsive element (ARE) in the promoter of an activin-responsive gene, *Mix.2* (Huang et al. 1995). The interaction between Smad2 and FAST-1 is direct and is mediated through the MH2 domain of Smad2 and a domain near the carboxyl terminus of FAST-1 termed the Smad interaction domain (SID; Chen et al. 1997). Association of Smad4 with FAST-1 requires sequences in the SID in addition to those required for Smad2 binding, but at this point there is no evidence for a direct interaction between Smad4 and FAST-1. Ectopic expression of FAST-1 is not sufficient to activate the ARE but is required for activin or TGF β stimulation of the ARE in tissue culture cells (Hayashi et al. 1997; Liu et al. 1997), indicating that FAST-1 is necessary for the targeting of the Smad2/Smad4 complex to this activin/TGF β responsive promoter element. An activin-regulated complex among Smad2, Smad4, and an as yet unidentified transcription factor has also been identified in frog embryos as binding to an element (unrelated to the *Mix.2* ARE) in the promoter of the activin early response gene *goosecoid*, indicating that there are probably multiple transcription factors mediating Smad2/Smad4 induction of mesodermal responses in early embryos (Candia et al. 1997).

Smad MH1 domains bind DNA

In addition to targeting promoters via transcription factors like FAST-1, Smads have an intrinsic, site-specific DNA-binding activity. This activity was first demonstrated for the MH1 domain of Mad, which binds a site in a Dpp-responsive element the gene *vestigial* (*vg*) in vitro (Kim et al. 1997). The isolated MH1 domain binds more potently than the full-length protein, suggesting that the MH2/linker regions may negatively regulate DNA-binding activity. Mad (or Medea) binding sites have been identified in several other Dpp responsive elements: a Dpp responsive enhancer in the mesoderm-response gene *tinman* is bound and synergistically activated by Medea and Tinman itself (Xu et al. 1998); on the other hand a Mad binding site in the minimal Dpp-responsive promoter for the *ultrabithorax* (*Ubx*) gene is not required for Dpp regulation in vivo (Kim et al. 1997), suggesting that DNA binding by Mad may not in all cases be necessary for Mad-mediated transcriptional regulation.

DNA-binding activities have also been demonstrated for vertebrate Smad3 and Smad4: An optimal target site for Smad3 or Smad4 has been defined both by examination of promoter sites and by oligonucleotide selection as containing a core GTCT (or its palindrome CAGA) (Dennler et al. 1998; Zawel et al. 1998); binding sites identified in promoters seem to vary significantly around this core. A concatamer of consensus Smad binding sites is sufficient to confer TGF β responsiveness on a reporter, indicating that direct Smad binding to DNA can be sufficient for transcriptional regulation by TGF β (Dennler et al. 1998; Zawel et al. 1998). In addition to binding to artificial consensus sites, Smad3 or Smad4 also binds to TGF β -regulated elements derived from the *collagenase* and *PAI-1* promoters. In the case of the *collagenase* pro-

motor, binding to a 31-bp sequence that contains an AP-1 site and confers TGF β responsiveness has been shown (Yingling et al. 1997). In contrast to the case of the *Drosophila* *vg* enhancer, however, elimination of the Smad3/Smad4 binding site from this 31-bp enhancer does not eliminate TGF β or Smad4 overexpression-mediated induction of an enhancer-reporter construct. The adjacent AP-1 site is necessary for TGF β - and Smad4-mediated activation, suggesting that AP-1 may be a component of Smad activation in this system (Yingling et al. 1997). In the *PAI-1* promoter, three separate Smad3/Smad4 binding sites have been identified; elimination of all three, but not any two, eliminates TGF β responsiveness of the promoter (Dennler et al. 1998). In the case of the *Xenopus* ARE, Smad4 also binds the DNA element directly, at a site distinct from the binding site for FAST-1. Mutation of the FAST-1 binding site eliminates responsiveness of the element, whereas mutation of the Smad4 binding site reduces, but does not eliminate, activin responsiveness (C.Y. Yeo, X. Chen, and M. Whitman, unpubl.).

Models for transcriptional regulation by Smads

Observations from *Xenopus* and *Drosophila* embryos, as well as vertebrate tissue culture cells, suggest several possible modes by which Smads regulate responsive promoter/enhancers (Fig. 2). First, they may form stable protein-protein complexes with other transcription factors, such as FAST-1; DNA recognition by this complex then results from the combined DNA-binding affinities of the Smads and the additional transcription factor (Fig. 2A). Second, Smads and other transcription factors may bind at nearby promoter/enhancer target sites, with protein-protein interactions providing cooperativity to the binding and thereby enhancing the specificity or affinity of promoter/enhancer recognition (Fig. 2B). Third, Smads and other regulatory factors may bind independently but synergistically regulate transcription (Fig. 2C), as has been suggested for regulation of *tinman* in the *Drosophila* mesoderm (Xu et al. 1998). Clearly these different modes may fall on a continuum rather than representing discrete alternatives, and the relative contributions of Smad and auxiliary transcription factor binding to specific DNA binding may vary substantially between specific promoters. Whereas the details of how the different Smad functions are applied in individual cases remain to be worked out, the intrinsic transcriptional activation function of Smads, their ability to interact specifically with other transcription factors, and their ability to directly target promoter/enhancer regulatory sites provide a general framework for investigating their roles in the regulation of gene expression.

Smad expression patterns in embryos

Expression of the signaling Smads appears to be widespread during embryogenesis. mRNAs encoding Smad1, Smad2, and Smad4 are present maternally and expressed ubiquitously in early frog embryos (Graff et al. 1996; Lagna et al. 1996), and in *Drosophila* early embryos, *Mad*

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Figure 2. Three mechanisms for Smad/transcription factor coregulation of promoter/enhancer elements. Shown is a schematic of three variations on coregulation of transcription at enhancer elements by Smads and other DNA-binding proteins. (A) Ligand-regulated protein-protein interactions drive association of Smads with other DNA-binding proteins to form a multiprotein complex that coordinately binds multiple target sites within an enhancer; (B) Smads and additional factors recognize target sites separately, but protein-protein interactions stabilize binding-to-target-site region, enhancing affinity/specificity; (C) Smads and additional factors bind target sites independently but act additively or synergistically in the activation of transcription.

and *Medea* RNAs are also expressed ubiquitously (Newfeld et al. 1996, 1997; Das et al. 1998; Hudson et al. 1998; Wisotzkey et al. 1998). In mice, Smad1 is expressed first in the early embryonic mesoderm, and later ubiquitously, whereas Smad2 and Smad4 are ubiquitous (in both embryonic and extraembryonic tissues) through day 8.5 of embryogenesis (Waldrup et al. 1998; Yang et al. 1998). During organogenesis in the mouse, increased Smad1 and Smad2 expression is seen in a variety of developing organs at sites of epithelial-mesenchymal interactions (Dick et al. 1998). Although expression patterns for several signaling Smads (e.g., Smads 3, 5, and 8) have not been reported, the broad spatial and temporal expression of signaling Smads examined so far would seem to indicate that in general, differential expression of these Smads is not a critical determinant of cell type-specific responses to TGF β superfamily signals. The possibility remains that additional tissue- or cell type-specific signaling Smads may be identified, as well as that tissue-specific enrichment of Smad expression is important in the development of distinct responses to TGF β superfamily signaling. In early development, however, cell type-specific responses to TGF β superfamily signals seem at this point likely to involve differential expression of mediators downstream of Smads (e.g., tissue or developmental stage-restricted transcription factors like FAST-1 or Tinman) rather than differential expression of the signaling Smads themselves.

Smad functions in embryos: loss of function in *Drosophila*

Three general types of experiments have provided our current picture of patterning steps regulated by Smads during early embryogenesis: (1) genetic loss-of-function experiments in *Drosophila*; (2) ectopic expression experiments in *Xenopus*; and (3) genetic loss of function in mice by homologous recombination. In *Drosophila*, Dpp is required for multiple steps in embryogenesis, oogenesis, dorsoventral patterning of the embryonic ectoderm, midgut, and adult appendages and eyes (for review, see

Gelbart 1989; Kingsley 1994). In both the wing imaginal disc and the embryonic ectoderm, Dpp appears to act as a morphogen, regulating embryonic pattern in a concentration-dependent manner (Ferguson and Anderson 1992; Wharton et al. 1993; Lecuit et al. 1996; Nellen et al. 1996). *mad* was originally identified as a genetic modifier of *dpp* (Raftery et al. 1995; Sekelsky et al. 1995); subsequent analysis has demonstrated that for each of the developmental events requiring patterning by Dpp signals, loss of Mad function results in a phenotype similar to loss of Dpp (Sekelsky et al. 1995; Newfeld et al. 1996; Wiersdorff et al. 1996). Mad is closest in sequence to Smad1, consistent with its role as a receptor-regulated Smad acting downstream of a BMP homolog. *Medea* is also necessary for dorsal-ventral patterning by Dpp, and loss of *Medea* cannot be compensated for by increase levels of Mad (Das et al. 1998; Wisotzkey et al. 1998), as might be expected from the Smad heterodimerization model developed in vertebrates. Decreased *Medea* function suppresses the phenotype associated with activation of the *dpp* type I receptor Sax, also consistent with the proposed vertebrate pathway (Das et al. 1998).

Although in general *Medea* appears to be necessary for Mad/Dpp signaling, detailed characterization of *Medea* loss-of-function alleles reveals several complications. Whereas decreased *Medea* function suppressed the effects of activated Sax, it did not suppress the phenotype associated with activation of Tkv, another type I Dpp receptor (Das et al. 1998). The differences in *Medea* requirements for Sax and Tkv may reflect simply distinct dosage sensitivities of the receptors to *Medea* levels but might also suggest the use of *Medea*-independent signaling mechanisms by the Tkv receptor. An additional puzzle arises from examination of mutant *Medea* clones in the developing wing disc: Mutant *Medea* clones that develop close to the source of Dpp at the anterior-posterior compartment boundary express a Dpp and Mad-dependent gene (*omb*) normally, whereas mutant *Medea* clones more distant from the Dpp source fail to express *omb* normally (Wisotzkey et al. 1998). Although the developmental characterization of *Medea* indicates that it

is generally required for Dpp signaling, this requirement may not be the same for all Dpp-responsive genes. The possible existence of a Smad4-like gene in *Drosophila* in addition to *Medea* has not been excluded, but these observations raise the possibility that Smad heterodimerization may not be as universally obligatory as the vertebrate data have implied.

Dpp is differentially expressed across the tissues it patterns (Wharton et al. 1993; Nellen et al. 1996), whereas Mad and Medea are expressed ubiquitously. Because the loss of Mad or Medea function results (with the exceptions noted above) in defects consistent with the absence of a Dpp signal, this would suggest a simple model (e.g., in the case of dorsal–ventral patterning of the early ectoderm) in which a Mad/Medea complex is activated in proportion to a graded Dpp signal, and this intracellular gradient of Mad/Medea activity specifies tissue-specific transcription patterns. That Mad can bind directly to response elements in Dpp-responsive promoters raises the possibility that Dpp patterned transcription is a direct readout from graded Mad or Mad/Medea promoter/enhancer binding. Similar gradients of Mad–Medea function may be used for tissue patterning at multiple stages in *Drosophila* (e.g., dorsoventral patterning of early ectoderm, anterior–posterior patterning of wing imaginal disc; morphogenesis of visceral mesoderm). The different Dpp/Mad/Medea transcriptional responses characteristic for each stage and tissue would then presumably be dictated by the presence of stage- or tissue-specific transcriptional activators or repressors that either help to target Mad/Medea to specific promoters/enhancers or bind independently of Mad/Medea and synergize with (or antagonize) Mad/Medea transcriptional regulation (Xu et al. 1998).

The genetic loss-of-function experiments in *Drosophila* strongly indicate that Mad and, in most cases, Medea, are necessary for Dpp-dependent patterning. If they are in fact the principal intracellular transducers defining patterned responses to *dpp*, it should also be possible to show that (1) they are activated in a gradient corresponding to the gradient of Dpp activity, and (2) that ectopic generation of Mad/Medea activity is sufficient to specify pattern as does Dpp. The demonstration that Dpp overexpression can cause nuclear accumulation of Mad in vivo (Newfeld et al. 1997) makes feasible the immunocytochemical examination of whether there is in fact differential or graded Mad activation in tissues in which patterning by Dpp is taking place. This has been examined in blastoderm embryos and wing discs; contrary to what might have been expected from the differential expression and activity of Dpp across the blastoderm, uniform cytoplasmic concentration of Mad was observed (Newfeld et al. 1997). The meaning of this negative result is not certain, but the interpretation most easily reconciled with other data would seem to be that in the blastoderm Mad protein is present in substantial excess over what is needed for endogenous signaling. In this case the quantity of Mad that is translocated to the nucleus in response to endogenous signals is too little to detect against the background of excess Mad, whereas an

ectopic, high-level Dpp signal may be sufficient to cause net nuclear accumulation of even the excess Mad. Examination of the spatial and temporal distribution of Mad activation remains an important test of its role in patterning, but additional tools (e.g., activation-specific Mad antibodies) may be necessary to test the prediction that Mad is differentially activated in Dpp-patterned tissues.

The second question, of whether direct activation of Mad can effectively mimic Dpp patterning signals should, if experiments with Smads in vertebrates are an appropriate guide, be possible to do by overexpression of wild-type or amino terminally truncated Mad. Ectopic expression of wild-type Mad and Medea in *Drosophila* does not, however, seem to be sufficient to perturb embryonic patterning (Hudson et al. 1998). Whether this is due to technical limitations (i.e., ectopic Smad protein expression in *Drosophila* is much less than can be achieved in *Xenopus*) or to more stringent post-translational regulation of *Drosophila* Mad/Medea than of vertebrate Smads is not clear. In any case, data from ectopic expression of Smads in *Xenopus* embryos have largely complemented the *Drosophila* loss-of-function experiments to address how activation of Smads can mimic endogenous patterning signals during development.

Ectopic expression in *Xenopus*

TGF β ligands may be morphogens

As in *Drosophila*, there is substantial evidence that TGF β superfamily ligands have a major role in patterning the early vertebrate embryo and that they may function as morphogens, specifying multiple tissue types as a response to graded concentrations of ligand. Most of this evidence has come from studies in *Xenopus*, in which it is relatively straightforward to apply (by mRNA injection into embryos or factor addition to isolated tissue) graded doses of ligands and assess their effects on developmental fate (Smith 1987; Green et al. 1992; Gurdon et al. 1994). Ectopic activin and BMP4 each induce a range of tissue types when added to early ectodermal cells, with activin inducing lateral mesoderm at low doses and more dorsal mesoderm at high doses (Green et al. 1992). BMP4 can induce dissociated ectodermal cells to form cement gland at low doses and epidermis at higher doses, and at still higher doses ventral mesoderm can also be induced (Dale et al. 1992; Jones et al. 1992). In the absence of BMP these cells form neural tissue (Wilson et al. 1997). In addition to these effects on isolated prospective ectoderm, BMP4 ventralizes the body axis when expressed in intact embryos—that is, expands the expression of ventral mesodermal tissues at the expense of dorsal mesodermal and neural tissues and structures (Jones et al. 1992; Dosch et al. 1997). Conversely, inhibition of BMP by addition of the antagonist Noggin can progressively induce cement gland at low doses and *cpl-1*, a dorsal forebrain marker, at higher doses (Knecht and Harland 1997), suggesting that gradients of inhibitors of signal-generating ligands may also help to establish early pattern. Inhibition of BMP signaling by ectopic expression of a dominant inhibitory BMP receptor dorsalizes

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ventral mesoderm (Graff et al. 1994; Suzuki et al. 1994), and expression of a dominant-inhibitory activin receptor that inhibits TGF β superfamily signaling in general (Schulte-Merker et al. 1994) can prevent the formation of mesoderm entirely (Hemmati-Brivanlou and Melton 1992). Although dominant-inhibitory receptors are not sufficiently specific tools to distinguish the role of individual ligands, these observations generally support a role for BMP and activin-like ligands in endogenous patterning.

Although the ectopic expression of TGF β superfamily ligands and of Smads in embryos is consistent with multiple morphogenic roles for these signals, the actual distribution of these signals in vivo is not clear (for review, see Conlon et al. 1996; Harland and Gerhart 1997). For the Smad2 signaling pathway, the identity of the upstream ligand or ligands most important for early patterning remains obscure. In *Xenopus*, *activin*, *Vg-1*, and several of the *nodal*-related genes may all lead to Smad 2 activation (Baker and Harland 1996; Chen et al. 1996; Graff et al. 1996; Nomura and Li 1998), and RNAs encoding these factors are present at embryonic stages consistent with at least some role in mesodermal induction and patterning (Weeks and Melton 1987; Jones et al. 1992; Dohrmann et al. 1993; Thomsen and Melton 1993; Oda et al. 1995; Lustig et al. 1996). It has not been possible to determine where and when the corresponding ligands may be secreted and active during development, leaving open the question of which ligands are responsible for Smad2 activation in vivo, as well as the temporal and spatial patterns of this activation.

In the case of BMPs, RNAs encoding BMP2, BMP4, and BMP7 are present maternally (Nishimatsu et al. 1992, 1993); BMP2 and BMP4 have been shown to be present throughout the ventral embryonic ectoderm and mesoderm in which they are proposed to act (Nishimatsu et al. 1993; Hemmati-Brivanlou and Thomsen 1995; Schmidt et al. 1995b). All three seem to have similar activities (Suzuki et al. 1997b), are thought to signal through Smad1, and may heterodimerize to act synergistically. Dorsalizing/neuralizing factors that antagonize the BMPs are expressed in the organizer (Re'em-Kalma et al. 1995; Piccolo et al. 1996; Zimmerman et al. 1996), leading to the proposal that a gradient of BMP signaling is established initially by the interaction of broadly expressed BMPs with a dorsally localized source of BMP antagonists. Consistent with this, the order in which different ectodermal derivatives are induced by increasing concentrations of BMPs corresponds roughly to their distance on the fate map of a gastrula embryo from the dorsal source of BMP antagonists (Wilson et al. 1997). A direct demonstration of a gradient of the proteins (either BMPs or their antagonists) has, however, not yet been technically feasible.

Smads can recapitulate morphogen function

Ectopic expression of Smads by mRNA injection suggests that differential tissue inductions observed with activin or BMP ligands can be mimicked by manipula-

tion of Smad activity. Overexpression of full-length Smad2 or its MH2 domain in prospective ectoderm is sufficient to induce relatively lateral mesodermal markers at low doses and dorsal mesodermal markers at high doses (Baker and Harland 1996; Eppert et al. 1996; Graff et al. 1996), whereas expression of Smad1 or Smad5 at low doses can, like BMP4 stimulation, induce formation of cement gland and at higher doses induce epidermis in dissociated animal pole cells (Suzuki et al. 1997a; Wilson et al. 1997). In intact embryos expression of Smad1 or Smad5, like BMP2, BMP4, or BMP7, ventralizes the embryo (Liu et al. 1996; Thomsen 1996; Suzuki et al. 1997b). The effects of ectopic Smad expression on patterning still occur even when the upstream receptors are inhibited by overexpression of dominant inhibitory receptor constructs, indicating that additional signals through these receptors are not necessary for ectopic Smads to mimic the patterning effects of their upstream ligands (Eppert et al. 1996; Graff et al. 1996). Carboxy-terminally truncated Smad4 can sequester receptor-regulated Smads, preventing the formation of viable signaling complexes. Overexpression of this truncated Smad4 can inhibit the induction of mesoderm by activin, supporting a role for Smads in mesodermal specification and/or patterning (Lagna et al. 1996).

Although graded overexpression of the receptor-regulated Smads can in some respects mimic graded ligand stimulation in the specification of early blastomeres, more detailed analysis may reveal significant differences in the effects of these different stimuli. As recently pointed out by Dyson and Gurdon (1998), doses of activin high enough to induce the dorsal mesodermal gene *gooseoid* inhibit expression of the mesoderm response gene *Brachyury*, which is induced by lower doses of activin (Latinkic et al. 1997). In contrast, high doses of Smad2 expression induce *gooseoid* and *Brachyury* concomitantly. This difference may be significant biologically in that in the gastrulating embryo, *Brachyury* expression is excluded from the dorsal-most region of the mesoderm, the *gooseoid*-expressing Spemann organizer. That graded overexpression of Smad2 does not recapitulate this pattern may reflect differences in timing of stimulation associated with ectopic expression of Smad2 by mRNA injection at the 1- to 2-cell stage versus ligand stimulation of dissociated cells at the 1000-cell stage, differences in function between overexpressed, presumably unphosphorylated Smad2 and endogenous Smad2 phosphorylated in response to ligand stimulation, or signals generated by ligand stimulation in addition to activation of Smad2. In any case, the difference between the effects of high-dose activin stimulation and high-dose Smad2 expression on *Brachyury/gooseoid* induction at least raise the possibility that a gradient of ligand stimulation in the early embryo may have more complex effects than simply the generation of a gradient of Smad activation.

The morphogen hypothesis: detecting endogenous gradients

The effectiveness of ectopic stimulation of the activin or

BMP4 signaling pathways by addition of ligands or overexpression of Smads to recapitulate mesodermal and ectodermal patterning is impressive, but several steps remain to establish the role of gradients of Smad activation in early embryonic patterning. The present technical limits of detection of endogenous secreted factor levels in the intercellular space make it very unlikely that a gradient of peptide factor across the embryo will be demonstrable anytime soon. Furthermore, recent work on BMP regulation suggests that the relevant gradient may not be of the factor itself, but of inhibitors of the factor, and possibly even inhibitors of the inhibitors (Piccolo et al. 1997). One way through the apparent technical morass surrounding the question of endogenous ligand gradients may be provided by our new understanding of the components of the activin and BMP signaling pathways. Because both the type I receptors and the Smads transducing these signals require site specific phosphorylation for activation, the possibility exists of making antibodies directed against these phosphorylated epitopes and using them as histochemical probes to assess endogenous spatial and temporal patterns of signal activation. Activation-specific antibodies have been generated for a variety of components of tyrosine kinase signaling pathways (Epstein et al. 1992), and antiphosphorylated MAP kinase antibodies have been used elegantly to demonstrate spatial and temporal patterns of MAP kinase activation during early embryogenesis (Gabay et al. 1997). Although comparable antibodies for the TGF β superfamily signaling pathway have not yet been reported, the nature of type I receptor and Smad activation should be compatible with this approach. Improved methods to examine endogenous signal activation would be invaluable tools for the study of signaling in development; the biochemistry of TGF β superfamily receptors and Smads may provide an approach to these methods.

Homologous recombination in mice

Why is Smad4 not necessary in the embryonic mesoderm?

Very recently, the knockout of Smad4 and Smad2 by homologous recombination in mice has provided our first information on the necessity of Smads for early vertebrate development, with some unexpected results. Homozygous knockout of Smad4, which previous work in tissue culture cells would predict a complete loss of TGF β superfamily signaling, resulted in mice that die early in embryogenesis (Sirard et al. 1998; Yang et al. 1998). These mice exhibit severe growth retardation during pregastrula development, abnormal visceral endoderm, and no signs of either mesoderm formation or gastrulation. More surprising, in light of previous work demonstrating the necessity of BMP4 (Winnier et al. 1995), type I BMP receptor (BMPRI) (Mishina et al. 1995), and Nodal (Conlon et al. 1994) for normal gastrulation and mesoderm patterning in the mouse, was the observation that the early mesodermal and gastrulation defects were rescued by aggregation of mutant embryos

with tetraploid wild-type cells (Sirard et al. 1998). These cells populate only the extraembryonic tissues; that these chimeras show normal early mesodermal development and gastrulation of Smad4 embryonic tissues implies that there is no cell-autonomous necessity for Smad4 in the early embryonic mesoderm. Embryos rescued with wild-type extraembryonic tissue develop substantial anterior defects after gastrulation, indicating that Smad4 is important in embryonic tissues for normal anterior development.

At this point, there are several possible interpretations of the lack of Smad4 requirement in embryonic tissues for early mesoderm specification and gastrulation. First, although BMP4, Nodal, and a type I BMP receptor are necessary for normal development through gastrulation, it is possible that the primary requirement for these signals, like the Smad4 requirement, is in the extraembryonic tissues. In this case, it would be a second set of non-BMP-, non-Smad4-mediated signals from the extraembryonic tissues that establish and pattern mesoderm in the epiblast prior to gastrulation. The principal argument against this possibility would seem to be that BMPRI is expressed primarily in the epiblast at early gastrulation (Mishina et al. 1995), in which it is required for normal cell proliferation, suggesting that this requirement is autonomous to the epiblast. Additional chimera studies will be necessary to establish more completely where BMP and Nodal signals must be generated and received for normal epiblast patterning. A second possibility is that BMP4/Nodal signaling is indeed required in the epiblast, but there is an as yet unidentified "Smad4a" that serves a function redundant with Smad4 in the early specification of mesoderm. A third possibility, raised by the observation of Medea-independent effects of Dpp (Wisotzkey et al. 1998), is that in some instances the receptor-regulated Smads can signal in the absence of a Smad4-like heterodimerization partner. A final possibility is that Smad signaling is simply not required for early mesoderm patterning and gastrulation in the mouse, and that the Nodal and BMP4 functions observed to be necessary can be transmitted through a different signaling pathway. Detailed characterization of TGF β /Smad responsiveness in cell lines derived from *Smad4*^{-/-} mice should begin to distinguish between these possibilities.

Smad2 knockout: another puzzle

Two independent knockouts of *Smad2* have been reported, and both result in severely defective early embryos that lack embryonic germ layers and recognizable anterior-posterior pattern (Nomura and Li 1998; Waldrip et al. 1998). Additional characterization of the two knockouts reveals, however, significant differences between them. Waldrip et al. (1998) report that *Smad2*^{-/-} embryos have relatively normal extraembryonic tissues, whereas the prospective embryonic tissue differentiates as extraembryonic mesoderm, expressing the mesodermal marker *Brachyury* throughout the epiblast at early gastrula stages. In contrast, Nomura and Li (1998) report severe defects in extraembryonic tissues in *Smad2*^{-/-} ho-

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mozygotes and no expression of *Brachyury* in the epiblast at gastrulation. In addition, *Smad2*^{-/+} heterozygotes generated by Waldrip et al. (1998) are phenotypically normal, whereas 20% of the heterozygotes generated by Nomura and Li (1998) exhibit severe gastrulation defects. The basis for these differences is unclear; similar genetic backgrounds were used, but different regions of the *Smad2* coding sequence were replaced in each case, and the possibility of the remaining exons encoding a weakly inhibitory or constitutively active form of *Smad2* may need to be examined.

In addition to examining the effects of loss of *Smad2* throughout the embryonic and extraembryonic tissues, Waldrip et al. (1998) use chimera analysis to examine the role of *Smad2* specifically in the extraembryonic tissues. ES cell injection experiments in which the epiblast of a *Smad2*^{-/-} embryo is populated largely with wild-type ES cells fail to rescue the observed defects, indicating that extraembryonic tissues require *Smad2* to generate signals critical for normal patterning of the embryo. The converse experiment, generation of chimeras in which only the embryonic tissues lack *Smad2*, has not yet been done, so the question of whether there is an embryonic as well as an extraembryonic requirement for *Smad2* in early patterning remains to be answered.

Synthesizing mouse knockouts and frog ectopic expression

Although important questions remain to be clarified for the *Smad2* and *Smad4* knockouts, both confirm that *Smad* signaling is critical for patterning of vertebrate embryos. What was perhaps not expected, however, is the apparent importance of *Smad* signals in the extraembryonic tissues for normal patterning of the early embryo. Models for the early establishment and patterning of mesoderm developed from experiments with frog embryos do not address the role of extraembryonic tissues because amphibians lack them, making it difficult to synthesize data obtained from ectopic expression of activated or dominant-inhibitory *Smads* in frogs with those obtained from knockouts in mice into a coherent picture of early mesodermal patterning. Although the general point can be made that *Smad* signaling is necessary in mice and sufficient in frogs for establishment or patterning of the early mesoderm, exactly where and when this *Smad*-linked patterning occurs in each organism is more difficult to compare. Additional characterization of the necessity for *Smads*, as well as for TGF β superfamily ligands and receptors, in embryonic versus extraembryonic tissues may help to clarify the similarities and differences in *Smad*-regulated patterning between mammalian and amphibian embryos.

Specificity, feedback regulation, and integration in TGF β signaling during development

The identification of *Smads* as TGF β signal transducers is important in large part because it provides a molecular handle on the dual problems of how different cell types

generate different responses to the same signal and of how different types of signals are integrated in their target cells. The fact that *Smads* can act as direct transcriptional activators, but induce different genes in different cell types, makes understanding of the interaction of cell type-specific cofactors with *Smads* to generate specific responses central to comprehension of the role of *Smads* in development. Furthermore, careful analyses of the components of the developmental regulation of individual genes and of the steps involved in specification of distinct tissues make clear that embryonic patterning involves the integration of multiple regulatory signals over time and space. Defining the mechanisms by which *Smad* signaling interacts with other pathways is therefore essential for understanding *Smad* function in patterning in vivo.

Specific transcriptional responses to *Smad* activation—FAST-1 and Tinman

The ubiquitous expression of signaling *Smads* during early embryogenesis, and their implication in the mediation of a broad range of responses that depend on the developmental history of the stimulated cell, raise the question of how cell type-specific responses to *Smads* are generated. The observation of a DNA-binding activity intrinsic to signaling *Smads* only highlights the question of how different promoters are activated by *Smads* in different cell types. A major component of the answer to this question presumably lies in other, cell type-specific factors binding to additional regulatory elements in the same promoter. Two examples of how this might work are provided by examination of activin regulation of the early response gene *Mix.2* in *Xenopus* embryos and of Dpp regulation of *tinman* in *Drosophila*.

As discussed above, in the early frog embryo the winged helix transcription factor FAST-1 targets *Smad2* and *Smad4* to an enhancer element necessary and sufficient for activation of *Mix.2* transcription (Huang et al. 1995; Chen et al. 1997; Liu et al. 1997). Levels of FAST-1 mRNA and the competence of embryonic cells to express *Mix.2* in response to activin decline over a similar time span (Chen et al. 1997; Rosa 1989), consistent with the possibility that early embryonic FAST-1 function is necessary for the responsiveness of the *Mix.2* enhancer to activin/*Smad2* activation; this function is reduced as development progresses, and activin regulation of this early embryo-specific response gene is lost. Ectopic activation of FAST-1 by fusion of its DNA-binding domain to a VP16 transcriptional activator domain activates not just *Mix.2* expression but also the full mesodermal transcriptional program regulated by activin (M. Watanabe and M. Whitman, in prep.). FAST-1 may therefore be responsible for targeting activated *Smad2*/*Smad4* to a broad set of promoters regulated by activin/TGF β signals specifically in early embryos, and activation of these promoters is lost as FAST-1 function declines. Although additional mechanisms are likely to be involved in loss of early developmental competence (Steinbach et al. 1997), the loss of early embryo-specific transcription fac-

tors that target Smads to early embryo-specific promoters may be one component of this process.

tinman is activated in a broad, mesoderm-specific pattern by the transcription factor Twist early in *Drosophila* development (Bodmer et al. 1990). During later mesodermal patterning, *tinman* expression is restricted to the dorsal mesoderm; this later expression is dependent on Dpp signaling (Frasch 1995). Dpp induces *tinman* in the mesoderm, but not in the ectoderm, in which Dpp is also clearly active in patterning. Mad and Medea bind to the *tinman* promoter in vitro, but these binding sites are not sufficient for induction in vivo (Xu et al. 1998). The basis for the mesoderm specific action of Dpp/Mad/Medea on *tinman* regulation appears to be Tinman protein itself, which binds at sites adjacent to the Mad/Medea sites and synergizes with Mad/Medea to activate *tinman* transcription. At this stage, there is no evidence that Mad or Medea interact directly with Tinman (as is the case for Smad2/Smad4 and FAST-1); synergy presumably occurs through interaction with the basal transcription apparatus. Ectopic expression of Tinman in the ectoderm results in Dpp induction of *tinman* transcription in ectoderm, confirming that Tinman is sufficient to act as a determinant of the normal mesoderm-specific response. In addition to the positive requirement for Tinman, there are additional negative regulatory sites that suppress *tinman* expression in the ectoderm, indicating that endogenous regulation involves tissue-specific repressors as well as activators. Although complete regulation of the spatial and temporal pattern of developmental genes is likely to involve complex sets of binding sites and transcription factors (Arnone and Davidson 1997), the undoubtedly simplified cases of regulation by FAST-1 and Tinman provide examples of how ubiquitous Smads transduce tissue-specific transcriptional responses.

Feedback regulation by inhibitory Smads

Following the discovery of Smads as signal transducers, several Smads were identified that act as inhibitors of TGF β superfamily signaling (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997a; Tsuneizumi et al. 1997). Smad6 and Smad7, and Dad, lack the carboxy-terminal phosphorylation sequence and have MH1 domains that are highly divergent from the signaling Smads. Overexpression of Smad6 or Smad7 can block signaling by multiple members of the TGF β superfamily (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997a; Hata et al. 1998). In mammalian tissue culture cells and amphibian embryos, Smad7 [a *Xenopus* Smad7 homolog has been independently named Smad8 (Nakayama et al. 1998)] blocks BMP signaling at low doses and activin/TGF β signaling at higher doses (Casellas and Hemmati-Brivanlou 1998). Conflicting results have been reported for the specificity of inhibition by Smad6. It may inhibit both BMP and TGF β signaling (Imamura et al. 1997), but inhibition of TGF β signaling is weak and may occur only at very high doses of Smad6 (Hata et al. 1998). Ectopic expression of *dad* in the *Drosophila* wing

disc inhibits signaling by Dpp and can block BMP signaling when expressed in *Xenopus* embryos (Tsuneizumi et al. 1997). Whether Dad is a selective inhibitor of BMP signaling or might also inhibit activin/TGF β signaling at higher doses has not been examined.

Two mechanisms have been proposed for the action of inhibitory Smads. Expression of Smad7 inhibits the receptor-regulated phosphorylation of Smad2 or Smad3, whereas Smad6 can inhibit receptor-regulated phosphorylation of Smad1 and Smad2 but not Smad3 (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997a) (Fig. 3). These observations indicate that Smad6 and Smad7 can inhibit transmission of regulatory signals between the type I receptors and the pathway-specific signaling Smads. This interpretation is supported by data showing that Smad6 and Smad7 can interact stably with type I receptors (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997a), suggesting that they function as competitive inhibitors of type I receptor phosphorylation of receptor-regulated Smads. For Smad6, however, an additional inhibitory function has been found in its ability to bind to Smad1, preventing the Smad1 from binding Smad4 and thereby sequestering it from its normal signaling function (Hata et al. 1998). Co-overexpression of Smad4 with Smad6 can rescue BMP signaling in COS cells, supporting the interpretation that in this system Smad6 acts to limit Smad4 availability (Hata et al. 1998). Data on both the function and mechanism of action of inhibitory Smads have come entirely from overexpression studies. At this point it is difficult to resolve to what extent the specificity of inhibition of different TGF β superfamily signaling pathways, or of the intracellular target for inhibition (i.e., receptor sequestration or Smad1 sequestration by Smad6), is a function of the levels of expression of the various transducing and inhibitory components of the signaling pathway. It is clear, however, that inhibitory Smads can differentially target

Figure 3. Multiple pathways for Smad regulation. Schematic of currently understood intracellular pathways for regulation of Smads. Green arrows denote positive regulatory interactions; red bars denote inhibitory interactions.

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distinct subsets of the TGF β superfamily of signals, and this seems likely to be significant for their function in vivo.

Several of the inhibitory Smads have been shown to be induced by the ligands they inhibit (Nakao et al. 1997a; Tsuneizumi et al. 1997; Nakayama et al. 1998), as well as by other stimuli (Topper et al. 1997). This implies that they function in a negative feedback loop, perhaps limiting the duration of ligand stimulation. In *Drosophila*, cells that would normally express Dad in the wing disc no longer do so when they lack upstream components of the Dpp signaling pathway, indicating that Dpp signals are necessary for expression of *dad* in vivo (Tsuneizumi et al. 1997). In *Xenopus* embryos, Smad7 (initially named Smad8) and BMP4 are coexpressed in the prospective ectoderm (Nakayama et al. 1998). Endogenous Smad7 expression is inhibited by blockade of endogenous BMP signaling, again demonstrating that BMP4 positively regulates Smad7 in vivo (Nakayama et al. 1998). Early ectodermal cells are sensitive to ectopic BMP stimulation, however, indicating that Smad7 is not present at levels sufficient to prevent BMP signaling in this tissue. Similarly, the Dpp-dependent expression of *spalt* and *omb* in the *Drosophila* wing disc is not prevented by endogenous Dad expression in the same cells.

Signal regulation by inhibitory Smads in vivo may well be more subtle than a simple feedback "off" switch: BMP signals during early embryogenesis may induce Smad7 as an attenuator of ectodermal sensitivity to subsequent signals, rather than as a strict inhibitor. Given the ability of inhibitory Smads to inhibit different TGF β signaling pathways at different doses, it is also possible that these Smads act as cell-autonomous modifiers to alter the relative strengths of different TGF β superfamily signals. In cases where Smad1 and Smad2 activation evoke quite different responses (e.g., early frog blastomeres), expression of a preferential inhibitor of one pathway (e.g., Smad6) might serve as an important modifier of developmental responses. In addition, if TGF β s signal through pathways in addition to those involving Smads (Yamaguchi et al. 1995), inhibitory Smads may modulate responses by inhibiting the Smad component, but not the non-Smad component, of the response.

During early embryogenesis, localized inhibition of TGF β superfamily signaling has emerged as a patterning mechanism as important as activation of signaling (for review, see Ferguson 1996). Extracellular inhibitors of BMPs, conserved structurally and functionally between vertebrates and *Drosophila*, pattern the dorsal-ventral body axis by local antagonism of BMP or *Drosophila* Dpp (Holley et al. 1995; Schmidt et al. 1995a; Piccolo et al. 1996). In some cases, these extracellular inhibitors can themselves be induced by TGF β superfamily signals (Green et al. 1994; Sasai et al. 1994). The identification of Smad6, Smad7, and Dad as inducible intracellular inhibitors of TGF β signaling suggests that pattern regulation could involve a comparable intracellular mechanism for localized signal inhibition. Further characterization of the specificity of inhibitory Smad action and the regulation of their expression should prove to be an important

component of understanding how TGF β signaling is patterned across developing tissues.

Cross regulation and integration of Smad signals

Patterning in vivo by TGF β s involves not only the induction of specific transcriptional responses by Smads but also the modulation of these responses by cross talk and integration with other signaling pathways. Numerous examples of interaction between signaling pathways in both vertebrate and invertebrate embryos are known; examination of Smad regulation provides an intracellular focal point for understanding how such cross talk works. Our present knowledge of intracellular regulation/integration of Smad signals can be divided into two general categories: (1) pathway cross talk in the cytoplasm and (2) signal integration at target enhancers/promoters.

Cross talk in the cytoplasm

Several examples of regulation of Smad signaling by interaction between signaling pathways in the cytoplasm have been reported: (1) cross interference between BMP and activin signaling in *Xenopus* embryos; (2) inhibition of Smad1 by EGF signaling; (3) inhibition of Smad1/Smad5 signaling by inhibition of TAK1; (4) activation of Smads by tyrosine kinase signaling; and (5) physical interaction of Smads with calmodulin. Given the relative youth of the Smad field this is undoubtedly only a foreshadowing of a much larger set of regulations yet to be found, but these examples begin to provide an indication of the range of possibilities.

Activin and BMP Smad1 and Smad2 signals have distinct and, to some extent opposed, effects in early *Xenopus* embryos. Smad1 overexpression/activation ventralizes embryonic mesoderm, whereas Smad2 overexpression/activation induces dorsal mesodermal markers (Graff et al. 1996). Inhibition of BMP signaling in the ventral marginal zone of early embryos results in ectopic formation of dorsal mesoderm (Graff et al. 1994; Suzuki et al. 1994), suggesting that endogenous BMP signaling in the lateral and ventral mesoderm antagonizes the specification of dorsal mesoderm by an activin/Vg-1 like signal. Given that BMPs, activin, and Vg-1 all share Smad4 as a common component of their respective signaling pathways, one possible point at which these pathways could cross interfere is through competition for Smad4. Candia et al. (1997) have demonstrated that the observed antagonism between BMPs and activin/Vg1 occurs at a postreceptor step and that this inhibition is reciprocal, that is, and BMP2/4 can inhibit expression from an activin/Vg-1 responsive reporter and, activin or Vg1 can inhibit expression from a BMP-responsive reporter. Expression of excess Smad4 relieves the inhibition of the activin/Vg-1 responsive reporter by BMPs, indicating that under some circumstances TGF β superfamily signals that use different pathway-specific Smads may compete for Smad4, and thus cross-modulate acti-

vation of downstream targets. The relief of cross-interference between BMPs and activin by Smad4 was observed under conditions of ectopic ligand addition—the extent to which endogenous signaling is in fact limited by Smad4 availability is unknown, as are the parameters that determine which TGF β signaling pathway “wins” under endogenous conditions of competition. In addition, it is clear that BMPs and activin/Vg-1 each induce genes that themselves can cross interfere with patterning specified by the other pathway (Cho et al. 1991; Gawantka et al. 1995; Latinkic et al. 1997); therefore, the observed antagonism in patterning is likely to occur at downstream steps as well as at the step of competition between pathways for Smad4.

EGF and Smad1 In addition to the carboxy-terminal sites in Smad1, Smad2, Smad3, and Smad5 identified as critical for positive regulation by type I receptors, Smads contain multiple additional potential phosphorylation sites, including consensus predicted MAPK sites. In the case of Smad1, several such sites in the region linking the MH1 and MH2 domains can be phosphorylated by MAP kinase after cell stimulation by EGF (Kretzschmar et al. 1997a). These linker phosphorylations inhibit nuclear localization of Smad1 and transcriptional activation by a Smad1–Gal4 fusion construct. Linker phosphorylation by MAP kinase occurs independently of BMP receptor regulation of carboxy-terminal phosphorylation and does not inhibit Smad1–Smad4 association. It does, however, prevent nuclear localization of the Smad1/Smad4 complex. The molecular basis for this inhibition by linker phosphorylation is not known. MAPK-dependent phosphorylation of the Smad1 linker provides a mechanism by which receptor tyrosine kinases or other agents that activate MAPK can antagonize BMP signaling. Physiological examples of such antagonism exist (see, e.g., Niswander and Martin 1993; Ganan et al. 1996; Neubuser et al. 1997), whether Smad1 linker phosphorylation is in fact a major mechanism mediating this antagonism in vivo remains to be shown.

TAK1 and Smads1/5 TAK1 (TGF β -activated kinase) was originally identified as a TGF β or BMP-activated MAP kinase kinase (MAPKKK) that can regulate a TGF β responsive promoter (Yamaguchi et al. 1995). In early frog embryos, ectopic activation of TAK1, like BMPs or ectopic expression of Smad1 or Smad5, can induce ventral mesodermal markers (Shibuya et al. 1998). Furthermore, expression of a kinase-dead inhibitory TAK1 (TAK1KN) construct inhibits gene induction by BMP or Smad1/5, but not gene induction by activin. The molecular basis for this functional interaction between TAK1 and Smad1/Smad5 is unknown. These observations do imply, however, that a positive regulator of a MAPK pathway is necessary for normal functioning of Smad1/Smad5 in the early embryo, in contrast to the inhibitory relationship between Smad1 and MAPK reported in the previous example (EGF and Smad1). The MAP/SAP kinase family is by now quite large, with a complex and incompletely understood set of regulators

and targets (Cohen 1997); understanding regulatory interactions between this family and the Smads will require a detailed knowledge of the sites on Smads phosphorylated by MAP/SAP kinases and the specific pathways and kinases regulating these phosphorylations in vivo.

HGF and Smad2 Still another link between MAPK signaling and Smad activation has come from the report that in several cell types HGF or EGF can activate Smad signaling, apparently by positive regulatory phosphorylation of Smad2 (de Caestecker et al. 1998). EGF, HGF, and TGF β all activate the standard TGF β reporter, 3TP-Lux, in a Smad4-dependent manner in the breast cancer cell line MDA-MB-468, indicating that all three factors require a Smad pathway for signaling to this promoter in these cells. All three factors stimulate phosphorylation of Smad2, but only TGF β stimulation leads to phosphorylation of the carboxy-terminal serines. The MEK1 inhibitor PD98059 inhibits the EGF and HGF, but not TGF β phosphorylation of Smad2, indicating that the tyrosine kinase receptors use a MAPK family member to activate Smad2. The sites of this activating phosphorylation has not been mapped, and therefore it is not yet known whether regulation through MAPK is direct. Like the studies of TAK1, these observations of the positive regulation of Smad2 by a MAPK contrast sharply with the observations that MAPK inhibits Smad1 but are certainly not mutually exclusive. There are a wide range of examples in which TGF β superfamily and MAPK-coupled signals synergize rather than antagonize one another (see, for examples, LaBonne et al. 1995; Oft et al. 1996); therefore, the potential physiological relevance of the positive effects of MAPK signaling on Smads seems at least as plausible as the inhibitory effects. At this point it is difficult to safely generalize about Smad regulation by MAPK: An activating signal for Smad1 may be inhibitory for Smad2; until we know more about the sites and structural effects of phosphorylation of Smads by MAPKs it will be difficult to draw general conclusions about how or when the MAPK pathway(s) modulate the Smad pathway. Nevertheless, the observed positive and negative MAPK/Smad regulations will be important targets for investigation of the physiological interactions between these pathways.

Calmodulin Recently, the identification of calmodulin as a Smad binding protein has suggested another potential mode for regulation of Smad signaling (Zimmerman et al. 1998). Calmodulin associates in vitro with Smad1–Smad4 in a calcium-regulated manner through a site near the amino terminus. In intact cells, inhibition of calmodulin enhances activin induction of the 3TP-Lux reporter, whereas calmodulin overexpression inhibits activin induction, suggesting that calmodulin might inhibit Smad function. The potential effects of calmodulin manipulation are sufficiently complex in vivo, however, that much additional work will be necessary to determine to what extent direct binding of signaling Smads by calmodulin modulates their function. Given the wide

range of stimuli that can regulate calmodulin function (Niki et al. 1996), direct regulation of Smads by calmodulin would open up a very broad set of potential interactions between TGF β s and other physiological signals.

Pathway convergence at a target promoter

Activin and Wnts In both *Drosophila* and *Xenopus*, TGF β and Wnt family-generated signals have been shown to interact during embryonic patterning (Grimm and Pflugfelder 1996; for review, see Harland and Gerhart 1997). In *Xenopus*, the cloning of the promoter for *goosecoid* provides an example of how this interaction works (Watabe et al. 1995). Two distinct promoter elements confer responsiveness to Wnt and activin signals, and act synergistically together to activate *goosecoid* transcription. In the case of the distal element (DE), which responds to activin, activin stimulates Smad2 and Smad4 association with an as yet unidentified transcription factor that binds to the element. Wnt signaling does not appear to affect the activation of the Smads or regulation of this element directly but, rather, regulates expression through the transcription factor complex of β -catenin/LEF-1, of the homeobox transcription factors Twin and Siamois (Brannon et al. 1997; Laurent et al. 1997). These factors then bind to a site, the proximal element, adjacent to the DE on the promoter (Laurent et al. 1997). The localization of *goosecoid* transcription to the organizer therefore appears to be regulated by the integration at the promoter of binding of a Smad2/Smad4-containing transcription complex and a Siamois/Twin transcription factor. Whether this binding is fully independent or, cooperative, to some extent, is not known.

Activin and FGF Induction of a range of early mesodermal responses by activin/Vg-1 signals, as well as by ectopically expressed Smad2, requires an endogenous FGF signal (Cornell and Kimelman 1994; LaBonne and Whitman 1994; M. Lee and M. Whitman, unpubl.). MAPK is a critical component of this requirement: Inhibition of MAPK mimics the block to FGF in preventing transcriptional induction by activin, ectopic activation of MAPK rescues activin induction of mesoderm in the presence of a block to FGF, and endogenous MAPK activity is blocked by blocking FGF signaling (LaBonne et al. 1995; LaBonne and Whitman 1997). MAP kinase activation does not appear either to stimulate or block Smad2 activation in early frog blastomeres but is required for downstream phenotypic effects of Smad2 expression (M. Lee and M. Whitman, unpubl.). Activin stimulation of the FAST-1-Smad2-Smad4 complex (ARF) occurs even in the absence of an FGF/MAPK signal and is unaffected by ectopic activation of MAPK (Huang et al. 1995). This permits the requirement for FGF/MAPK signaling in this case to be mapped downstream of (or parallel to) Smad activation. Interestingly, transcriptional activation of the ARE reporter, to which ARF binds, is unaffected by blockade of FGF/MAPK, even though activin regulation of the larger *Mix.2* promoter from which the

ARE was isolated is blocked by inhibition of FGF signaling (M. Watanabe and M. Whitman, in prep.). This would seem to indicate that signals from the activin and FGF signaling pathways are integrated at discrete regulatory elements in the *Mix.2* promoter, with the ARE mediating a Smad2-linked signal and an as yet unidentified element mediating a signal from MAPK. Comparison of the role of cross talk between MAPK and activin signals in early mesoderm induction to the MAPK interactions with Smad1 signaling in COS cells (Kretschmar et al. 1997a) and Smad2 signaling in MDA-MB-468 cells (de Caestecker et al. 1998) suggests that there are multiple points at which the MAPK and Smad pathways can interact.

Conclusions

In the context of the morphogen hypothesis, the fact that manipulation of Smad signaling pathways is sufficient to specify multiple tissue types is intriguing, but other evidence from early embryos indicates that there is much more to developmental specification than gradients of Smad signaling. Graded stimulation by individual ectopic signals in vitro may mimic processes that are specified in vivo by the intersection of multiple signals: High levels of Smad2 signaling are sufficient to induce genes specific to the Spemann organizer in isolated ectoderm (Green et al. 1992; Baker and Harland 1996; Graff et al. 1996), but a variety of experiments indicate that formation of the endogenous organizer depends on the intersection of several signals, including both Wnt/ β -catenin and activin/Vg-1 Smad2-like signals (Watabe et al. 1995), as well as the local inhibition of BMP signals (for review, see Harland and Gerhart 1997). In addition, there is a great deal of evidence indicating that patterning involves temporally complex, often labile, steps. In the case of the *Xenopus* cement gland, for example, several studies have demonstrated that a defined dose of BMP4, Smad1, or Smad5 is sufficient to specify this structure. However, a long history of dissection and transplantation experiments in *Xenopus* gastrulae have elegantly demonstrated that specification of endogenous cement gland is a highly spatially and temporally dynamic process that is not easily explained by a simple standing gradient of Smad1 activation (Sive et al. 1989; Bradley et al. 1996). Similarly, Knecht and Harland (1997) have shown that although Noggin inhibition can induce a dorsal brain marker in a graded fashion, patterning of this marker in explants is a function of both the age-dependent competence and cell motility of the target tissue, suggesting multiple dynamic components to neural patterning at this tissue. Manipulation of Smads by ectopic expression, null mutation, and knockout have proven to be powerful tools for establishing what Smads can do and what they are required for in early development, but this will be only a first step toward elucidating how they participate in the multicomponent, temporally dynamic processes that establish the pattern of the early embryo. Characterization of how feedback pathways and regulation by non-TGF β signals (summarized in Fig. 3) actually

modulate Smad signals in vivo, identification of localized transcription factors that confer tissue specific responses to Smads, and the development of methods to measure the spatial and temporal distributions of activated Smads in embryos will be critical components of a full understanding of how TGF β s may function as morphogens during early embryogenesis.

Acknowledgments

I thank Carole LaBonne, Paul Wilson, Laurel Raftery, Michelle Lee, and anonymous reviewers for valuable comments on the manuscript. The author is supported by grants from the National Institute of Child Health and Development.

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Smads and early developmental signaling by the TGF β superfamily

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Genes Dev. 1998, **12**:

Access the most recent version at doi:[10.1101/gad.12.16.2445](https://doi.org/10.1101/gad.12.16.2445)

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