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PRINCIPAL INVESTIGATOR: Richard Padgett, Ph.D.

CONTRACTING ORGANIZATION: Rutgers, The State University
of New Jersey
Piscataway, New Jersey 08854-8010

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Richard Padgett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Rutgers, The State University of New Jersey
Piscataway, New Jersey 08854-8010

E-MAIL:
padgett@waksman.rutgers.edu

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One of the most potent inhibitors of epithelial cell growth is TGFβ. Understanding the connection between TGFβ and cell cycle control is an important avenue of experimentation towards treating breast cancers. We are utilizing two complementary approaches in *C. elegans* to find cell cycle regulatory genes that respond to TGFβ signaling. First, in genetic screens using a cell cycle reporter gene, *ribonucleotide reductase*, we are looking for mutations in genes that release dauer animals (a TGFβ induced developmental stage) from their cell cycle arrest. Secondly, we are using DNA microarrays probed with RNA from arrested animals and from animals released by TGFβ to identify genes that are transcribed differentially. To carry out these experiments, we have constructed a new reporter gene for use in our genetic screen, and have worked out conditions to synchronize and harvest sufficient quantities of animals for making mRNA for the microarray experiments. During the next year, we will be identifying genes that are candidates for regulatory control by TGFβ, which will offer insights into how TGFβ control this aspect of the cell cycle.

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Richard W Padgett 7/13/00

PI - Signature Date

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INTRODUCTION

More than 80% of breast cancers are composed of epithelial cells. One of the most potent inhibitors of epithelial cell growth is TGFβ. TGFβ signaling plays a major role in the normal development of the breast and in the progression of breast cancers by controlling exit from the cell cycle. Thus the regulation of these cell cycle exit controls constitute a promising avenue towards treating breast cancers (Catzavelos *et al.*, 1997). We are utilizing two complementary approaches in *C. elegans* to find cell cycle regulatory genes that respond to TGFβ signaling. First, in genetic screens using a cell cycle reporter gene, *ribonucleotide reductase*, we will look for mutations in loci that release dauer animals (a TGFβ induced developmental stage) from their cell cycle arrest. Secondly, we are taking a complementary molecular approach to find genes regulated by the *C. elegans* TGFβ pathway. New DNA chip technology allows one to assay the level of expression of large numbers of genes in a single hybridization experiment. Using RNA from arrested animals and from animals released from dauer arrest, we will probe chips containing the 13,500 genes of *C. elegans* to identify the ones that are transcribed as animals are released from TGFβ induced arrest. These two approaches should provide us with candidate genes that connect the TGFβ pathway with specific regulators of cell cycle arrest. To extend our findings, we will look in mammals for homologs of genes we discover in our experiments. This information will increase our understanding of how cell cycle regulation is achieved, and provide reagents for the design of novel therapeutics.

BODY

Are there connections between TGFb and cell cycle? There are clear connections between TGFb and cell cycle regulation in mammals (see Figure below) and invertebrate model systems, such as Drosophila and *C. elegans*. For example, in dauer arrested animals, cell divisions cease, but if the activity of a p27 homolog is reduced (by RNA interference experiments), then some tissues resume cell division (Hong et al., 1998). This is most easily seen by an S-phase specific reporter gene (the promoter of *ribonucleotide reductase* fused to the green fluorescence protein).

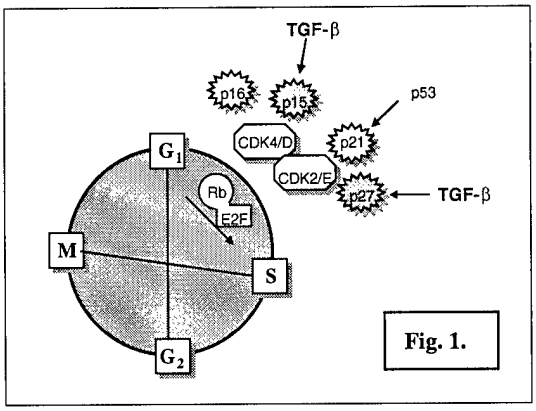

Fig. 1.

Given that the TGFβ pathway and particularly its interface with cell cycle machinery is evolutionarily conserved, *C. elegans* genetics can provide a quick way towards the

discovery of genes that connect TGFβ to the cell cycle. Below are listed our primary aims:

Aim #1. Dauered animals are arrested at the L2 stage, when all divisions cease. We will genetically suppress these animals and, rather than look for non-dauer animals, we will score for the expression of an S phase reporter gene. This type of suppressor screen has not been previously performed. It promises to be exquisitely sensitive, since we do not have to suppress the dauer phenotype in the entire animal and thus, it differs from previous screens. We should be able to identify loci that have not been previously recovered. Since this screen uses green fluorescence protein as a reporter, we can do these screens in live animals, which will allow large numbers of mutants to be examined.

Aim #2. As a complementary approach to our genetic screens, we will also take a molecular approach to find genes regulated by the *C. elegans* TGFβ pathway. We will use the newly emerging DNA chip technology to identify genes which are turned on when animals are released from the dauer state. We will collect RNA from animals that are arrested and not arrested and use this RNA to probe a collection of over 10,000 *C. elegans* genes.

Aim #3. While the above experiments are ongoing, we will examine the effect of newly uncovered putative cell cycle regulator genes on the ability to induce our S-phase cell cycle reporter gene in dauered animals. In addition, we will examine the relationship between our new mutations and known cell cycle inhibitors to determine if our new mutants are acting upon or through known regulatory molecules.

EXPERIMENTS IN PROGRESS.

Generate strains for genetic screens. We are using a green fluorescent protein (*gfp*) as a reporter to detect progression of the cell cycle. Several genes modulate their expression during the cell cycle, and have become useful molecular markers for monitoring the phase of cell cycle. We obtained a strain of worms construct that contain an integrated construct that incorporates the promoter of the *ribonucleoside reductase gene* fused to *gfp* for use in these experiments. Unfortunately, its expression levels are low, making its use questionable for our screen and the construct acted like a recessive, rather than a dominant reporter.

Suspecting that the low level of expression was an aberration of that particular construct, we re-engineered a new construct with this promoter, employing one, two, or three copies of the promoter fused to *gfp*. Multiple copies of the promoter could lead to higher levels

of expression. This new construct is much brighter and does not appear to exhibit the recessive properties of the previous reporter construct (see Figure below). We feel that the expression levels are sufficient to proceed with developing it for use in our genetic screens. At present, we are integrating the array into the chromosomes, so the genetic manipulations will be easier. Afterwards, we will test the new strain for its ability to detect cell cycle progression under the conditions of our screen. We feel that this strain will work well for our screen, but as a backup, we have designed another reporter with the PCNA promoter, if the need arises.

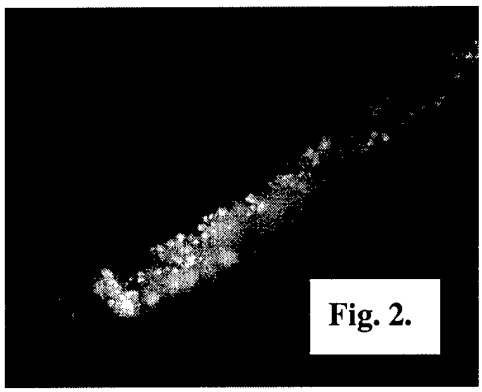

Fig. 2.

Perform pilot screen to optimize conditions. Although we needed to re-engineer the reporter gene for our genetic screen, we thought it useful to do a small pilot screen to work out the conditions of the screen. We performed a small pilot screen and examined the animals under the compound microscope and the dissecting microscope. This helped us gain some experience in screening procedures and will help us when we switch to our new *gfp* strains.

Do differential hybridizations with DNA chips. In these experiments, we plan to compare two populations of RNA to find genes that are differentially expressed. Large numbers of animals have to be grown in order to obtain enough polyA RNA. We have gone through all the stages of preparing the worms to collecting the mRNA, and have attempted to optimize the various steps.

First, we need large quantities of worms to obtain sufficient amounts of RNA. The simplest method is to grow them in liquid culture to a high density to obtain enough animals to seed plates. Bleaching the animals releases eggs, which are resistant to the bleach. Eggs are allowed to hatch, thus providing a synchronized population of L1 animals. These animals are grown on plates to the appropriate stage (L2) and harvested. RNA is immediately made from the worms, followed by an mRNA purification step.

We have gone through each of these individual steps several times to gain proficiency. Potential problems include fungal contamination of the liquid culture and the growth advantage of spontaneous mutations. Under the conditions that we used, neither of these presented a problem to us. Growth in liquid culture requires much bacteria as a food source. Fortunately, we have a fermentation facility in our Institute, and they have grown much of the necessary bacteria for these experiments. Now each growth step is optimized

and we are beginning to grow the worms to collect RNA. Our collaborators at Stanford University will be able to perform the hybridizations quickly, once they receive RNA.

Our goals are to compare the RNA populations of wild-type animals and animals entering the dauer phase. In our first round of experiments, we will use a temperature-sensitive allele of *daf-4* to arrest the animals. Wild-type (N2) and *daf-4* animals will be grown at 25°C and harvested at the time of dauer transition, at the L2 stage. This stage can be visually detected by observation under a dissecting microscope. In later experiments, we may be able to shift the animals to the non-permissive temperature and collect RNA. However, animals enter the dauer phase slowly and this could present a problem with generating discrete sets of RNAs. In future experiments, we will examine this possibility. However, all the genes that we expect to find in this approach should be detectable by this method.

KEY RESEARCH ACCOMPLISHMENTS

1) examined the promoter regions of several genes to find best candidate gene for use as a cell cycle reporter gene
3) generated new *gfp* reporters for doing a large scale screen
3) integration of new *gfp* reporters underway
4) pilot screen done to test screening conditions
5) test dauer animals to determine which time points are best for making RNA
6) tested procedures for making RNA from large pools of animals
7) begun to scale up the strains from which RNA will be made

REPORTABLE OUTCOMES

In the first year, we optimized our experimental protocols for the two major aims of our proposal. As a result, our results are not ready for publication. However, we have written three peer reviewed articles about TGFb signaling that cite the Dept. of Defense grant. These are included in the Appendix.

1. Das, P., L.L. Maduzia, and R. W. Padgett (1999) Genetic Approaches to TGFβ Signaling Path*ways,* **Cyto. and Growth Factor Reviews** 10:179-186.

2. Patterson, G. I. and R.W. Padgett (2000) TGFβ-related Pathways: Roles in *C. elegans* Development, **Trends in Genetics** 16:27-33.

3. Zimmerman, C. and R.W. Padgett (2000) TGFβ Signaling Mediators and Modulators, **Gene** 249:17-30.

CONCLUSIONS

During our first year, we have made substantial progress toward our goals, so that in the coming year, we will be able to collect some of our data. We have made an improved version of our reporter gene that will allow us to more efficiently perform our genetic screen. Our pilot screen has allowed us to refine our screening procedures. With regards to the DNA microarray experiments, we have modified various protocols so that we can grow enough worms to make the mRNA necessary for the hybridizations. RNA preps have been done three times and the RNA tested by PCR for its complexity. We have just received a temperature sensitive *daf-4* mutant, which we will use to make our RNA pools. In the coming months, we anticipate that we will begin to carry out our experiments.

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TGFβ-related pathways

roles in *Caenorhabditis elegans* development

Genetic and molecular analysis in *Caenorhabditis elegans* has produced new insights into how TGFβ-related pathways transduce signals and the developmental processes in which they function. These pathways are essential regulators of dauer formation, body-size determination, male copulatory structures and axonal guidance. Here, we review the insights that have come from standard molecular genetic experiments and discuss how the recently completed genome sequence has contributed to our understanding of these pathways.

In recent years, rapid progress has been made in understanding how transforming growth factor-β (TGFβ) and related ligands signal, in part because of a wealth of genetic and developmental information previously available on the pathways in which these ligands function. Model genetic systems show us how TGFβ-related pathways signal, how they are regulated and what cellular processes they control. As the *Caenorhabditis elegans* genome is completely sequenced and the tools to analyse these basic cellular processes is expanding, *C. elegans* will continue to play a major role in elucidating these functions and networks. In this review, we discuss the genetics and developmental biology of *C. elegans* TGFβ signaling.

The TGFβ superfamily plays critical roles in several important processes, such as cell proliferation, embryonic patterning and cell-type specification[1–6]. Biochemical identification of serine-threonine kinase receptors as mediators of TGFβ signaling was an important advance in the field, as was the identification of cytoplasmic and nuclear effectors belonging to the Smad family (a fusion of *sma* and *Mad* gene names). In *Drosophila*, Mothers against dpp (Mad) was genetically identified as part of the Decapentaplegic (dpp) pathway, and its cDNA sequence indicated it is a cytoplasmic protein, which is consistent with a role as a mediator of receptor signaling[7]. Work in *C. elegans* revealed three Smads that function in the same TGFβ signaling pathway, suggesting that multiple Smads might be required in other pathways[8]. Cloning of mammalian homologs demonstrated that these genes are conserved across diverse metazoan phyla[8,9]. Furthermore, developmental studies in *Xenopus* led to the identification of Smad2, a potent mesoderm inducer[10]. These discoveries spurred a flurry of Smad cloning and database harvesting.

Garth I. Patterson
patterson@waksman.rutgers.edu

Richard W. Padgett*
padgett@waksman.rutgers.edu

Department of Molecular Biology and Biochemistry, 604 Allison Road, Rutgers University, Piscataway, NJ 08854, USA.
*Waksman Institute, 190 Frelinghuysen Road, Rutgers University, Piscataway, NJ 08854, USA.

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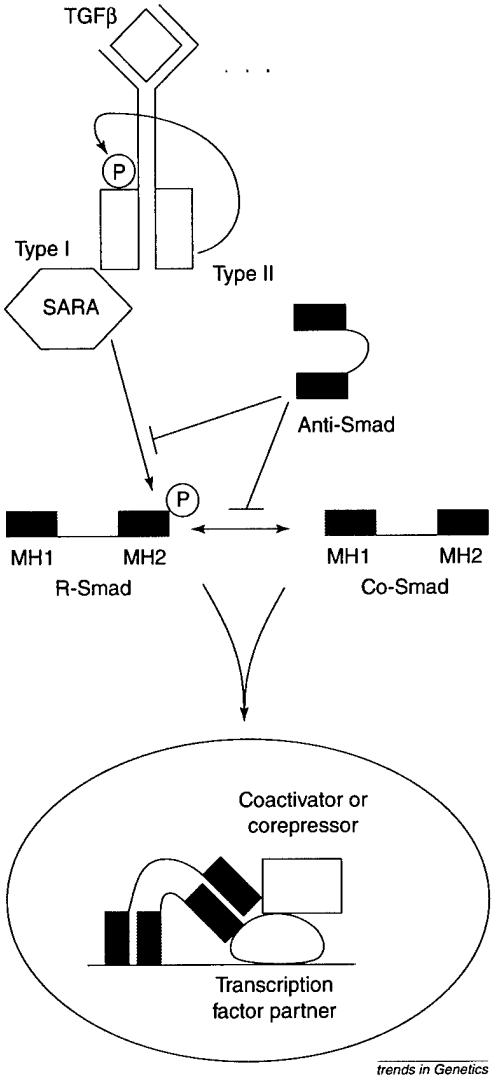
FIGURE 1. Model for TGFβ signal transduction



The Smads are depicted as two blocks connected by a linker region. The MH1 domains of the R-Smads and Co-Smads are shown in green and the MH1 domain of the Anti-Smad is shown in red. The MH2 domain of all the Smads is shown in blue. After ligand binding to the cellular receptors, Type I and Type II, the R-Smads are phosphorylated by the Type I receptor with the aid of Smad anchor for receptor activation (SARA). The phosphorylated R-Smad now complexes with the Co-Smad. Both enter the nucleus, where they bind specific DNA sequences. Because the Smads bind DNA weakly, their activity in the nucleus is altered by other transcription partners and other coactivators or corepressors. In most TGFβ pathways, an Anti-Smad is transcribed in response to signaling, which attenuates TGFβ signaling by binding to the receptors and/or Smads.

Smads come in three varieties

A general model for TGFβ superfamily signaling has been elucidated[1,3]. Ligand binding causes the type II receptor to phosphorylate the type I receptor (see Fig. 1). This event activates the type I receptor, which then, with the help of the SARA (for Smad anchor for receptor activation) protein[11], phosphorylates a Smad on C-terminal residues. Smads are defined by two conserved domains; MH1 (for Mad homology domain 1), which mediates DNA binding and some protein–protein interactions, and MH2, which mediates transcriptional activation and interactions with other transcription factors. R-Smads, which physically interact with distinct receptor complexes to generate signaling specificity, are activated when the type I receptor phosphorylates a conserved SSXS sequence at the C-terminus. These Smads interact with a Co-Smad, and the complex translocates to the nucleus where it, along with other factors, activates the transcription of target genes. The R-Smad or Co-Smad, or both, can interact with DNA via the MH1 domain. The Smad complex can activate or repress transcription, depending on whether it binds to a transcriptional activator or transcriptional repressor. In *Drosophila* and vertebrates, a single Co-Smad is used in all of the known pathways[1–4,12]. The Anti-Smads are a third class of Smads that are induced by TGFβ signaling and they block the phosphorylation of R-Smads and/or the interaction of R-Smads with Co-Smads.

Are Smads responsible for all signaling by the TGFβ family of receptors? The answer is 'perhaps'; the phenotypes of Smad mutants in *Drosophila* and *C. elegans* are virtually identical to the phenotypes of receptor mutants[7,8]. In addition, over-expression of Smads in *Xenopus* can mimic the effects of ligand and receptor, even in the presence of dominant-negative receptor constructs[4]. Thus, in these systems, Smads are necessary and sufficient for the major characterized effects of the receptors. However, this does not rule out the possibility of other molecules participating in signal transduction from the receptors, and it remains to be seen how substantially other molecules contribute to downstream events.

C. elegans dpp/BMP-related pathways

Now that the *C. elegans* genome has been sequenced, it provides a unique opportunity to examine the entire repertoire of TGFβ-related pathways in a way that has not been previously possible. Three TGFβ superfamily pathways have been genetically characterized in *C. elegans* (the dauer, Sma/Mab and *unc-129* pathways). The components of these pathways are most closely related to bone morphogenic protein (BMP) and dpp pathway components found in vertebrates and *Drosophila*. In addition, DAF-4, the only type II receptor in *C. elegans*, functions as a type II BMP2 receptor *in vitro*[13]. Therefore, we will refer to the *C. elegans* pathways as BMP-like pathways. This review discusses the elucidation of these three pathways, models for how signals in these pathways are transmitted, from production of the ligand to transcriptional changes in target cells, the role of these pathways in controlling development, and prospects for further understanding of this signal transduction pathway.

Dauer pathway

C. elegans, like many soil nematodes, can choose one of two larval developmental pathways. This developmental decision was the subject of a recent review[14], so we will focus on highlights that are relevant for an understanding of BMP-like signaling in this pathway. Chemosensory neurons measure food availability and competition for food resources from other nematodes[14]. Scarce food and high pheromone (a chemosensory cue that indicates population density) promote 'dauer' development. When this pathway is chosen, the worms arrest in the third larval

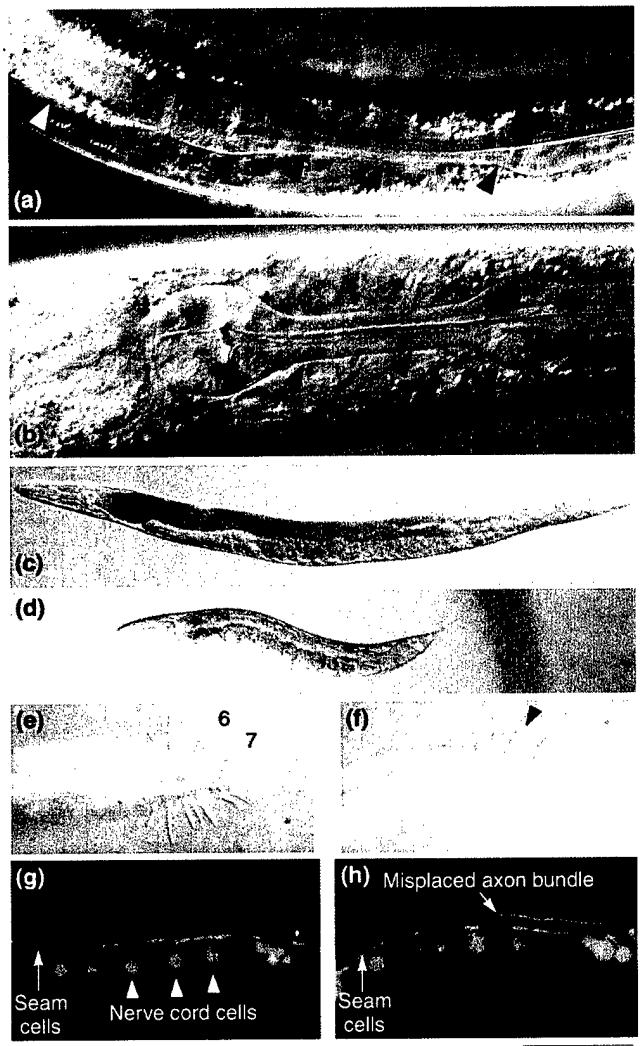
stage as a dauer, which has morphological, behavioral and physiological specializations that allow survival and dispersal from conditions in which food resources are inadequate to allow reproduction (Fig. 2a, b).

A BMP-like pathway plays a key role in the control of the dauer decision. Mutants that disrupt normal regulation of the dauer decision are of two types: dauer constitutive, which develop as dauers even under conditions that are appropriate for reproductive growth, and dauer defective, which develop as non-dauers under conditions that are appropriate for dauer development[14]. Careful analysis of phenotypes and epistasis relationships of the Daf mutants nicely predicted the functional relationships of the cloned genes[14,15]. In particular, a group of dauer-constitutive genes that constitute a BMP-like pathway was identified (Fig. 3). The genes encode a ligand (*daf-7*), two receptors (*daf-1* and *daf-4*), and two Smads (*daf-8* and *daf-14*)[2,14]. The biochemical relationships of these gene products have not been studied, but comparison with vertebrate gene products has led to a model that DAF-7 binds and activates the serine-threonine kinase receptor complex, which in turn phosphorylates the two Smads, DAF-8 and DAF-14. These Smads have an amino acid sequence that is related to the conserved SSXS motif that has been shown to be a target of the receptor kinases in other systems, but otherwise are highly diverged from other Smads (Table 1). DAF-8 and DAF-14 differ from R-Smads in the MH1 domain. DAF-8 is highly diverged in this domain[14], and is missing highly conserved residues that contact DNA and other residues that play important structural roles in the hydrophobic core of the MH1 structure[3]. DAF-14 is unique among Smads in that it has no MH1 domain[16].

The expression of the BMP-like ligand gene, *daf-7*, is controlled by cues received by chemosensory neurons. Chemosensory neurons in the amphid sensillum are critical for regulation of dauer[14]. Expression of a *daf-7*::green fluorescent protein (GFP) reporter is reproducibly seen only in the amphidal sensory neuron pair called ASI, and expression is activated by food and repressed by pheromone and high temperature[17–18]. Thus, coupling of environmental cues to regulation of the BMP-like pathway might be fairly direct; sensation of food and pheromone could take place in the sensory endings of ASI and be transduced to the nucleus to regulate *daf-7*. Alternatively, sensation could act in other amphid neurons and be transduced via interneurons or hormonal signals to ASI. Analysis of *ttx-3* mutants indicates that the temperature input for thermotaxis and dauer formation both use the same neuronal pathway[19], which does not include ASI; therefore, the effect of temperature on *daf-7* expression might be indirect. Mosaic analysis and expression of DAF-4 from cell-type specific promoters indicates that DAF-4 functions in the nervous system to control dauer formation[16]. DAF-7 ligand produced in ASI may bind DAF-4 receptor on nearby neurons to regulate the production of a hormonal signal that controls the dauer development of hypodermis, intestine, gonad and other cell types.

Two genes, *daf-3* and *daf-5*, were identified as dauer-defective mutations that suppress the dauer-constitutive phenotype of mutations in the five genes above[14]. This epistasis suggests that *daf-3* and *daf-5* act downstream or in parallel; in either event, the dauer-defective phenotype suggests that *daf-3* and *daf-5* are antagonized by the putatively receptor-activated Smads. *daf-5* has not been cloned, but *daf-3* encodes another Smad[20]. The fact that

FIGURE 2. Phenotypes exhibited by animals mutant for the BMP-like pathways

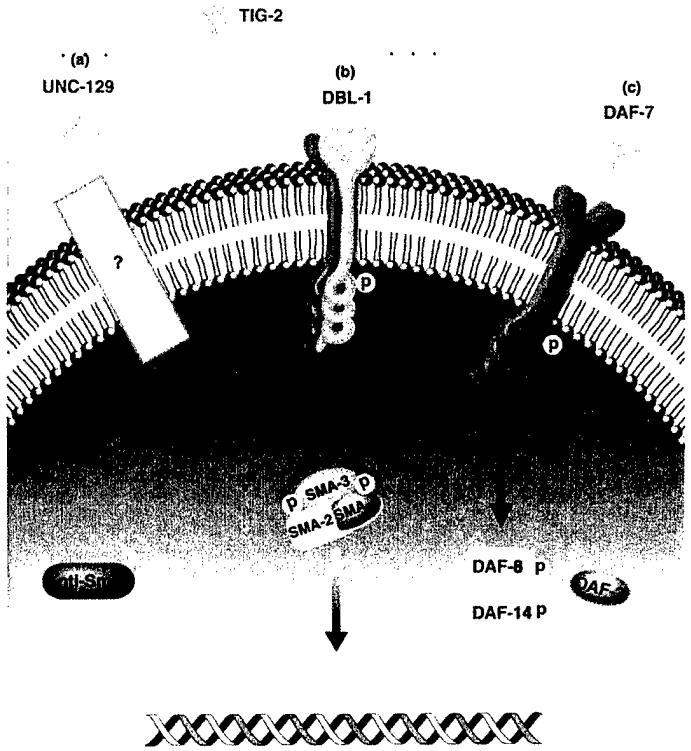


trends in Genetics

(a) Anterior of *daf-7* mutant induced to form dauer, (b) *daf-7, daf-3* mutant non-dauer L3. Note that the width of the body is much less in the dauer than in the L3 animal, as is the pharynx (solid arrowheads show the outline of the pharynx). The dauer also has a sparkly intestine (open arrowhead), probably owing to the accumulation of storage granules. (c) A wild-type worm. (d) A *sma-6* mutant at the same magnification. (e) A wild-type male tail. Rays 6 and 7 are marked. (f) A *sma-6* tail. The arrowhead shows the fat ray produced by the fusion of rays 6 and 7. (g) and (h) represent the midbody region of a wild type and *unc-129* mutant, respectively. Ventral cord cells are expressing green fluorescent protein. Note that the *unc-129* animal has an ectopic nerve bundle due to misdirected axon migrations. Panels c–f are reproduced with the permission of Company of Biologists Ltd[29]. Panels g–h are reproduced with permission from Academic Press[42].

daf-3 is antagonized by the BMP-like pathway genes is unique. Anti-Smads have been described, but their function is to antagonize the receptors and receptor-activated Smads, and they have no known function in the absence of the receptors or Smads. By contrast, *daf-3* is antagonized by the receptors and Smads, and functions to induce dauer formation when the receptors or Smads are missing in mutants. DAF-3 is like other Smads in structure (Table 1),

FIGURE 3. *C. elegans* BMP-like pathways



The pathways used in (a) axon pathfinding, (b) body size/male tail development, and (c) dauer formation. Ligands are shown outside the cell in pink. Receptors are shown in green (receptors for UNC-129 are unknown).

despite its functional distinctiveness – it has both MH1 and MH2 domains[20] and it can bind DNA[21].

A model to explain these unique features has been proposed[20], in which phosphorylation of DAF-8 and DAF-14 by the receptors leads to their physical interaction with DAF-3. To explain the epistasis relationship, this interaction is suggested to inactivate DAF-3. When the DAF-8 and DAF-14 Smads are not phosphorylated, or are not present in mutants, DAF-3 would be free to regulate gene expression. It is also possible that the DAF-8 and DAF-14 Smads signal in parallel to DAF-3 without direct interaction, and that the two pathways are mutually antagonistic[14].

daf-12 mutates to a dauer-defective phenotype, and is epistatic to mutations of the *daf-7* BMP-like pathway. *daf-12* encodes a nuclear hormone receptor homolog, and is most closely related to the vertebrate Vitamin D receptor and a *Drosophila* orphan receptor, DHR96, which is ecdysone induced[22]. Smad3 and Vitamin D receptor have recently been shown to physically interact and to coactivate Vitamin D-responsive promoters[23]. So DAF-12 could well be a cofactor of the DAF3 Smad. However, *daf-12* mutations have heterochronic effects (those affecting developmental timing) that are not shared by *daf-3* and *daf-5*, in that *daf-12* mutants repeat second larval stage patterns of cell division and migration during the third larval stage[24]. In addition, *daf-12* is epistatic to *daf-28*, whereas *daf-3* and *daf-5* are not[25]. These facts, and the homology of DAF-12 to a *Drosophila* protein that binds ecdysone and that might be involved in molting[22], suggest that *daf-12* might not be an integral part of the BMP-like pathway. Rather, *daf-12* might be involved in progression through the L2 stage to L3, and *daf-12* mutants might have a defect in carrying out third larval-stage fates that includes an inability to form the specialized L3 dauer. A precedent can be found for heterochronic mutations affecting dauer formation in a manner that is apparently independent of BMP-like signals. The gene *lin-4* is similar to *daf-12* in that it is heterochronic, unable to form dauers and epistatic to *daf-7* (Ref. 26). The function of *lin-4* is to negatively regulate *lin-14*, a novel gene that has no known connection to BMP-like signaling.

Sma/Mab pathway

As described above, studies on the dauer pathway established the existence of BMP-like signaling in *C. elegans*. Interestingly, *daf-4*, which encodes a type II BMP2/BMP4 receptor[13], has mutant phenotypes not exhibited by the other *daf* genes. In addition to producing dauer constitutive animals, mutations in *daf-4* also cause a small body size (Sma; see Fig. 2c, d), crumpled spicules, and male sensory tail ray defects (Mab; see Fig. 2e, f)[8]. The spicules are male copulatory organs that aid sperm transfer, and the male sensory rays are used for locating the vulva. A model to explain the multiple mutant phenotypes of *daf-4* is that DAF-4 propagates two distinct BMP-like signals in *C. elegans* to control the dauer pathway and the Sma/Mab pathway (Fig. 3). This model requires that two sets of ligands and type I receptors act with DAF-4 to transduce signals via distinct Smads, resulting in different developmental responses. Cloning of genes with a Sma/Mab phenotype demonstrated this to be true[8,27–29].

Three genes (*sma-2*, *sma-3* and *sma-4*) that mutate to cause a small body size, crumpled spicules, and male tail ray defects were found to encode proteins similar to each other[8]. Moreover, they were homologs of the *Mothers against dpp* (*Mad*) gene from *Drosophila*[7], the first identified member of this family. If these Smads are regulated by DAF-4, then there should be another type I receptor which forms a complex with DAF-4. A polymerase chain reaction screen using primers similar to sequences of *Drosophila* BMP-like type I receptors[29] identified a new type I receptor that was a candidate partner for DAF-4. This receptor corresponds to *sma-6*, which mutates to a Sma/Mab phenotype similar to that of *daf-4*, *sma-2*, *sma-3* and *sma-4*. These results confirm that *daf-4* sends two independent signals and provides *in vivo* evidence that a given type II receptor can interact with different type I receptors (Fig. 3). A mutant screen for additional small animals and a reverse genetic approach showed that null mutants in the BMP-like ligand gene *dbl-1* (also known as *cet-1*) exhibit the same mutant phenotypes as the Smads and the type I receptor, *sma-6* (Refs 27, 28). A *dbl-1*::GFP fusion is mainly expressed in the amphid neurons, neurons of the ventral nerve cord and neurons and glial cells in the tail, which must serve as the source of the signal for some or all of the three developmental processes in which it is involved.

This BMP-like pathway appears to regulate body size by a novel mechanism. Newly hatched *Sma* mutant animals are normal length, but *Sma* mutant adults are about

TABLE 1. Comparisons of dauer pathway Smads with other known Smads

	MH1 domain	MH2 domain	SSXS phosphorylation target	Relationship to receptors	Refs
R-Smads (includes Smad1, 2, 3, 5, 8 from vertebrates; Mad from *Drospholia* and SMA-2 and SMA-3 from *C. elegans*)	Yes	Yes	Yes	Activated by receptors	1–6
Co-Smads (includes Smad4 from vertebrates, MEDEA from *Drosophila*, and SMA-4 from *C. elegans*)	Yes	Yes	No	Assists R-Smads in transducing signal	1–6
Anti-Smads (includes Smad6, 7 from vertebrates and Dad from *Drosophila*)	[a]Diverged	Yes	No	Antagonizes receptors	1–6
DAF-8	Diverged	Yes	Yes	Activated by receptors?	14
DAF-14	No	Yes	Yes	Activated by receptors?	16
DAF-3	Yes	Yes	No	Antagonized by receptors	19

[a]The diverged MH1 domain in Anti-Smads and DAF-8 is not likely to bind DNA, because it is missing structural residues that form the hydrophobic core and contact DNA.

half the size of normal adults[27,28]. Examination of adult nuclei has not shown any differences in number from wild type, strongly suggesting that the small body size is not caused by a decrease in cell number[27,28]. This would indicate that all the expected cells are present but some or all are physically smaller than in the wild type. An interesting possibility is that the controls that regulate cell growth and mitosis have been uncoupled in the *Sma* mutants so that the cells divide before their normal dividing volume is reached. The receptors and Smads might control the production of a signal that regulates cell division, or might function cell autonomously in various cells to control the size of those cells. The broad expression reported for *daf-4* and *sma-6* are consistent with either model[20,29].

Null mutations in the Sma/Mab pathway result in transformations of rays and often fusion between adjacent rays. The rays that are transformed in Sma/Mab mutants are mostly dorsal rays 5, 7 and 9, which adopt the fate of their anterior neighbor and often fuse with it, creating a fatter ray[27–29]. The basis for these phenotypes appears to be an improper migration of the cells that comprise the ray. Mutations in the Sma/Mab genes also disrupt a cellular migration necessary to form the spicule, a copulatory structure necessary for mating[30].

Do *C. elegans* BMP-like pathways pattern the embryo?

BMP and dpp pathways are essential for vertebrate and *Drosophila* development; mutants die as embryos. It has been suggested that *Drosophila* and vertebrates have homologous BMP pathways that control dorsoventral patterning in the embryo[31]. The defects in axon outgrowth in *unc-129* mutants and in male tail rays in Sma/Mab mutants can be interpreted as defects in dorsoventral patterning. However, no BMP-like pathway mutants disrupt the basic dorsoventral axis of *C. elegans* in the manner of *Drosophila* or vertebrates. Is it possible that the *C. elegans* BMP pathways function in dorsoventral patterning in early embryogenesis? We think this is unlikely, for two reasons. First, one of the *daf-4* mutant alleles has a stop codon that is predicted to eliminate the last 45 amino acid residues of the kinase domain[13]. This allele of *daf-4* probably encodes a kinase-inactive protein, yet it has no defects other than the Daf and Sma/Mab phenotypes we have discussed. There is no other type II receptor in the *C. elegans* genome sequence that could function in the place of DAF-4. In addition, analysis of *sma-6* and *sma-3* mutants also indicates that molecular null alleles of these genes have been isolated, and no embryonic phenotypes have been observed. Analysis of double mutants of BMP-like pathway genes provides the second reason for our suggestion that there is no embryonic function for these pathways. Our reasoning is as follows. It is possible that the embryonic function of the genes is not apparent in the single mutants because the screens that led to their isolation were for larval phenotypes. These screens might have led to the isolation of hypomorphs only. However, if this were the case, we expect that the embryonic function might be revealed when two of these putative hypomorphs are combined. Many double mutants have been made between alleles of multiple genes in the dauer pathway[15] and Sma pathway[29], as well as double mutants that include mutations in both pathways[27,29], and none of these double-mutant combinations show any enhancement of known phenotypes or any new embryonic phenotype.

What does the lack of embryonic function for the BMP-like pathways in *C. elegans* imply about the evolution of these pathways? It is possible that the common ancestor of *C. elegans*, *Drosophila* and vertebrates had a BMP pathway functioning in dorsoventral patterning, and the pathway was lost in an ancestor of *C. elegans*. It is also possible that *Drosophila* and vertebrates share a common ancestor more recently than with *C. elegans*, and that the BMP pathway function arose in the *Drosophila*/vertebrate line after its divergence from the *C. elegans* line. It is not possible to be certain which model is true; the relationship of nematodes to other phyla is uncertain, because of a lack of fossil evidence and the rapid rate of sequence evolution in many nematode species[32]. A recent analysis of 18S RNA sequences suggests that arthropods and nematodes are more closely related to each other than either is to vertebrates[33], which would imply that the first model above is correct.

Orphan genes

Mutations in *unc-129* cause defects in axonal guidance, and *unc-129* encodes a BMP-like ligand[34]. *unc-129* mutants do not have Daf or Sma/Mab phenotypes, and examination of *daf-1*, *sma-6* and *daf-4* mutants revealed no axon guidance defects. The genome sequence reveals no additional receptors or Smads that could constitute an *unc-129* signal transduction pathway. Thus, *unc-129* is not likely to signal in the conventional way. It has been suggested that UNC-129 might function by binding

UNC-5 directly or by acting on a signal transduction pathway unlike the conventional BMP-like pathways. The genome sequencing consortium has identified one other Smad in the *C. elegans* genome with an unknown function; it is most closely related to an Anti-Smad, but its biochemical properties have not been examined. The genome sequence also reveals an orphan ligand of unknown function, *tig*-2. All of the receptors and other Smads in the genome participate in Sma/Mab or Daf pathways, so it seems more likely that the new Anti-Smad and orphan ligand function in either or both of the characterized pathways, rather than in a new, undiscovered pathway. The genome sequence reveals a SARA homolog. The characterized vertebrate SARA proteins function with the activin/TGFβ pathway specific Smad2 and Smad3 (Ref. 11). The existence of SARA in *C. elegans*, which has only BMP-like pathways, suggests the possibility that these pathways in vertebrates use a different isoform of SARA that remains to be discovered and characterized.

Smad cofactors

The forkhead protein FAST1 forms a DNA-binding complex with Smads in vertebrates[1–4], and there are three *C. elegans forkhead* homologs that regulate the same processes as the Sma/Mab and dauer BMP-like pathways. The *daf-16* gene mutates to a dauer-defective phenotype that is epistatic to the dauer-constitutive phenotype of mutations of the DAF-2 insulin receptor and associated signaling molecules. DAF-16 is a forkhead transcription factor[35,36], and therefore might interact directly with DAF-3. It should be noted that DAF-16 is not orthologous to FAST1, but to AFX and FKHRL-1. These two proteins have recently been shown to be regulated by the mammalian homolog of AKT-1 and AKT-2 (Refs 37, 38), which were genetically identified as regulators of *daf-16* (Ref. 39). A second forkhead protein, LIN-31, has been implicated in the formation of the spicule. *lin-31* and Sma/Mab pathway mutants have identical phenotypes in spicule development; the migration of a tail cell and the resulting production of a cellular mold for the spicules, is abnormal[30]. Thus, LIN-31 might be a cofactor of SMA-2, SMA-3 and SMA-4 in controlling this migration. *lin-31* mutants do not affect the ray or body-size phenotypes seen in *sma-2*, *sma-3* and *sma-4* mutants, so any interaction is tissue specific.

The Smad DAF-3 and the forkhead protein PHA-4 regulate expression of a pharyngeal promoter, but these two proteins might not be cofactors. PHA-4 is required during embryogenesis for organogenesis of the pharynx, and induces expression of the myosin gene, *myo*-2 (Refs 40, 41). By contrast, DAF-3 plays no detectable role in embryonic development of the pharynx, but does bind an element from the *myo-2* promoter and represses expression in adults[21]. DAF-3 could exert its repressive effect by binding and inactivating PHA-4.

Conclusions and future prospects

Many of the major players in these pathways have recently been identified. Probably the most important questions to answer next are: where and when do these pathways act, and how do these pathways interact with other pathways? In particular, which cells receive the signals, and how do they respond? Additional mutations that disrupt each of the three pathways have been isolated, and cloning these genes will help to answer the outstanding questions. The identification of Smads in the dauer and Sma/Mab pathways suggests that a major output is transcriptional, and it will be important to identify the Smad target genes. An additional promising approach to identifying genes transcriptionally regulated by the Smads is to use a microarray that contains sequences corresponding to all of the *C. elegans* coding regions, similar to the sort of array that has been used to study the expression of genes in yeast. The existence of the genome sequence also makes it possible to screen for target genes '*in silico*', by identifying genes that have Smad-binding sites upstream. Given that every gene in the TGFβ pathway is conserved between phyla, new insights are likely to come from studying the developmental processes involving TGFβ-like pathways in *C. elegans*. The coming years should continue to provide new insights into how these signaling pathways function, and perhaps they will provide a fairly complete picture of all TGFβ superfamily signaling in a single intact organism.

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Drosophila in cancer research

an expanding role

In recent years, *Drosophila* researchers have developed powerful genetic techniques that allow for the rapid identification and characterization of genes involved in tumor formation and development. The high level of gene and pathway conservation, the similarity of cellular processes and the emerging evidence of functional conservation of tumor suppressors between *Drosophila* and mammals, argue that studies of tumorigenesis in flies can directly contribute to the understanding of human cancer. In this review, we explore the historical and current roles of *Drosophila* in cancer research, as well as speculate on the future of *Drosophila* as a model to investigate cancer-related processes that are currently not well understood.

In 1916, decades before *Drosophila* would become one of the most popular models for studying many aspects of modern biology, the discovery of melanotic tumor-like granules in mutant larvae by Bridges and Stark first suggested that flies could develop tumors[1]. Later, spontaneous mutations were identified that caused animals to die at larval stages with overproliferation of certain internal tissues[2,3]. Subsequent screens for such a phenotype were highly successful as dozens of genetic loci were recovered in *Drosophila* at a time when few human tumor suppressors had been identified[2,4–6]. Most of the tumor-causing mutations that were identified during this time were defined as tumor suppressor genes because they behaved as recessive loss-of-function mutations[7]. Molecular characterization of some of these fly tumor suppressor genes pointed to the importance of cell–cell communication in the regulation of cell proliferation[3,8,9] (Table 1).

Despite very promising beginnings, the fly has not received much attention as a model system for cancer research. Several factors might have contributed to this outcome. Although the over-proliferated larval tissues and melanotic tissues that were observed in the fly mutants had some characters resembling those of human tumors, they lacked the appearance of the massive *in situ* overproliferation that is commonly associated with most mammalian tumors. Second, the molecular characterization of these early fly tumor suppressors did not demonstrate a similarity to the tumor suppressors that had been identified in humans[10,11]. Furthermore, characterization of these fly tumor suppressor genes did not provide an obvious connection to the contemporary understanding of the processes that are involved in tumor formation, such as regulation of the cell cycle. Finally, the indiscreet classification of some *Drosophila* genes as tumor suppressors also contributed to the state of neglect by the general cancer research community. For example, inactivation of neurogenic genes causes hypertrophy of the nervous system. However, they are not tumor suppressors because the phenotype is caused by conversion of epidermal cells into neurons and not by overproliferation of neuronal tissues[12].

Christopher J. Potter
christopher.potter@yale.edu
Gregory S. Turenchalk
gregory.turenchalk@yale.edu
Tian Xu
tian.xu@yale.edu

Howard Hughes Medical Institute, Department of Genetics, Yale University School of Medicine, Boyer Center for Molecular Medicine, 295 Congress Avenue, New Haven, CT 06536-0812, USA.

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Mini-review

Genetic approaches to TGFβ signaling pathways

Pradeep Das, Lisa L. Maduzia, Richard W. Padgett*

Waksman Institute and Department of Molecular Biology and Biochemistry, and Cancer Institute of New Jersey, Rutgers University, Piscataway, NJ-08854, USA

1. Introduction

At the turn of the century, when Thomas Hunt Morgan started using the fruitfly, *Drosophila melanogaster*, for genetic experiments, the degree of conservation of many basic cellular processes between diverse metazoan species had not yet been realized. Later, as the various biological disciplines developed, it became clear that this was indeed the case. This insight spurred Sydney Brenner in the late 1960s to develop the nematode, *Caenorhabditis elegans*, into a model organism for studying nervous system structure and development as an avenue to understanding these processes in higher organisms [1]. Even when Nüsslein-Volhard and Wieschaus [2] did their pioneering work in identifying patterning genes in *Drosophila* in the late 1970s, it was not yet widely accepted that similar genes would be used to pattern other metazoa. We now know that the overwhelming majority of cellular and developmental processes are highly conserved, and that *C. elegans* and *Drosophila* are excellent model systems to study these processes.

The widespread use of *C. elegans* and *Drosophila* is attributable to the fact that both are multicellular organisms which can be manipulated with a number of sophisticated molecular and genetic tools, making the identification and characterization of mutant loci a practical option. Further, the genome sequencing projects in the two organisms have proved to be a valuable asset. While over 99% of the *C. elegans* genome has been sequenced and published [3], the *Drosophila* genome project is expected to reach completion shortly. Genome sequencing provides easier access to gene identification, and has reinforced the finding that many genes and pathways are evolutionarily conserved.

In this review, we provide a summary of the genetic analysis of TGFβ signal transduction, as well as its role in various human diseases and mouse models. We also use discoveries in the TGFβ pathway as an example to highlight some of the techniques used in the invertebrate world of *C. elegans* and *Drosophila* to further our understanding of this, and other, signaling systems. The roles of such techniques in elucidating diverse signaling pathways, as well as pathways of human disease genes, will become more important as the information from the genome projects increases and as the development of genetic tools to analyze them becomes more powerful. Given the conservation of signaling mechanisms, there will be increasing synergy between studies in invertebrates and vertebrates in future years for solving different cellular pathways.

2. Genetic analysis of TGFβ signaling

2.1. Superfamily ligands

TGFβ superfamily members are secreted growth factors that possess several important functions in cell proliferation, differentiation, and adhesion (reviewed in [4]). The TGFβ family is divided into three primary groups, namely the TGFβs, activins, and bone morphogenetic proteins (BMPs), each comprised of ligands with specific roles in development and homeostasis.

The *Drosophila* gene *decapentaplegic* (*dpp*) was identified as a TGFβ member [5] soon after the first vertebrate members of the superfamily were cloned. *dpp* was known to have several critical functions in *Drosophila* development, and had been genetically well

* Corresponding author. Tel.: +1-732-445-0251; fax: +1-732-445-5735.

E-mail address: padgett@waksman.rutgers.edu (R.W. Padgett)

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characterized [6–8]. It was also shown that vertebrate BMP4 and *Drosophila dpp* could functionally substitute for each other, providing important evidence for the conservation of structure and function within the superfamily [9,10]. These results gave credence to the use of the *Drosophila* system for analysis of the pathway. In recent years, several other homologs have been identified from both *Drosophila* and *C. elegans* [11–15] and as is discussed below, the study of these invertebrate ligands has been crucial in providing important breakthroughs in understanding TGFβ signaling in other organisms.

The recent innovation of some genetic tricks in *Drosophila* have permitted the testing of various models of ligand function. These innovations include the ability to induce clones of cells that are either mutant for a particular gene [16], or over-express it in tissues where it is not normally expressed, using the FLP/FRT and the UAS/GAL4 techniques [17,18]. Similar tools have been used in a series of elegant experiments to demonstrate that *dpp* and *wingless* function as classical morphogens [19–21].

2.2. Receptors

TGFβ superfamily ligands have been shown to signal via serine/threonine (S/T) kinase receptors (reviewed in [22]). These receptors were first cloned from vertebrates [23], and were later shown to belong to two related groups — the type I and II groups. It was observed that these genes were homologous, to *daf-1*, a previously cloned orphan S/T kinase receptor in *C. elegans* [24]. *daf-1* had been isolated as a gene that functions to control entry into the dauer stage, wherein the animals become resistant to harsh environmental conditions. A type II receptor gene, *daf-4*, has since been cloned from *C. elegans*, and shown to function with the type I receptor, *daf-1*, in the dauer pathway [25]. Curiously however, *daf-4* mutants were observed to have phenotypes that were not displayed by *daf-1* mutants, leading to the hypothesis that *daf-4* may function in two distinct signaling pathways to control different patterning events [26]. Such genetic analyses are a powerful tool in studying signal transduction, and can be of enormous help in identifying partner molecules. The hypothesis stated above, based on the examination of mutant phenotypes, was borne out by subsequent discoveries [26], and is examined in more detail below.

It has been demonstrated that the TGFβ ligands may signal as either homo- or hetero-dimers, by binding both type I and type II receptors. The type II receptor phosphorylates and activates the type I receptor, which then transduces the signal to downstream target molecules [27]. In *Drosophila*, the *dpp* receptor genes *punt* (type II), *thick veins*, and *saxophone* (both type I) have been cloned and shown to be critical for *dpp* signaling [20,28–32]. As these genes function together to pattern the same morphological structures, animals mutant for any one of them display similar phenotypic defects. The *dpp* pathway provides a good case study of another kind of genetic trick — looking for loci that mutate to similar phenotypes, and then cloning the genes responsible. *Schnurri*, for example, was identified by first scrutinizing various loci for ones that mutated to yield a ventralized embryo, a characteristic of *dpp* alleles, and then cloning the gene molecularly [33,34].

2.3. Smads

After the characterization of the ligands and receptors, further elucidation of the TGFβ pathway was stalled. Various molecular and biochemical tools, such as two-hybrid and immunoprecipitation experiments, failed to yield any convincing downstream signal transducers. The breakthrough in this area was facilitated by the genetic analysis of TGFβ signaling in both *Drosophila* and *C. elegans*. Screens designed to identify mutants that enhanced the morphological defects of *dpp* alleles [35,36], resulted in the isolation of *Mothers against dpp* (*Mad*), a gene later shown to encode a novel protein [36]. Similar studies in *C. elegans* resulted in the isolation of three genes, *sma-2, sma-3*, and *sma-4*, with mutant phenotypes similar to alleles of the type II receptor gene, *daf-4*. Upon cloning, all three loci proved to encode molecules homologous to *Drosophila Mad* [26]. The identification of these four related genes, now called the Smad family [37], as conserved TGFβ signal transducers from two different invertebrate organisms, led to the cloning and biochemical characterization of several vertebrate homologs. It is now clear that the Smads form an integral part of the transduction process (reviewed in [38,39]).

It has been shown that the Smads are phosphorylated by the activated ligand-receptor complex. They then translocate to the nucleus, where they regulate the expression of target genes in conjunction with specific transcription factors. Smad4, the first vertebrate Smad to be functionally characterized, was shown to be mutant in several different tumorigenic tissues [40–43]. Without the invertebrate data linking these genes to TFGβ signaling, the role of Smad4 in cancer would have been unclear.

3. TGFβ signaling defects in mouse models

Targeted disruptions of the vertebrate Smads over the past few years have also yielded insights into the functions of these molecules in TGFβ signal transduc-

Table 1
Loss-of-function phenotypes associated with various TGFβ pathway components

Organism	Mutated or deleted TGFβ component	Defect
Human	MIS	Müllerian Duct Syndrome [58,59,71]
	MIS type II receptor	
	CDMP-1 (GDF-5)	Brachydactyly [72], abnormalities in limb bone development [73,74]
	TβRII	Colon, head and neck cancers [54,75]
	TβRI	Metastatic breast cancers [76], colon, gastric and prostatic cancers, AIDS-related kaposis sarcoma (reviewed in [54]), chronic lymphocytic leukemia [77]
	ALKI	Human hereditary hemorrhagic telangiectasia (HHT) [55–57]
	Endoglin	
	Smad2	Colorectal and lung cancers [61,62]
	Smad4	Breast, colorectal, esophageal, head and neck, lung, ovarian, pancreatic, prostatic, and gastric cancers [40–43,78,79], juvenile polyposis [80], biliary duct carcinoma [81]
Mouse	TGFβ1	Abnormal modulation of immune system [82], multifocal inflammatory lesions [83]
	BMP2	Abnormal cardiac development, malformation of amnion/chorion [84]
	BMP4	Defects in gastrulation and mesoderm formation [85]
	BMP5	Defects in skeletal and soft tissue development [86,87]
	BMP7	Abnormalities seen in skeletal, kidney and eye development [88,89]
	Smad2	Embryonic lethal (E7.5–E10.5), defective mesoderm formation [44–46]
	Smad3	Defective immune function [48], develop colorectal cancers [47,49]
	Smad4	Embryonic lethal (die before E7.5), fail to initiate gastrulation, in combination with APC mutations, aids in the progression of colorectal tumors [50,51,90]
	Smad5	Embryonic lethal (E9.5–11.5), defects in heart development [53]
Drosophila	Dpp pathway	Immaginal disc and dorsal-ventral patterning defects [6–8]
	Activin pathway	Defects in cell growth and proliferation [91,92]
C. elegans	Sma/Mab pathway	Small body size, male tail ray fusions, crumpled spicules [26,93]
	Dauer pathway	Defective in entering or exiting a developmentally arrested state [94]

tion (Table 1), and have allowed the testing of the models derived through in vitro studies.

Homozygous *Smad2* mutants die as early embryos due to defective mesoderm formation and other defects [44–46]. This is a more severe phenotype than its putative upstream activators, such as TGFβs and activins. In contrast, *Smad3* mutants are normal at birth and go on to become slightly smaller adults than their litter-mates. They do, however, exhibit a compromised immune system, and die from immune-related complications between one and eight months of age [47,48]. Many Smad3 deficient mice also develop metastatic colorectal carcinoma [49]. Biochemically, Smad2 and Smad3 behave in very similar ways, except for the observation that Smad2 is unable to bind DNA, while Smad3 does. Now these in vivo data from mouse knock-outs support the fact that they may have different functions.

Homozygous *Smad4* mutant mice have the most severe phenotypes of all Smad knock-out mice, a logical result given the evidence pointing to its central role as a Co-Smad in all TGFβ superfamily signaling. They fail to initiate gastrulation and die as early embryos [50,51]. They show defective mesoderm formation and growth retardation, which have been observed in *BMP4*- and *activin*-deficient mice. They also show visceral endoderm abnormalities. The defects in mesoderm formation can be rescued by aggregating the mutant embryos with wild-type tetraploid cells, which can only contribute to the development of extraembryonic tissue [50]. This suggests that Smad4 may not have a function in BMP signaling in early embryonic stages, or that another BMP-specific homolog of Smad4 has not yet been identified. These data are in contrast to the biochemical studies that have demonstrated the requirement for Smad4 in BMP, TGFβ, and activin signaling. However, the recent finding that in *Xenopus*, two forms of Smad4 exist, each with its own unique characteristics [52], supports the idea that the mouse may have multiple Smad4 genes with different functionalities.

Mutations in *Smad5* lead to lethality in late embryogenesis [53]. These embryos undergo normal gastrulation, and begin organogenesis before dying. The loss of Smad5, which has been biochemically determined to function in the BMP pathway, leads to phenotypes that are less severe than those of mutant BMP ligands or receptors. Interestingly, Smad 5 homozygotes show defects in heart development, much like BMP-2 deficient mice [53]. It has been shown in vitro that

Smad5 may act with Smad1 and Smad8 in BMP signaling, and this overlap of function may explain the reduced severity of the mutations.

4. TGFβ mutations in human diseases

Given the varied and important functions of TGFβ pathways in the development and homeostasis of different tissues, it is not surprising that many connections have been found to link mutations in these components to the formation of human diseases (Table 1).

One of the first connections between TGFβ signaling and cancer was drawn from the identification of mutations of the TGFβ type II receptor. These mutations are found in a significant percentage of hereditary or somatic forms of nonpolyposis colorectal cancer with microsatellite instability [54]. The mutations make cells refractive to TGFβ signals, and lead to an enhancement of the tumorigenic state of cells. This type of cancer accounts for about 10% of all colorectal cancers.

More recently, mutations in *endoglin*, a TGFβ accessory protein, or in *ALK-I*, a type I receptor, have been shown to lead to hereditary hemorrhagic telangiectasia (HHT), a disease in which the vascular structure develops abnormally, and where a tendency is seen for the nasal mucosa to rupture frequently [55–57]. Type III receptors are thought to only help present ligand to the receptor, and are thought of as being critical components of the pathway. In vitro experiments have shown that endoglin can only weakly bind any of the known ligands, suggesting that this disease state is caused by the disruption of signaling of an as-yet unidentified ligand.

Müllerian Inhibiting Substance (MIS), a more distant member of the TGFβ superfamily, is required for the normal regression of the Müllerian duct during male development. In instances where MIS is disrupted, this regression fails to occur, and leads to pseudo-hermaphroditism in males. The disease, known as the Müllerian duct syndrome [58,59], can be caused in both humans and mice by mutations in the ligand or its receptor [60].

The intensive research into Smads in the recent past has uncovered a number of connections with various cancers. Smad4, previously called *DPC4* (deleted in pancreatic carcinoma, locus 4), is found to be mutant in over 50% of all pancreatic carcinoma [40]. In addition, it is also mutated in a significant percentage of colorectal carcinoma, and less frequently in other cancers such as prostatic, ovarian, and breast [41–43]. This difference in the frequency of Smad4 mutations in various cancers may reflect a difference in the role of TGFβ signaling in the homeostasis of these tissues. Smad2 has also been found to be mutated in a small number of colorectal cancers [61,62]. From these results, some interesting observations may be made, such as the fact that Smad4 appears to be the only Smad mutated in an overwhelming majority of cases. This may reflect the key nature of the role in TGFβ signaling, or may reflect some aspect of TGFβ function in tissues. The answers to such questions should lead to other exciting observations as well.

5. Strategies in invertebrate TGFβ signaling

5.1. Genetic screens

As described above, screens for novel TGFβ pathway components, based on the mutant phenotypes observed for ligands or receptors in *Drosophila* and *C. elegans*, have resulted in a rapid elucidation of not only these invertebrate developmental signals, but also of their human counterparts. In the context of TGFβ signaling, screens have also been performed in *C. elegans* and *Drosophila*, for genes that mutate to a similar phenotype as known components, and it is expected that the cloning of these genes will further define the mechanism of signaling by identifying additional components. Similar strategies can be adopted for almost any signaling system. For example, Forrester et al. [63] describe screens for genes involved in regulating the migration of developing neurons, wherein they are able to score for those morphological traits that are frequently associated with neuronal migration defects.

In addition to the general strategies outlined above, model systems make available a range of phenotypes and elegant genetic tricks that can be benefited from. For example, over-expression of the *dpp* receptor *thick veins*, causes the mis-patterning of wing or eye tissues, and these phenotypes may be enhanced or ameliorated by removing one copy of a gene that normally functions in the pathway and is necessary for the activity of *thick veins*. These 'modifier' screens have also been used to elucidate other signaling systems, such as the MAP kinase pathways (e.g. see [64]).

One key feature of *C. elegans* biology is that the lineage of each cell in the adult worm is completely known. Every hermaphrodite worm is born with 588 cells that divide to give adults with 959 cells. It is possible to trace the 'family tree' of each of these cells in the adult, and to determine the exact nature of the defect in worms with morphological defects, therefore providing the opportunity to study complex phenotypes at a very basic level (e.g. see [65]). In addition, over- or mis-expression of a gene may cause subtle phenotypic defects that can be used to study aspects of the gene that may not otherwise have been possible. These phenotypes can then be used for the further isolation of partner molecules.

The complete sequencing of the *C. elegans* genome has emphasized the need for the development of new tools for reverse genetics. The quick generation time of the nematode adds to its attraction as a good model organism, and has made the development of these tools simple to accomplish. Two ideas that show great promise are discussed below. First RNA interference (RNA-i) can be used for transient "knock outs", and secondly, reverse genetics using various PCR-based assays can be performed to identify mutations in the gene of interest. One of the popular techniques for reverse genetics utilizes transposable elements [66], and another involves the use of chemical mutagenesis ([67] and G. Moulder, R. Barstead, C. Johnson, personal communications).

5.2. RNA interference (RNA-i)

Although the sequence of the *C. elegans* genome is available, this effort has only yielded the molecular identities of the many loci in the genome. The functions of the proteins encoded by each of those loci, however, still need to be determined. A recently developed technique, referred to as RNA interference (RNA-i), has proven to be an invaluable tool in this regard (Fig. 1). Double-stranded RNA from the coding region of a gene injected into an adult *C. elegans* gonad interferes with endogenous gene activity causing a mutant phenotype specific for that particular RNA [68]. Previously, single stranded antisense RNA has been used, with limited success, to interfere with gene activity. Extremely high concentrations of RNA are needed to reveal even a modest phenotype. Fire et al. [68] have shown that injection of double-stranded RNA at much lower concentrations produces a more severe phenotype, similar to phenotypes observed in animals with null mutations, indicating specificity of interference. The actual mechanism by which double-stranded RNA interferes with gene activity is unclear. This powerful technique has been successfully used to identify the function of a variety of genes in *C. elegans*. It has recently been successfully extended to *Drosophila* as well, where double-stranded RNA was injected into syncitial blastoderm embryos to show that the *frizzled* and *frizzled 2* genes are a part of the *wingless* pathway [69], and to show that the MyoD homolog, *nautilus*, was essential for muscle formation [70]. In addition to a simple demonstration of their role in the pathway, it was shown that the patterning defects were not caused by the injection of either RNA alone, but required the injection of both RNAs together. Such a hypothesis would previously have required the generation of mutants in both genes to be proven. The extension of this technique to other organisms will further increase the value and acceptability of this method for studying gene structure and function.

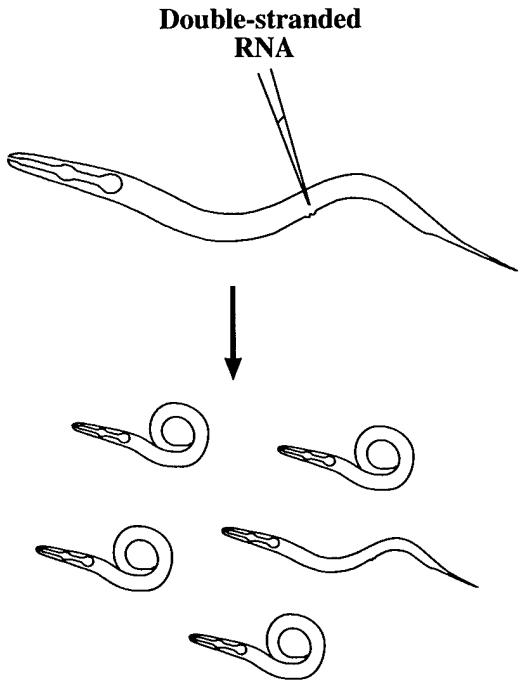

Fig. 1. RNA-interference (RNA-i): a technique used to determine the mutant phenotype of a gene. Double-stranded RNA, comprising both sense and antisense strands, is injected into animals. Interference with wild-type gene function causes a mimicking of the mutant phenotype of the gene. This technique, pioneered in *C. elegans*, is applicable to some other organisms.

5.3. Reverse genetics in C. elegans

Once a gene of interest has been identified, it is necessary to obtain mutants to further characterize its function. One method of generating mutants that has become popular recently, is based upon the use of ethyl methanesulphonate (EMS) or trimethylpsoralen (TMP), chemicals that cause random deletions within the genome at low frequencies. The worms are first mutagenized, then distributed onto agarose plates with an appropriate food supply and grown until the population is dense. Next, DNA is harvested from a subset of each population and analyzed by PCR. PCR is performed using nested primers 1–3 Kb apart. Animals in which the gene of interest has incurred a deletion will yield PCR products smaller than those observed from wild-type worms (Fig. 2). PCR-positive reactions identify the original plate, from which the worms are recovered and re-plated at a lower density. This process is repeated until a single mutant worm is identified. Usually, 1×10^6 mutagenized genomes result in the identification of targeted deletions approximately 50% of the time.

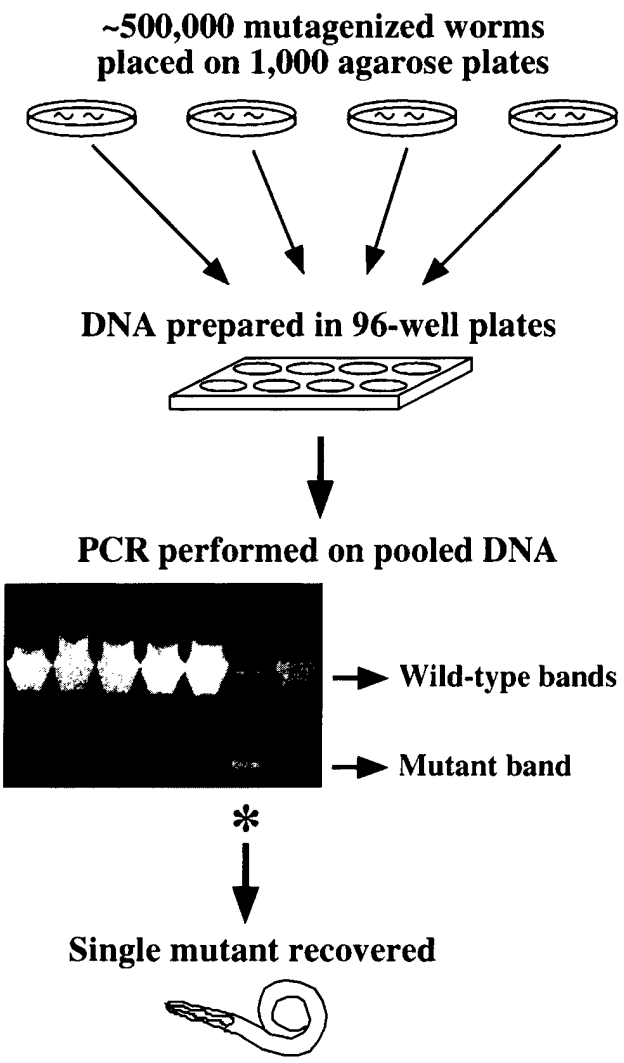

Fig. 2. A novel approach to reverse genetics. A mutagen is used to generate deletions in the genome, and a PCR-based strategy is used to screen for, and isolate, the worm carrying a deletion in the gene of interest.

6. Conclusions

The genetic analysis of TGFβ and other pathways in invertebrates provides an effective way to quickly identify partner molecules, and characterize their roles in signaling. Genetics in mice and zebrafish complement these studies, while biochemical studies in cell culture add functionality.

In summary, screens and related genetic strategies have been very rewarding in the elucidation of the TGFβ signal transduction pathway. These strategies can also be used to study genes involved in a variety of important processes, such as axonal migration and differentiation, neuronal degeneration, apoptosis, and cell cycle. Invertebrate model systems can also be used to study important human disease genes. For example genes involved in Huntington's or Alzheimer's diseases may be studied by expressing wild-type or mutant forms of the protein to produce phenotypes that could then be used in modifier screens. The validity of such approaches has gained widespread acceptance with several biotechnology companies and individual laboratories using genetics in invertebrate organisms to gain insights into the functions of human genes.

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Review

Transforming growth factor β signaling mediators and modulators

Cole M. Zimmerman, Richard W. Padgett *

Waksman Institute, Department of Molecular Biology and Biochemistry, and the Cancer Institute of New Jersey, Rutgers University, Piscataway, NJ 08854-8020, USA

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Abstract

Transforming growth factor β is a multi-functional growth and differentiation factor responsible for regulating many diverse biological processes in both vertebrate and invertebrate species. Among the most dramatic of TGFβ's effects are those associated with specification of cell fates during development and inhibition of cell cycle progression. The core TGFβ signaling pathway has now been described using a synergistic combination of genetic and biochemical approaches. Transmembrane receptors with intrinsic protein serine kinase activity bind ligand in the extracellular milieu and then phosphorylate intracellular proteins known as Smads. Phosphorylated Smads form heterooligomers and translocate into the nucleus where they can modulate transcriptional responses. More recent studies indicate that many other proteins serve as modulators of Smad activity, and utimately define specific cellular responses to TGFβ. Here we describe both the simplistic core TGFβ signaling pathway and the growing number of proteins that impinge on this pathway at the level of Smad function to either enhance or inhibit TGFβ responses

Keywords: Receptor serine kinases; Signal transduction; Smads; Transcription factors; Tumor suppressors

1. Introduction

The transforming growth factors β (TGFβs) are extracellular peptides that regulate many diverse biological processes. TGFβs were initially identified and named based on the observation that they stimulate cellular proliferation of fibroblasts in culture. However, it was shown later that these molecules are among the most potent known inhibitors of cellular growth and division in many other cell types. In fact, TGFβs are now the most well-studied and widely known family of growth inhibitory proteins; molecular components at each step in the signaling pathway have been identified as tumor suppressors. TGFβs also have roles in regulating cellular differentiation, adhesion, motility, and death (Massagué, 1990; Roberts and Sporn, 1990, 1993; Alexandrow and Moses, 1995).

Many other peptide growth factors are structurally related to TGFβ and are, therefore, members of the TGFβ-superfamily of ligands. These include the activins, the bone morphogenetic proteins (BMPs), and the growth and differentiation factors (GDFs) among others. In addition, homologues have been identified in *Drosophila melanogaster* (*dpp*, *Gbb/60A*, *screw*, and *dActivin*), and in *Caenorhabditis elegans* (*daf-7*, *dbl-1*, *unc-129*, and *tig-2*). These factors have essential roles in embryonic patterning, organogenesis, immune system function, and tissue homeostasis (Kingsley, 1994; Raftery and Sutherland, 1999; Patterson and Padgett, 2000).

Remarkable progress has been made over the last several years in elucidating the molecular mechanisms by which cells translate the presence of TGFβ in the

Abbreviations: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CBP, CREB-binding protein; dpp, decapentaplegic; EGF, epidermal growth factor; FAST, Forkhead activin signal transducer; Gbb, glass bottom-boat; GDF, growth and differentiation factor; HDAC, histone deacetylase; HGF, hepatocyte growth factor; Mad, mothers against dpp; MH1, Mad homology domain 1; MH2, Mad homology domain 2; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; RSK, receptor serine kinase; SARA, Smad anchor for receptor activation; TGF, transforming growth factor.

* Corresponding author. Tel.: +1-732-445-0251; fax: +1-732-445-5735.
E-mail address: padgett@waksman.rutgers.edu (R.W. Padgett)

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extracellular milieu into discrete changes in gene expression. A combination of biochemical, cell biological, and genetic analyses were not only exploited, but were absolutely necessary, in developing the current models for TGFβ signal transduction. These studies have defined a novel signaling strategy in which ligand-regulated transmembrane receptor serine kinases (RSKs) modulate the activity of transcriptional regulators called Smads. This work, and the resulting models, has recently been well-reviewed both comprehensively (Massagué, 1998), and with specific focus on the role of these factors in the process of development (Whitman, 1998), and in genetic model organisms (Raftery and Sutherland, 1999; Patterson and Padgett, 2000). Therefore, this review will describe the core TGFβ signaling pathway in brief historical context, and then focus on recent work that demonstrates the importance of co-factors and cross-talk among different signaling pathways in modulating TGFβ signal transduction.

2. TGFβ signal transduction

The first milestone in the molecular analysis of TGFβ signaling pathways was the identification and cloning of a type II receptor for activin, and soon after for TGFβ (Mathews and Vale, 1991; Lin et al., 1992). These discoveries were of special importance because each of the receptors contained an intracellular protein kinase domain with predicted serine/threonine specificity. This observation suggested the existence of a novel intracellular signaling strategy because all previously characterized receptor protein kinases were tyrosine specific. Subsequent cloning of type II receptors for other members of this ligand superfamily, including homologues in *Drosophila* and *C. elegans*, proved that these receptors are also members of a highly conserved protein family, the type II RSKs (Georgi et al., 1990; Childs et al., 1993; Baarends et al., 1994; Kawabata et al., 1995).

2.1. RSKs

Analysis of a panel of mutagenized mink lung epithelial cell lines strongly suggested that another cell surface binding protein for TGFβ played an essential role in signal transduction (Wrana et al., 1992, 1994). These proteins were cloned using PCR strategies based on the conserved sequences within the kinase domains of the type II receptors. The most comprehensive description of this group of related molecules designated them activin receptor-like kinases (ALKs); therefore, this nomenclature has been used until functional names could be assigned (Franzén et al., 1993; ten Dijke et al., 1993, 1994). Although these proteins share homology with type II RSKs, they clearly belong to a distinct family and have unique structural features, which now define type I RSKs. Most notable is the presence of a conserved glycine- and serine-rich sequence motif (GS domain) between the transmembrane and kinase domains that contributes to the activation state of the receptors.

Demonstration that type II and type I RSKs form ligand-induced heteromeric complexes at the cell surface was the first step in defining the mechanism of RSK activation and signal initiation (Wrana et al., 1992). Subsequently, unidirectional phosphorylation of the type I RSKs at the GS domain by type II RSKs was shown to be required for signal propagation (Wrana et al., 1994). The fortuitous identification of an activating point mutation (T204D in ALK5, T206E in ALK4) in type I receptors proved very useful in confirming the order of events in RSK activation (Wieser et al., 1995; Willis et al., 1996). For example, these activated type I receptors induce ligand-independent signaling in the absence of functional type II RSKs, and in the presence of dominant-negative type II RSK mutants (Wieser et al., 1995; Attisano et al., 1996; Willis et al., 1996). Therefore, type I receptors are downstream of type II receptors in RSK-mediated signaling. (This stands in sharp contrast to the mechanism of activation of receptor tyrosine kinases, in which ligand induces homomeric receptor complexes, and bidirectional transphosphorylation of receptor subunits initiates signaling.)

Therefore, the following model for initiation of RSK-mediated signaling has been proposed. A TGFβ-family ligand induces heteromeric RSK complex formation at the cell surface. Type II RSKs phosphorylate type I RSKs at serines within the GS domain, which results in activation of the type I kinase and subsequent phosphorylation of intracellular signal mediators.

Because there appear to be many more ligands within the TGFβ-superfamily than identified RSKs, and because some overlap has been observed in ligand binding by RSKs, the question of ligand-receptor specificity is still being investigated. Initially, type II receptors were thought to determine the ligand binding specificity of the RSK complex; TβRII, for example, binds only to TGFβ (Lin et al., 1992). However, other type II RSKs bind to multiple ligands in combination with different type I receptors. ActRII binds to activin in combination with ALK4, but also binds both BMP2 and BMP7 in the presence of BMP type I receptors (Cárcamo et al., 1994; Yamashita et al., 1995; Willis et al., 1996). In addition, ActRIIs have been shown to have functional roles in mediating both activin and BMP signals in *Xenopus* (Chang et al., 1997).

Recently, the combination of genetic analysis and completion of the genome sequence of *C. elegans* has provided significant insight regarding this issue. The key observation was that worms mutant for type I RSK *daf-1* displayed only a subset of the mutant phenotypes observed when the type II RSK *daf-4* was mutated (Georgi et al., 1990; Estevez et al., 1993). This suggested

that *daf-4*-mediated signals in addition to those propagated via *daf-1*. Would these additional signals be independent of type I receptors, or could *daf-4* pair with other type I receptors in addition to *daf-1*? The answer came with the cloning and analysis of *sma-6*, a type I RSK in worms that accounted for all of the *daf-4* phenotypes not associated with *daf-1* (Krishna et al., 1999). This proved that a single type II RSK could function in parallel pathways with different type I RSKs in worms. Furthermore, genome sequencing indicates with near certainty that these are the only available RSKs in *C. elegans*. Given the high degree of conservation among TGFβ pathway components between nematodes and vertebrates, it is quite likely that type II RSKs function similarly in mammalian systems.

2.2. Smad proteins

While the mechanism of signal initiation and ligand-receptor specificity was being investigated, an intensive search was also being carried out to identify and clone molecular mediators of RSK signaling. Because the predicted substrate specificity of the RSKs differs from that of the well-characterized receptor tyrosine kinases, the mediators of RSK signaling were expected to be novel. Genetic analysis in both *Drosophila* and *C. elegans* yielded the first bona fide members of this signaling pathway, which are now known as Smads (Fig. 1).

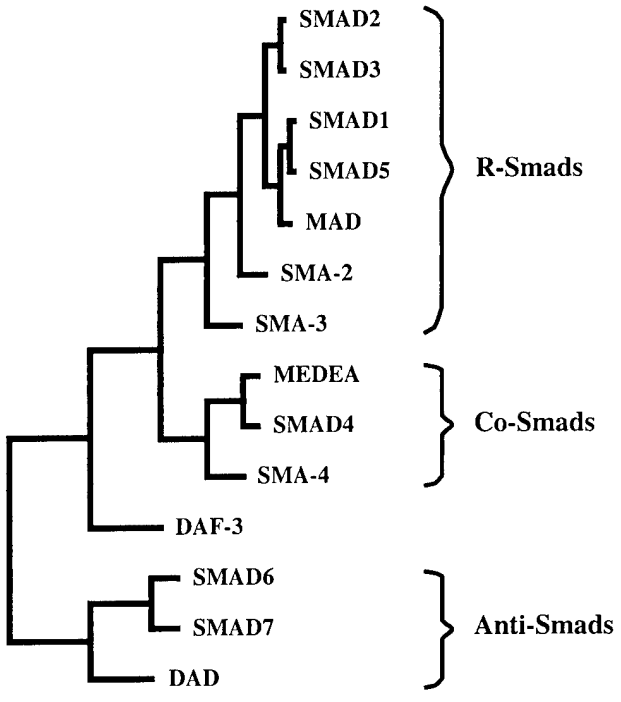

Fig. 1. Phylogenetic relationships among Smad proteins. Smad proteins fall into three classes based on sequence homology, which correlate with their observed functional characteristics. The only exception is DAF-3; its sequence more closely resembles that of Co-Smads, and its function is divergent.

The observation that different mutations in the *Drosophila* BMP homologue, *dpp*, result in phenotypes of graded severity was exploited in screens designed to isolate modifiers of weak *dpp* alleles. Two maternal effect enhancers of a weak *dpp* phenotype were isolated and named *Mothers against dpp* (*Mad*) and *Medea* (Raftery et al., 1995; Sekelsky et al., 1995). Molecular cloning and sequencing of *Mad* indicated that the gene encoded a protein with no previously described functional motifs, although the sequence was homologous to three predicted open reading frames in the *C. elegans* genome. Subsequent cloning of the worm genes (*sma-2*, *-3*, and *-4*) based on shared mutant phenotypes of small body size and male tail defects with type II RSK *daf-4* confirmed the presence of these homologous proteins, and demonstrated that multiple Smads are required in this signaling pathway (Savage et al., 1996). Although this protein family does not share homology with other known proteins, each member does contain highly conserved N- and C-terminal domains separated by a linker of variable length and sequence. The N-terminal domain has been called Mad-homology domain 1 (MH1), and the C-terminal domain has been designated Mad-homology domain 2 (MH2) (Fig. 2). Because these proteins lacked identifiable hydrophobic signal sequences or transmembrane domains, and because genetic mosaic analysis indicated that *sma-2* is required cell autonomously in the same cells as *daf-4*, they were proposed to function as intracellular mediators of RSK signaling (Savage et al., 1996).

Following the genetic identification of these molecules, several vertebrate homologues were rapidly cloned (Fig. 1). One of the first human *Mad* homologues, *DPC4*/Smad4, was cloned independently in a search for genes homozygously deleted in pancreatic carcinoma; this observation led to the suggestion that these genes may function as tumor suppressors (Hahn et al., 1996). In addition, a murine homologue was cloned based on its functional capacity to change cell fate in *Xenopus* from ectoderm into mesoderm (Baker and Harland, 1996). A unified nomenclature was soon adopted in which the original gene names from *C. elegans* (*Sma*) and *Drosophila* (*Mad*) were combined; the proteins are now known as Smads (Derynck et al., 1996).

Functional analysis of Smads in *Drosophila* and in *Xenopus* further elucidated their role in mediating RSK signals downstream of receptors. In addition to the observations made in worms, genetic evidence for placing Smads downstream of receptors was provided by the demonstration that *Mad* mutants can suppress the effects of dominant activating mutations in the type I RSK *thickveins* (Hoodless et al., 1996; Wiersdorff et al., 1996). Analysis of Smad function in *Xenopus* animal cap explants demonstrated that overexpression of Smad1 resulted in ventralization reminiscent of treatment with BMPs; conversely, overexpression of Smad2 resulted in

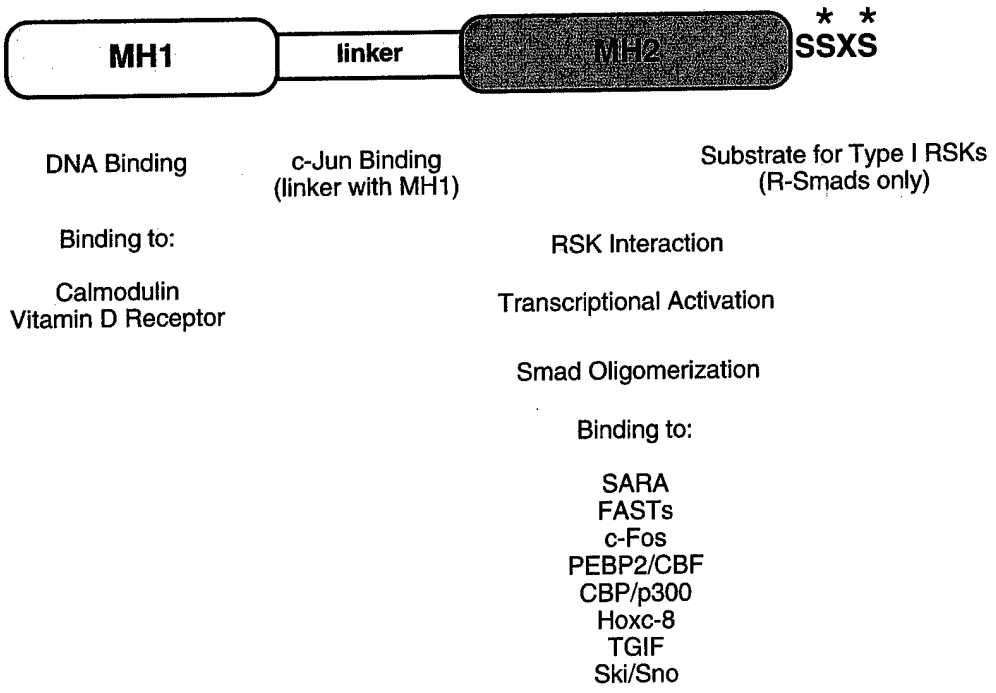

Fig. 2. Structural and functional characteristics of the Smad proteins. Highly conserved *Mad* homology domains 1 and 2 are shown in schematic form. Stars mark the type I RSK phosphorylation sites within the R-Smad C-terminal sequence. Functional roles of each domain are listed below the schematic representation.

activin-like mesodermal dorsalization (Baker and Harland, 1996; Graff et al., 1996). Observation of similar effects for Smad 1 and Smad2 in the presence of dominant-negative receptors provides additional evidence that Smads act downstream of receptors (Graff et al., 1996). These results not only demonstrated that Smads mediate TGFβ-family signals, but also provided the first evidence that different Smads are responsible for transducing different ligand-specific responses (Table 1).

Rapid progress in the biochemical analysis of Smad proteins followed their identification and biological characterization. The first question to be addressed, based on the predicted enzymatic activity of the receptors, was whether Smads become phosphorylated in response to receptor activation. In fact, one class of Smads is phosphorylated directly by type I RSKs on extreme C-terminal serines within the terminal sequence motif SSXS (Hoodless et al., 1996; Macias-Silva et al., 1996; Souchelnytskyi et al., 1996; Kretzschmar et al., 1997b). This group of Smads is known as the 'receptor-regulated' class (R-Smads) and, when phosphorylated, physically associates with another class of Smads known as the 'common' Smads (Co-Smads) (Lagna et al., 1996). Co-Smads lack the SSXS motif and are not phosphorylated by RSKs in response to ligand (Zhang et al., 1996). Therefore, ligand-induced phosphorylation of R-Smads results in heteromeric complex formation with Co-Smads in the cytoplasm.

Specificity between TGFβ-family signaling pathways is also maintained at the level of R-Smad phosphorylation (Table 1). Smads 1, 5, and 8 are phosphorylated only by BMP type I receptors (Hoodless et al., 1996),

Table 1
Molecular mediators of TGFβ-family signaling pathways. Known components of selected ligand-initiated cascades are listed. This is not a comprehensive list, but rather an ordering of the most well-characterized pathways

Ligand	Type II RSK	Type I RSK	R-Smad	Co-Smad
TGFβ	TβRII	ALK5	Smad2 Smad3	Smad4
Activin	ActRII ActRIIB	ALK4	Smad2 Smad3	Smad4
BMP 2/4	BMPRII ActRII	ALK3 ALK6	Smad1 Smad5 Smad8	Smad4
dpp	*punt*	*thickveins* *saxophone*	*Mad*	*Medea*
dActivin	*punt*	*baboon*	*dSmad2*	*Medea*
daf-7	*daf-4*	*daf-1*	*daf-8* *daf-14*	*daf-3* (?)[a]
dbl-1	*daf-4*	*sma-6*	*sma-2* *sma-3*	*sma-4*

[a] *Daf-3* is structurally similar to the Co-Smads, but functions differently as described in the text.

whereas Smads 2 and 3 are phosphorylated specifically by TGFβ and activin type I receptors (Eppert et al., 1996; Macias-Silva et al., 1996; Zhang et al., 1996). This specificity is the result of conserved recognition sequences within both the type I RSKs and the R-Smads (Feng and Derynck, 1997; Chen et al., 1998; Lo et al., 1998).

The next clue as to the possible function of these unique signal mediators was the observation that RSK signaling resulted in nuclear accumulation of phosphorylated R-Smad and Co-Smad heteromers (Macias-Silva et al., 1996; Liu et al., 1997; Nakao et al., 1997b). Analysis using Smad4-deficient cell-lines indicated that association with Smad4 is not required for nuclear translocation of R-Smads (Liu et al., 1997). However, Co-Smads do not accumulate in the nucleus without a phosphorylated partner, demonstrating that Co-Smads require an activated R-Smad for nuclear entry (Liu et al., 1997; Das et al., 1998). Therefore, if RSK activation and R-Smad phosphorylation results in nuclear accumulation of heteromeric Smad complexes, then it was reasonable to expect that Smads would have some function in the nucleus.

Once again, a combination of experimental systems was exploited to demonstrate a functional role for Smads inside the nucleus. First, the MH2 domains of both Smad1 and Smad4 were shown to activate transcription in cultured cells when tethered to DNA as GAL4-DNA binding domain fusions (Liu et al., 1996). In addition, full-length Smad1 exhibited BMP-induced transcriptional activity in the same assay. These observations not only supported the idea that Smad proteins could be direct transcriptional regulators, but also suggested that Smads may be the only required mediators of TGFβ-family signals between RSKs and the nucleus.

The hypothesis that Smads might be physiological

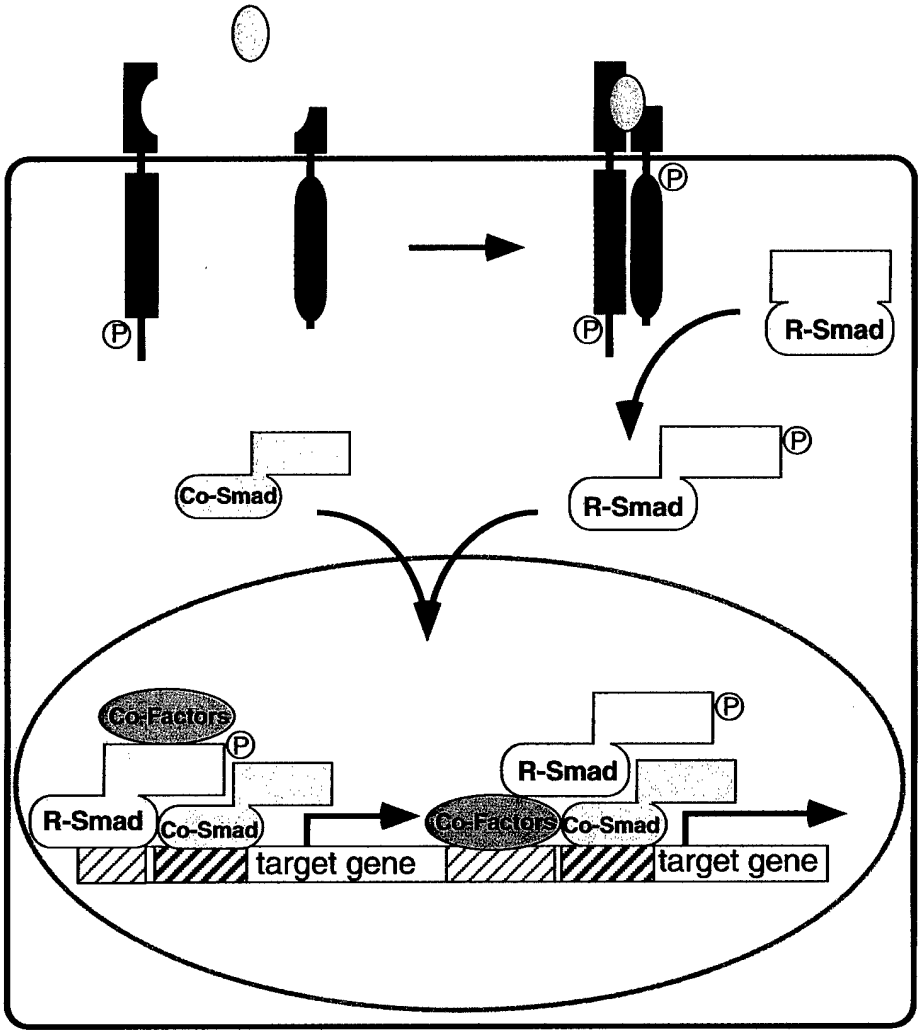

Fig. 3. Model for RSK signal transduction from the membrane to the nucleus. Ligand-bound phosphorylated type I RSKs (I) phosphorylate R-Smads, which associate with the Co-Smads and move into the nucleus. TGFβ-family target genes are induced either by direct binding of Smad complexes to the promoter, or by association of Smad complexes with other DNA binding proteins, such as FAST1. This represents a minimal 'core' TGFβ signaling pathway.

regulators of transcriptional responses was supported by the finding that Smad2 is one member of an activin-induced DNA-binding complex on the promoter of a *Xenopus* immediate–early response gene, *Mix.2* (Chen et al., 1996). The primary DNA binding protein within this activin-dependent complex was shown to be a novel winged-helix transcription factor called Forkhead activin signal transducer (FAST1); Smad4 was also identified as a member of the complex (Chen et al., 1997). Furthermore, TGFβ-induced transcriptional activation was observed in mammalian cells using a reporter gene controlled by the activin responsive element (ARE) from the *Mix.2* promoter, but only in the presence of exogenous FAST1 (Weisberg et al., 1998; Zhou et al., 1998). These findings confirmed that Smads can function as transcriptional regulators within a ligand-induced sequence-specific DNA binding complex.

Proof that Smads function as transcriptional regulators was provided by the observations that the MH1 domain of *Drosophila Mad* can bind directly to specific DNA sequences in the quadrant enhancer of the *vestigial* (*vg*) gene, and that this binding is required for *dpp*-dependent *vg* transcription in vivo (Kim et al., 1997). Subsequently, mammalian Smads 3 and 4 were also shown to bind DNA in vitro. Optimal Smad binding sequences were identified by iterative oligonucleotide selection, and tandemly repeated copies of this sequence [Smad binding element (SBE)] are sufficient to confer TGFβ-responsiveness on a minimal promoter in cell culture (Zawel et al., 1998). The optimal SBE is palindromic (GTCTAGAC) and is found in the promoters of many TGFβ-responsive genes with only minor modifications. Interestingly, Smad2 does not bind to DNA like Smads 3 and 4 due to additional amino acids near the DNA-binding domain present only in Smad2 (Dennler et al., 1999).

Based on these observations and others, discrete functions have been ascribed to the highly conserved Smad MH1 and MH2 domains (Fig. 2). Additionally, the crystal structures of isolated MH1 and MH2 domains have been solved, allowing functional descriptions to be interpreted in light of structural observations (Shi et al., 1997, 1998). The MH1 domain is primarily responsible for binding to Smad-specific DNA-binding elements. The MH2 domain, however, mediates a host of protein–protein interactions, functions as a transcriptional activation motif, and in R-Smads serves as a substrate for type I RSKs.

In summary, Smad proteins mediate RSK-initiated signals from the cell membrane into the nucleus. RSKs are activated via ligand-induced complex formation between type II and type I RSKs. Type II receptor-dependent phosphorylation of the type I receptor at the GS domain activates the type I receptor kinase domain, which then phosphorylates R-Smads on C-terminal serine residues. Phosphorylated R-Smads associate with Co-Smads in the cytoplasm and translocate into the nucleus where transcriptional activation occurs via direct DNA binding, or by association of the Smad complex with other DNA-binding proteins (Fig. 3).

3. Modulation of TGFβ signaling

More recent studies have been directed at understanding intracellular regulation of the Smad pathway, and have focused on how a host of Smad binding proteins modulate TGFβ signaling. In addition, other signaling pathways have now been shown to impinge on the Smad pathway and can either potentiate or inhibit Smad mediated signals. The initially striking simplicity of the core RSK/Smad pathway is quickly giving way to a much more complex view of cellular regulation by TGFβ.

Positive regulators of TGFβ signals include both an upstream accessory protein, known as Smad anchor for receptor activation (SARA), and several downstream effectors that function as either general or tissue-specific transcriptional regulators.

3.1. Positive modulators and effectors

3.1.1. SARA

The first direct cytoplasmic Smad accessory protein to be discovered was called SARA, based on the predicted function of this R-Smad binding protein (Tsukazaki et al., 1998). SARA was identified in a screen for Smad2 MH2 domain interacting proteins, but also appears to bind to Smad3. Conversely, SARA does not bind to Smad 1, nor does it bind to Smad4. Although Smad4 is not a substrate for RSKs and, therefore, may not require a SARA-like partner, the current prediction is that homologous SARA family proteins do exist that bind specifically to R-Smads 1, 5, and 8 and mediate BMP-specific responses. In fact, SARA homologues are present in both *Drosophila* and *C. elegans* genomes, which provides genetic tools for analyzing SARA functions.

In addition to the Smad-binding domain, SARA also contains a sequence motif known as the 'FYVE' domain (Tsukazaki et al., 1998). This is a double zinc finger structure that has been shown to bind phophatidylinositol-3-phosphate and mediate membrane association in yeast and mammalian systems. Direct membrane localization of SARA via the 'FYVE' domain has not been demonstrated, although SARA is present at the membrane and does associate with RSK complexes independent of Smad binding. Further experiments are needed to prove the role of the 'FYVE' domain within SARA.

SARA binds unphosphorylated Smad2, which is released upon ligand-induced phosphorylation and then associates with Smad4 in the cytoplasm; Smad2–Smad4

and Smad2–SARA complexes appear to be mutually exclusive (Tsukazaki et al., 1998). Furthermore, mutations introduced into the 'FYVE' domain of SARA result in different patterns of Smad2 subcellular localization, and also interfere with TGFβ-dependent transcriptional activation. These observations have led to the following model for SARA function. SARA resides at the plasma membrane by virtue of its lipid-binding 'FYVE' domain. Unphosphorylated Smad2 is recognized and concentrated by the SARA Smad-binding domain at the membrane, in close proximity to RSK complexes. RSK activation results in Smad2 phosphorylation, dissociation of SARA from Smad2, and formation of transcriptionally active Smad2–Smad4 heterocomplexes.

The initial discovery and characterization of SARA raises some interesting questions for future investigations. First, how important is SARA's role in TGFβ signaling? Is SARA absolutely required, or is it an enhancer of signaling efficiency in specific cell-types or tissues? Is there a large family of RSK-specific SARA proteins that interact with specific Smads, or can some TGFβ signals be transmitted independently of these adapters? Does SARA function as a scaffolding for assembly of a larger protein complex, and what other protein members might participate? The genetic and biochemical experiments to elucidate these issues are certainly well under way.

3.1.2. *FAST proteins*

The first nuclear Smad-binding protein was identified as part of an activin-inducible DNA-binding complex in *Xenopus* (Chen et al., 1996). The protein was labeled FAST1, and is a member of the large and diverse winged-helix family of transcription factors. This discovery not only confirmed a biological role for Smads inside the nucleus, but was also the initial observation that the overwhelming majority of direct Smad binding proteins are transcriptional regulators.

As described, FAST1 was identified and cloned based on its activin-induced capacity to bind directly to the promoter of the activin-regulated gene, *Mix.2* (Chen et al., 1996). Subsequently, Smads 2, 3, and 4 were also shown to be present in the complex; Smad2 associates directly with FAST1, whereas Smad4 probably does not bind FAST1 but contributes additional DNA binding specificity and transcriptional activation functions to the complex (Chen et al., 1997; Labbe et al., 1998). It is not known whether FAST1 has any transcriptional activity of its own.

FAST1 homologues have been identified in several species, including mouse and human. Studies using human FAST1 have defined an optimal DNA-binding sequence for these factors (TGT G/T T/G ATT) that is also present in the ARE of the *Xenopus Mix.2* gene. In addition, the importance of adjacent Smad binding sequences was demonstrated in the context of transcriptional activation by the human FAST1 complex (Zhou et al., 1998). Cloning and analysis of a murine homologue, FAST2, indicates that transcriptional regulation by these factors may be more complex. Like the FAST1 complex on the *Mix.2* promoter, FAST2–Smad2 complexes activate transcription from the *goosecoid* (*gsc*) promoter in cultured cells. However, FAST2–Smad3 complexes inhibit transcription from the same promoter (Labbe et al., 1998). This represents the first suggestion that Smad2 and Smad3 may have opposing roles in selected systems.

Whether FAST-like molecules also mediate BMP-initiated signals remains to be determined. To date, no BMP-specific FAST proteins, which would be expected to interact with R-Smads 1, 5, and 8, have been described. However, the Forkhead family of transcription factors is large and homologues are present in *Drosophila* and *C. elegans*. The sheer number of Forkhead family proteins and the limited homology outside the DNA-binding domain make the sequence-based prediction of FAST–Smad combinations quite difficult. Genetic analysis, however, may provide clues as to the identity of any functional FAST–Smad partners.

3.1.3. *AP-1 family members*

Another group of transcriptional activators with which Smads have now been shown to interact physically are the AP-1 family members, c-Jun and c-Fos. These AP-1 factors have long been suspected to play a role in TGFβ gene regulation based on the requirement of intact AP-1 binding sites in the promoters of several TGFβ-responsive genes, including PAI-1. c-Jun binds directly to amino acids within the variable linker region of Smad3, whereas c-Fos binds within the conserved MH2 domain of Smad3. This ligand-induced complex consisting of Smad3, Smad4, c-Jun, and c-Fos then binds to either overlapping or adjacent AP-1 and Smad binding sequences in target promoters. Transcriptional activation resulting from these cooperative interactions has been demonstrated using the classical TGFβ-responsive promoter PAI-1 (Zhang et al., 1998; Wong et al., 1999).

3.1.4. *TFE3*

TFE3 is a basic helix–loop–helix transcription factor that has also been shown to cooperate with Smads 3 and 4 to induce PAI-1 transcription in response to TGFβ. All three proteins can bind directly to DNA sequence elements within the PAI-1 promoter, and contribute to maximal TGFβ-dependent transcriptional activation (Hua et al., 1998). Recent data demonstrate that Smad3 binds directly to TFE3, and that this interaction is enhanced when the C-terminal serines of Smad3 are phosphorylated (Hua et al., 1999). The DNA bind-

ing sites employed by TFE3 in cooperation with Smads are distinct from those used by AP-1 family factors, indicating another apparent mechanism for generating specificity at Smad-responsive promoters. Whether TFE3 interacts with AP-1 family members, either functionally or physically, via Smads on this promoter has not been reported.

3.1.5. Vitamin D receptor

Another transcriptional regulator that specifically interacts with Smad3 is the vitamin D receptor (VDR). VDR is a vitamin D-regulated nuclear receptor, similar to the steroid-hormone receptors, that functions with co-activators of the steroid receptor coactivator 1/transcriptional intermediary factor 2 (SRC-1/TIF2) family. This VDR–Smad3 interaction is unique in that VDR appears to bind the MH1 domain of Smad3; most other Smad protein–protein interactions are mediated via the Smad MH2 domain. Additionally, activation of TGFβ signaling results in Smad3-mediated enhancement of VDR-dependent transcription (Yanagi et al., 1999; Yanagisawa et al., 1999). Therefore, Smad3 transcriptional effects are not limited to direct TGFβ target genes, but are also manifest in co-operative enhancement of other signal-regulated pathways.

3.1.6. Polyoma virus enhancer binding protein 2/core binding factor (PEBP2/CBF)

A more recently identified transcription factor that partners with R-Smads to effect changes in gene expression is the PEBP2/CBF, which forms complexes in vivo with both TGFβ and BMP-regulated R-Smads. Interaction with Smad3 requires the MH2 domain, and results in transcriptional activation of the germline Ig Cα promoter. In addition, binding sites for both Smads and PEBP2/CBF are absolutely required for transcriptional regulation. The mechanism of transcriptional control for PEBP2/CBF and Smads may be unique in that PEBP2/CBF appears to interact with all of the R-Smads, and that Smad binding sites appear to play a more direct and necessary role in regulation of the Ig Cα promoter (Hanai et al., 1999).

3.1.7. Olf-1/EBF associated zinc finger (OAZ)

A search for transcription factors that regulate the BMP-responsive gene Xvent-2 in *Xenopus* identified a protein known as OAZ, which was previously identified as a transcription factor involved in the development of rat olfactory epithelium and pre-B lymphocytes. OAZ binds to the BMP-responsive element of the Xvent-2 promoter, and mediates induction of an artificial reporter gene under the control of the BRE (BMP-responsive-element). Furthermore, OAZ can be found in BMP-dependent complexes with both Smad4 and Smad1. The interaction with Smad1 is direct via the MH2 domain, whereas the interaction with Smad 4 is not direct and probably results from the ligand-dependent association of Smad1 with Smad4 (Hata et al., 2000). The identification of a role for OAZ in this context supports the hypothesis that tissue-specific transcription factors may play a prominent role in specifying the cellular effects of Smads, which are ubiquitous TGFβ signal mediators.

3.1.8. MSG1

In addition to acting as TGFβ-regulated transcriptional co-factors, Smads also bind to more ubiquitous co-activators and recruit these accessory proteins to specific promoters. For example, MSG1 is a small nuclear protein with strong transcriptional activity that lacks any identifiable DNA-binding capacity. MSG1 is thought to be important in differentiation and development, although its specific role has not been well-defined. MSG1 was shown by two-hybrid analysis to interact with the MH2 domain of Smad4. TGFβ-dependent transactivation of a GAL4 DNA-binding domain–Smad4 fusion was significantly enhanced by co-expression of MSG 1. This transcriptional enhancement was suppressed by overexpression of the Smad4 MH2 domain, which could sequester MSG1 away from the GAL4–Smad4 fusion (Shioda et al., 1998). Although these results rely on an artificial transcriptional system, they do suggest that Smads can tether more potent transcriptional activators to target genes.

3.1.9. CREB-binding protein (CBP)/p300

More physiologically relevant results supporting this idea are provided by studies of the well-known transcriptional co-activators CBP and p300. These co-activators enhance transactivation by a host of unrelated transcription factors through two different but related mechanisms. First, CBP/p300 brings sequence-specific transcription factor complexes into close proximity to the basal transcriptional machinery, which is required for transactivation. Second, CBP/p300 has intrinsic histone acetylase activity, which modifies chromatin structure and can directly effect the availability of specific promoter sequences to binding proteins.

A number of laboratories have now shown that CBP/p300 binds directly to the phosphorylated MH2 domain of both Smad2 and Smad3. Furthermore, exogenous CBP/p300 augments TGFβ-induced transactivation in a Smad4-dependent manner. Enhancement of Smad transcriptional activity has been shown for a variety of TGFβ-responsive reporter genes in cell culture, including p3TP-Lux-, SBE-Luc-, PAI1-Luc-, and GAL4-based systems. The specificity and requirement for CBP/p300 in these assays was confirmed by showing that the adenoviral protein E1A, an inhibitor of CBP/p300 transcriptional enhancement, blocks the observed increases in Smad-dependent transactivation in the presence of this co-activator (Nishihara et al., 1999). Therefore, Smad proteins provide a critical func-

tion to cell type-specific transcriptional complexes by linking these complexes to the general transcription apparatus through ubiquitous co-activators such as a CBP/p300 (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Topper et al., 1998).

3.1.10. Homeodomain proteins

One other mechanism of transcriptional activation by Smad1 has recently been reported based on the observation that Smad1 physically associates with one of the homeobox DNA-binding proteins, Hoxc-8. BMP signaling leads to phosphorylation of Smad1, association of Smad1 with Hoxc-8, and transcriptional induction of a Hoxc-8 target gene, osteopontin. However, BMP-induced Smad1–Hoxc-8 complex formation was shown to prevent Hoxc-8 from binding to its target site in the osteopontin promoter. Therefore, Hoxc-8 appears to be a transcriptional repressor, which is inhibited by binding to Smad1 (Shi et al., 1999). This represents the first evidence that Smads may allow for transcriptional activation indirectly by physically interfering with functional transcriptional repressors.

The idea that TGFβ-family signals may de-repress selected genes via Smad-mediated events is also suggested by studies of the *daf-3* Smad in *C. elegans*. Genetic analyses clearly demonstrate that *daf-3* is antagonized by RSKs and other Smads in *C. elegans*. In the absence of TGFβ signaling, *daf-3* causes worms to enter an alternative developmental stage known as dauer; accordingly, RSK and Smad mutants undergo *daf-3*-induced dauer formation [Patterson et al., 1997; reviewed in Patterson and Padgett (2000)]. An intact activated TGFβ signaling pathway, however, blocks *daf-3*-induced dauer arrest. Furthermore, *daf-3* has been shown to bind directly to DNA and to repress transcription from a pharynx-specific promoter element. In *daf-3* mutants, repression is relieved and pharynx-specific reporter expression is restored (Thatcher et al., 1999). Whether there are additional protein partners in a *daf-3* complex is not yet known.

However, there is now clear precedent for future investigations of transcriptional de-repression mediated through TGFβ-initiated Smad-dependent processes. There are also several other transcription factors that may cooperate with Smads to induce gene transcription, but which have not been shown to interact physically with Smads. For example, SP1-family transcription factors have been implicated in TGFβ signal mediation by virtue of the requirement of intact SP1-binding sites in the p15 and p21 CKI promoters for TGFβ-responsiveness (Datto et al., 1995; Moustakas and Kardassis, 1998). Similarly, the *Drosophila* protein SCHNURRI plays some role in *dpp* signaling and gene regulation, but confirmation that it cooperates with Smads has been elusive (Arora et al., 1995; Greider et al., 1995).

These many examples clearly demonstrate that protein partners for TGFβ-regulated Smad proteins are indispensable for appropriate activation and modulation of Smad target genes. Furthermore, it appears that Smads regulate transcriptional responses in a number of related ways. Smads can bind to other sequence-specific DNA-binding proteins and provide a transactivation function. Smads can bind DNA at defined target sequences and tether other transcriptional co-activators to these promoters. Finally, Smads may be able to activate transcription by either direct or indirect de-repression of silenced genes. In any case, Smad proteins are clearly very versatile TGFβ-sensitive transcriptional modulators.

3.2. Negative modulators and inhibitors

In addition to Smad co-factors that positively regulate or enhance transcriptional outputs, a number of other proteins have been discovered that attenuate TGFβ signaling by interfering with Smad functions. These negatively acting Smad partners are required to prevent the inappropriate activation of TGFβ pathways, and to turn off the pathway following normal activation.

Although there are many ways by which TGFβ signals are inhibited, including the extracellular sequestration of ligands by proteins such as follistatin, chordin, and noggin, and the intracellular regulation of RSK activation by FKBP12, we will focus primarily on direct Smad regulators. The molecules highlighted here function at different levels within the signaling pathway: Anti-Smads competitively inhibit Smad functions, transcriptional repressors block activation of Smad target genes in the nucleus, and ubiquitin-dependent proteases degrade Smads and attenuate TGFβ signaling. Interestingly, the most logical of potential Smad inhibitors based on activation by RSK phosphorylation — a phosphatase — has not yet been discovered.

3.2.1. Anti-Smads

The first Smad inhibitors to be described were another class of divergent Smad proteins known simply as inhibitory Smads or Anti-Smads. These Smads contain divergent MH1 domains but share the conserved MH2 domain with R-Smads and Co-Smads; however, they lack C-terminal phosphorylation sites. Smads 6 and 7, along with a *Drosophila* homologue, *Daughters against dpp* (*Dad*), function as negative feedback inhibitors of TGFβ-family signaling. Anti-Smads are transcriptionally induced by the ligands that they ultimately inhibit, as well as by shear stress in vascular endothelium (Nakao et al., 1997a; Topper et al., 1997; Tsuneizumi et al., 1997; Nakayama et al., 1998).

Two mechanisms have been proposed for how Anti-Smads exert their inhibitory function. Each of the Anti-Smads can bind to RSK complexes and prevent phosphorylation of R-Smads (Hayashi et al., 1997; Inamura

et al., 1997; Nakao et al., 1997a; Tsuneizumi et al., 1997). This, in turn, prevents Smad heteromer formation and nuclear accumulation and explains the non-specific inhibition observed when Anti-Smads are overexpressed in cell culture. An alternate mechanism that accounts for the specific inhibition of BMP signaling by Smad6 is based on the observation that Smad6, when expressed at lower levels, does not prevent phosphorylation of Smad1, but instead competes with Smad4 for binding to phosphorylated Smad1 (Hata et al., 1998). Additional analyses of endogenous Anti-Smads will be required to refine these models.

3.2.2. Co-repressors

Another mechanism by which Smad transcriptional activity can be inhibited is via direct binding to transcriptional co-repressors, or binding to intermediary proteins that recruit such repressors. Two prominent examples of this type of Smad inhibition have been described. First, another homeodomain protein, 5′ TG 3′ interacting factor (TGIF), was identified as a Smad2-binding protein using the yeast two-hybrid system. Although the function of TGIF is not well understood, this study showed that it can function as a transcriptional repressor at Smad-responsive promoters. Furthermore, inhibitors of histone deacetylases (HDACs) were shown to reduce the TGIF-dependent repression, suggesting that HDACs may be recruited to Smad-responsive promoters via interaction with TGIF–Smad complexes (Wotton et al., 1999). However, Neither a specific HDAC protein nor direct interaction with TGIF was conclusively demonstrated.

Because Smads have been shown to enhance transcription by association with histone acetylases such as CBP/p300, the possibility that repression of Smad-mediated transactivation occurs through histone deacetylation is rational. The role of histone acetylation in transcriptional regulation has been well-documented; acetylation of core histones disrupts the packing structure of DNA thus increasing accessibility to DNA-binding proteins, whereas deacetylation allows orderly packing and excludes transcription factors (Lee et al., 1993; Wolffe, 1996). Confirmation that HDACs play a role in repressing Smad-regulated promoters has been provided by the observation that Smads interact directly with the related HDAC-binding oncoproteins Ski and Sno. Both Ski and Sno have been found in human tumor cell lines and both can transform cells in culture; this implies that these proteins function oppositely to Smads. TGFβ-dependent interaction of Smads 2, 3, and 4 with Ski and Sno results in transcriptional repression of several different Smad-responsive promoters. Repression was shown to be dependent on the presence of the nuclear hormone receptor co-repressor (N-CoR) within the Smad–Sno/Ski complex, and the N-CoR-associated HDAC. In these studies, the presence of HDAC1 in the Smad complex was demonstrated. In addition, exogenous Ski was shown to reduce Smad-associated histone acetylase activity (presumably due to CBP/p300) in a dose-dependent manner (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999a,b). Finally, both TGIF and Ski compete with CBP/p300 for binding to Smad complexes (Akiyoshi et al., 1999; Wotton et al., 1999). Together, these observations demonstrate that Smad transcriptional activity can be inhibited by physical association with co-repressors that tether HDACs to Smad-responsive promoters.

3.2.3. Smad ubiquitination regulatory factor 1 (Smurf1)

The large number of potential Smad-binding partners implies that the amount of Smad protein available inside the cell is a key determinant of ultimate Smad function. Consistent with this hypothesis, the directed proteolysis of BMP-responsive R-Smads has been reported. Smads 1 and 5 were shown to interact with a novel ubiquitin ligase discovered as a Smad1-binding protein using two-hybrid screening. Smurf1 shares sequence homology with the Hect subclass of E3 ubiquitin ligases, and specifically targets Smads 1 and 5 for ubiquitination and subsequent degradation. Smurf1 function was confirmed in *Xenopus* embryos, where ectopic expression inhibits Smad1-induced ventralization (Zhu et al., 1999). Ubiquitination and degradation of Smad1 was independent of BMP receptor activation, leaving open the question of how Smad proteolysis is regulated in vivo.

Ubiquitin-dependent degradation of nuclear Smad2 has also been recently demonstrated (Lo and Massagué, 1999). In this case, the nuclear localization of Smad2 appears to be the key determinant of ubiquitination and degradation, rather than phosphorylation per se. However, because RSK-mediated phosphorylation precedes nuclear accumulation of Smads in vivo, it is reasonable to hypothesize that TGFβ signals are attenuated in part by degradation of activated R-Smads.

3.2.4. Calmodulin

Calmodulin, the primary mediator of calcium signaling, has also been shown to interact physically with R-Smads and Co-Smads in vitro, and to inhibit TGFβ signaling in cultured cells. Calmodulin binds two different amphiphilic α-helices in the MH1 domain of R-Smads in a strictly calcium-dependent manner; calmodulin is one of only two proteins that interact with Smads through the MH1 domain. Exogenous calmodulin also inhibits Smad-mediated transactivation from multiple TGFβ-responsive promoters. Furthermore, inhibition of calmodulin function allows increased expression of Smad-dependent reporter genes (Zimmerman et al., 1998). Because calmodulin interacts with a large number of other proteins, and because calcium signaling regulates a diverse set of cellular

processes, more work is required to determine the physiological significance of these observations. However, the roles of other signaling pathways in modulating TGFβ responses are now also being elucidated.

4. Cross-talk with other signaling pathways

Signaling by interferon-γ is mediated by cytokine receptors that activate JAK tyrosine kinases and, subsequently, STAT proteins. Interferon-γ inhibits TGFβ signaling by the direct STAT-mediated transcriptional induction of Smad7. Smad7, of course, is an Anti-Smad that prevents R-Smad phosphorylation, R-Smad and Co-Smad complex formation, and nuclear accumulation of the complexes (Ulloa et al., 1999). Similarly, the inhibition of TGFβ signaling by TNFα has also recently been shown to rely on Smad7 induction, in this case by NF-κB/Rel A (Bitzer et al., 2000).

Conversely, cooperation between STAT3 and Smad7 in primary fetal neural progenitor cells has been demonstrated (Nakashima et al., 1999). In these cells, leukemia inhibitory factor (LIF) activates STAT3, which synergizes with BMP2-activated Smad1 to effect differentiation into astrocytes. This apparently results from the physical association of both STAT3 and Smad1 with p300 (described previously) and subsequent transcriptional modulation. Therefore, the relationship between Smads and STATs is complex and the ultimate effects of simultaneously activating these factors may be highly cell-type specific.

In a similar way, the classical MAP kinase pathway has been implicated in both positive and negative regulation of TGFβ signaling at the level of Smad function. Positive regulation of Smads results from EGF and HGF receptor activation, which causes phosphorylation of Smad2 at sites independent of the C-terminal serines targeted by RSKs. EGF- and HGF-dependent phosphorylation of Smad2 results in activation of Smad2-responsive reporter genes, suggesting that MAP kinases may be able to potentiate Smad signaling in addition to RSKs (de Caestecker et al., 1998; Brown et al., 1999). However, EGF and HGF stimulation of MAP kinases has also been shown to inhibit Smad signaling. Phosphorylation at consensus MAP kinase sites within the variable linker region of R-Smads prevents nuclear accumulation of Smad complexes, and transcriptional induction of Smad-dependent reporter genes (Kretzschmar et al., 1997a, 1999). The reason for the striking discrepancy between these reports is not clear. Could these opposing effects be the result of cell-type specific differences, or could EGF and HGF stimulation differentially modify Smad-binding proteins to change signal outputs? There are several complex possibilities, but only more work will distinguish between them.

What is clear is that Smads integrate a variety of signal inputs, and interact with a host of cellular proteins to modify transcriptional responses.

5. Conclusion

In summary, Smad proteins mediate TGFβ-initiated RSK signals from the plasma membrane into the nucleus. Activation of RSKs occurs through ligand binding and complex formation between type II and type I receptor subunits. Type II receptor-dependent phosphorylation of the type I receptor at the GS domain activates the type I receptor kinase, which phosphorylates an R-Smad on C-terminal serines. The phosphorylated R-Smad associates with a Co-Smad in the cytoplasm and then moves into the nucleus, where transcriptional regulation occurs via direct DNA binding by the Smad complex, or by association of the Smad complex with other DNA-binding proteins (Fig. 3).

Smad functions can be enhanced or inhibited by a large variety of partner binding proteins. DNA-binding proteins, transcription factors, and transcriptional co-activators all participate with Smads in transactivation. Conversely, Anti-Smads, transcriptional repressors, and elements of the protein degradation machinery all antagonize Smad functions. The core TGFβ signaling pathway is certainly a simplified description of this critically important regulatory network.

Future investigations will focus on clarifying many of the issues discussed here, and on several other interesting questions. Learning more about the genes regulated by TGFβs will be a necessary step in understanding how these factors modulate such a diverse range of biological processes. In addition, clarifying the role of a limited set of receptors for a larger group of ligands will be important. Another open question is whether the Smads are the only TGFβ signal mediators. The sheer diversity of ligand functions implies that other mediators may be present, and there is suggestive evidence that other mediators exist. The models and tools are now available to address these issues, and to generate the next set of interesting questions.

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