Development 134, 3565-3569 (2007) doi:10.1242/dev.005926

TGFβ superfamily signaling: notes from the desert

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The TGFβ pathways play crucial roles in many developmental events, as well as contributing to many disease states. To provide a venue for both signaling and developmental research on TGFβ, a FASEB-sponsored bi-annual meeting was initiated six years ago, the fourth of which was organized by Caroline Hill and Michael O'Connor and took place this July in Tucson, Arizona. The meeting highlighted major advances in our understanding of the structural and biochemical aspects of TGFβ superfamily signaling, its intersection with other pathways, and its contribution to disease.

Introduction

Members of the transforming growth factor β (TGFβ) superfamily of ligands initiate signaling by binding to, and bringing together, type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal through the phosphorylation of Smad proteins. Activated Smad complexes accumulate in the nucleus where they regulate the transcription of target genes (Fig. 1). In this manner, the members of the extended TGFβ superfamily of signaling molecules control a broad array of cellular processes, including cell proliferation, cell-cell and cell-matrix interactions, differentiation and apoptosis, both during development and in adult tissues, in organisms ranging from flies and worms to mammals.

Major themes

New clues to morphogen gradient formation

TGFβ ligands function as classical morphogens in many developmental contexts. Three groups reported on efforts to understand how TGFβ ligand gradients form and what these gradients mean mechanistically and how to mathematically model these events. Marcos Gonzalez-Gaitan (University of Geneva, Switzerland) examined the Decapentaplegic (Dpp) gradient in flies and made comparisons with Wingless (Wg) gradients. By using the FRAP (fluorescence recovery after photobleaching) technique, he found that ~40% of Dpp molecules move through the tissue and that endocytosis is required for Dpp spreading. Wg spreading is short range, and differences in degradation and diffusion account for many of the differences between the Wg and Dpp gradients.

Chip Ferguson (University of Chicago, Chicago, IL) examined aspects of Dpp gradient formation in the early fly embryo. Dpp is expressed in a broad dorsal swatch, but signals in only a few dorsal cells. Using perivitelline injections of antibodies into embryos, the formation of the Dpp gradient could be observed. Further information was obtained by expressing tagged Dpp molecules from the *eve* stripe 2, which encircles the embryo perpendicular to the normal Dpp expression. Using these two tools, Ferguson tested Dpp movement and gradient formation in various genetic backgrounds to assign functions to several genes necessary for efficient gradient formation. Interestingly, the extracellular transport of the ligand is not sufficient to attain a refined stripe on the dorsal side of the embryo. He was able to identify additional players that participate in refining the pattern. Arthur Lander (University of California, Irvine, CA) reported progress in modeling the Dpp gradient. Modeling biological processes is gaining momentum, particularly as a tool for understanding and predicting the behaviors and functions of morphogen gradients. Lander explained how relatively simple models of gradients tend to be 'fragile': even small perturbations can have large effects on the position of the gradient. However, nature has designed these gradients to be robust. How is this achieved? Lander suggested that a host of other factors, including co-receptors and feedback regulation of morphogen, receptor and co-receptor synthesis, are required to provide robustness to the many different kinds of perturbations that organisms encounter in real life.

MicroRNAs modulate TGFβ pathways

Two years ago, only a single presentation touched on the microRNA (miRNA) control of TGFβ signaling. This year, three presentations illustrated that miRNAs control different aspects of TGFβ signaling. Alex Schier (Harvard University, Cambridge, MA) reported that *dicer* knockouts in zebrafish (which remove all mature miRNAs) are surprisingly well developed, indicating that most signaling pathways must be active (Giraldez et al., 2005). Adding back a single miRNA, mir-430, corrected most of the early defects. Possible targets of mir-430 included several TGFβ signaling components (Choi et al., 2007). Using frogs, Stefano Piccolo (University of Padua, Padua, Italy) isolated small RNAs and tested them for activity in early embryos. From this, he identified a miRNA that specifically targets a TGFβ-like receptor and helps set the size of the organizer (Martello et al., 2007). Using *Drosophila*, Richard Padgett (Rutgers University, NJ) presented evidence for miRNA regulation of Mad in the Dpp pathway. In two of these cases, the data suggest that miRNAs do not pattern axes, but rather refine global mRNA and protein expression levels and possibly add robustness to developmental events. Given the number of miRNAs and the large number of predicted targets, additional connections between TGFβ pathways and miRNAs are likely to emerge.

New pathway genes and new twists on development

Michael O'Connor (University of Minnesota, Minneapolis, MN) reported on the roles of bone morphogenetic protein (BMP) in postnatal synaptic remodeling in mice (Sun et al., 2007). BMP signaling was modulated by using genetic mutations of the BMP inhibitor chordin, or by perfusing recombinant BMP pathway components onto hippocampal slices. chordin-null mice showed increased presynaptic neurotransmitter release from hippocampal neurons. These mice learned faster in water-maze tests and open-field tests. Perfusion of BMP ligands onto hippocampal slices produced the same effect on neurotransmitter release, findings that together indicate that BMP contributes to synaptic plasticity and learning.

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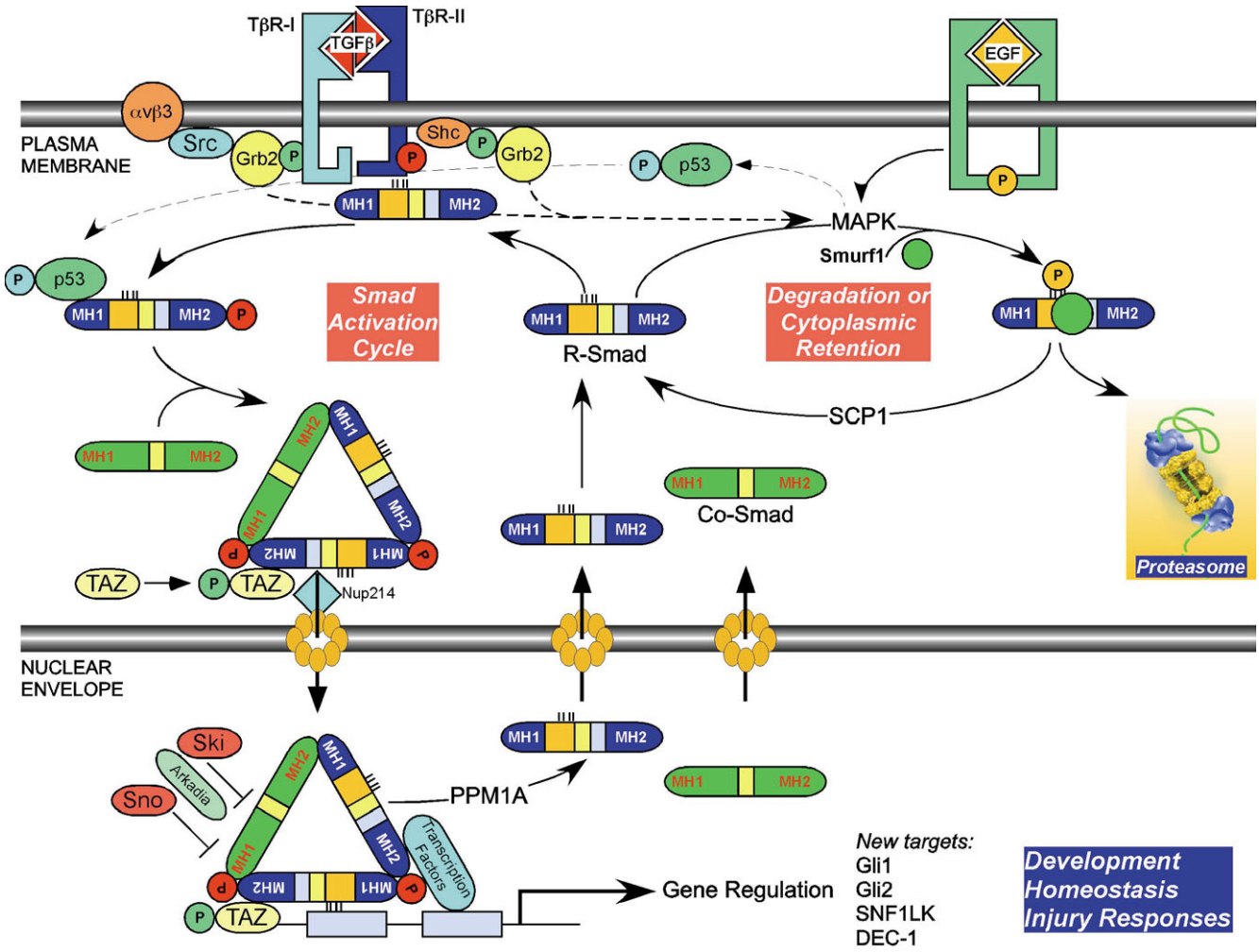

Fig. 1. Modulation of Smad signaling. TGFβ/Smad superfamily signal strength is regulated and modulated at several different levels, both intrinsically and through cross-talk with other signaling pathways. R-Smads and co-Smads continuously shuttle between cytoplasm and nucleus. Following TGFβ ligand-mediated engagement of the type II/type I receptor complex and R-Smad activation, C-terminally phosphorylated R-Smads are preferentially retained in the nucleus, where they initiate a specific transcription program. Signal termination at the level of receptors results in rapid dephosphorylation of activated R-Smads in the nucleus by the nuclear phosphatase, PPM1A. In addition, cells adapt to their persistent exposure to TGFβ ligands by eliminating activated R-Smads by proteasomal degradation. This adaptive response may be mediated by TGFβ-initiated non-Smad-dependent activation of MAP kinase pathways, which phosphorylate specific tyrosine residues in the Smad linker regions. These phosphorylation events appear to trigger proteasomal degradation and prevent nuclear transport of activated R-Smads, and are turned off by SCP-type phosphatases. Similar mechanisms centered on R-Smad linker regions appear to be utilized by mitogenic and stress stimuli to counterbalance the effects of TGFβ signals. Conversely, MAP kinase-mediated N-terminal phosphorylation of p53 enables its interaction with activated R-Smads, thereby enhancing their cytostatic functions. This pathway may serve as a negative feed-forward loop to counterbalance excessive mitogenic signaling.

Kavita Arora (University of California, Irvine, CA) and Cathy Savage-Dunn (Queens College, Flushing, NY) provided updates on *schnurri* (*shn*) signaling from studies in *Drosophila* and *C. elegans*. *Drosophila shn* primarily functions to repress *brinker*, which represses *dpp* target genes (Torres-Vazquez et al., 2001). Arora and co-workers showed in *Drosophila* that *Drosophila* Shn activates transcription of the *Xenopus* BMP target *XVent2* through conserved cis-regulatory elements. In *C. elegans*, *schnurri* (*sma-9*) has an essential role in *dbl-1* signaling, but nematodes do not have the *brinker* gene, and *schnurri* functions as both a transcriptional activator and a repressor in male tail patterning (Liang et al., 2003). One issue that remains is whether vertebrate schnurris are associated with TGFβ activities. Of the three vertebrate homologs, Shn2 (Hivep2) interacts with Smads to mediate the BMP-dependant activation of peroxisome proliferator activated receptor γ2 (Pparγ2) in mouse adipogenesis (Jin et al., 2006), and Shn1 (Hivep1) can activate or repress transcription depending on the cellular context. The work in these systems establishes a convincing reason to revisit the role of vertebrate schnurris in the context of TGFβ signaling. Even though TGFβ-like components are found in essentially all animals, from sponges to vertebrates, they are not present in yeast or plants. Jerry Thomsen (State University of New York, Stony Brook, NY) presented evidence for the existence of BMPs and activins in the sea anemone *Nematostella vectensis*, a new model for studying TGFβ. He and his principal collaborators, Mark Martindale and Dave Matus, have seen unusual patterns of gene expression between TGFβ ligands and known regulators, such as chordin and noggin. One surprising finding is that, unlike in the fly and frog,

where chordin and BMP genes are expressed in opposing domains across the blastopore at gastrulation, the anemone genes are expressed in a coincident domain, asymmetrically located on one side of the blastopore. Thomsen plans to use cnidarians to elucidate ancient developmental processes that are affected by TGFβ signaling and to understand the pathway's evolution. Richard Padgett reported on a new gene, isolated from genetic screens in *C. elegans*, which contains a transmembrane domain, but, given its very short cytoplasmic tail, is unlikely to transduce a signal. Mutations in the gene result in small animals, a phenotype also observed in mutations of other core signaling components. The gene product binds to the type I TGFβ receptors and is necessary for proper signal transduction. Since it is conserved in *Drosophila* and in vertebrates, it will be of interest to determine its function in other animals and how it enhances signaling.

TGFβ-induced Smad-mediated and non-Smad signaling

The regulation and modulation of Smad signaling was a major focus of this meeting. Joan Massagué (Sloan-Kettering Institute, New York, NY) discussed mechanisms whereby mitogenic and stress response pathways modulate TGFβ and BMP signaling strength. Mitogen-activated protein kinases (MAPKs) catalyze inhibitory phosphorylation in the Smad1 linker region. The Massagué laboratory showed that linker phosphorylation restricts Smad1 activity by enabling Smad1 to be recognized by the HECT-domain ubiquitin ligase Smurf1. Besides causing Smad1 polyubiquitination, Smurf1 binding inhibits the interaction of Smad1 with the nuclear translocation factor Nup214. Consequently, MAPK-dependent Smurf1 binding leads to Smad1 degradation or cytoplasmic retention. Together, Smad1 linker phosphorylation and Smurf1 act as interdependent inputs to control BMP signaling during mouse osteoblast differentiation and *Drosophila* neural development. Thus, the interplay between linker phosphorylation, Smurf-dependent ubiquitination and nucleoporin exclusion enables BMP signaling to be regulated by diverse signals and biological contexts (Sapkota et al., 2007).

Using *Drosophila* as a model, Laurel Raftery (Massachusetts General Hospital, Boston, MA) addressed the importance of Mad linker region phosphorylation events in development. She reported that mutation of all four serine phosphorylation sites in the Mad linker region to alanines gives rise to a phenotype that is nearly as severe as that seen in flies that express a constitutively active Dpp type I receptor called Thickveins (Tkv), whereas mutation to aspartic acid mimics the wild-type phenotype. This finding is consistent with the notion that phosphorylation events in the Mad linker region serve to downregulate Dpp signal strength. Additional presentations also reported that phosphorylation events in the Mad linker region provide an important negative-feedback function for Dpp signaling during wing development.

Stefano Piccolo discussed the integration between mitogenic pathways, p53 and Smad signaling. It turns out that RTK/Ras/MAPK activity induces p53 N-terminal phosphorylation, enabling the interaction of p53 with TGFβ-activated Smads. This mechanism confers mesoderm specification in *Drosophila* embryos and promotes TGFβ cytostasis in human cells. Thus, this pathway might serve as a negative feed-forward loop to modulate excessive mitogenic signaling. Conversely, cancer-associated p53 mutations might eliminate the capacity of TGFβ to attenuate the mitogenic growth stimulus and help derepress cell proliferation (Cordenonsi et al., 2007). Xiao-Fan Wang (Duke University, Durham, NC) pointed out that substantial differences exist in steady state levels of Smad3 across cell types, suggesting that basal levels of Smad3 might predetermine the intrinsic ability of cells to respond to TGFβ. He showed that Smad3 is significantly less stable than Smad2, and is subject to constant turnover by the proteasome pathway. Furthermore, Smad3 physically associates with several members of the APC–β-catenin–axin–glycogen synthase kinase 3 beta (GSK3β) complex. In addition, GSK3β phosphorylates Smad3 at Thr66 in its MH1 domain (see Fig. 1) and is essential for Smad3's proteasomal degradation.

Caroline Hill (Cancer Research UK, London, UK) presented interesting new findings that indicate that Smad2 does not move freely through the cell by diffusion, but that an intact microtubule network and kinesin ATPase activity are required for Smad2 phosphorylation and nuclear accumulation in response to activin (inhibin β)/Nodal in early vertebrate embryos and to TGFβ in mammalian cells. Smad2 interacts with the kinesin light chain 2 (KLC2) subunit of the kinesin complex. Interfering with kinesin activity in *Drosophila* and zebrafish embryos phenocopies loss of Nodal signaling. These results reveal that kinesin-mediated transport of Smad2 along microtubules to the receptors is an essential step in ligand-induced Smad2 activation (Batut et al., 2007). Just as phosphorylation events within the Smad linker region are important ways for other signaling pathways to modulate TGFβ signaling, it is equally important to understand the mechanisms by which TGFβ signals are turned off. Using a biochemical screen of all known serine-threonine phosphatases, Xin-Hua Feng (Baylor College of Medicine, Houston, TX) reported the identification of SCP family phosphatases (SCP1, SCP2 and SCP3, also known as CTDSP1, CTDSP2 and CTDSPL, respectively) as the phosphatases that dephosphorylate Smad3 at serines 204, 208 and 213 (in addition to threonine 8 in the MH1 domain). SCP1 blocks epidermal growth factor (EGF)-dependent inhibition of TGFβ signaling. William Schiemann (University of Colorado Health Sciences Center, Aurora, CO) and Rik Derynck (University of California, San Francisco, CA) provided new evidence linking TGFβ receptors directly to non-Smad signaling pathways. Schiemann showed that Src phosphorylates the TGFβ type II receptor, TβR-II (Tgfβr2) (see Fig. 1) on Y284 both in vitro and in vivo. This Src-mediated phosphorylation of Y284 creates a docking site for the SH2 domains of growth factor receptor binding protein 2 (Grb2) and Src homology domain 2 (Shc2) on the type II receptor, thereby providing a direct biochemical link between the TGFβ signaling receptors and MAPK activation (see Fig. 1). Importantly, a Y284F-TβR-II mutant abrogated breast cancer cell invasion induced by alpha(v)β(3) integrin (Itgα5β3) and TGFβ, and partially restored their cytostatic response to TGFβ. Equally important, expression of the Y284F-TβR-II mutant also prevented TGFβ-dependent stimulation of breast cancer growth and pulmonary metastasis in vivo. These findings provide the first direct evidence for a direct biochemical link between the canonical TGFβ receptor signaling module and another cellular signaling pathway. Given that both the type I and type II TGFβ receptors undergo auto- or heterophosphorylation at a number of sites, it is likely that additional similar connections will be uncovered that connect the TGFβ pathway to other signaling pathways (Galliher and Schiemann, 2007).

Continuing on the theme of non-Smad signaling by TGFβ, Rik Derynck reported that TGFβ induces Erk MAP kinase phosphorylation within 5 minutes. TGFβ also induces rapid phosphorylation of the adaptor protein Shc (Shc1) on both Ser and Tyr. Shc rapidly associates with TβR-I (Tgfβr1) in the TGFβ receptor complex following ligand addition, and phosphorylation of Shc is direct, i.e. by the TGFβ-activated TβR-I (see Fig. 1).

Moreover, Derynck showed that TGFβ induces the association of Grb2 with Shc, as well as with SOS1, thereby activating the Erk MAP kinase pathway. A dominant-negative Shc mutant, as well as Shc knockdown by siRNA, blocks TGFβ-induced Erk MAP kinase phosphorylation, demonstrating that Shc phosphorylation on Tyr initiates the activation of the Erk MAP kinase pathway in response to TGFβ. Thus, both Schiemann and Derynck provided new evidence for direct TGFβ receptor-mediated signaling events that act in parallel to the Smad pathway and provide cross-talk with other cellular signaling pathways. Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden) presented new information on SNF1-like kinase (SNF1LK), a member of the AMPK family of kinases, and the mammalian ortholog of the *C. elegans* body size regulator KIN-29 (Maduzia et al., 2005). Both TGFβ and BMPs rapidly induce SNF1LK transcription. In siRNA-SNF1LK knockdown experiments in mammalian cells, the cytostatic and tissue injury genetic programs induced by TGFβ were amplified, in association with an increase in Alk5 (Tgfβr1) protein levels. Further experiments showed that SNF1LK specifically associates with Smad7 within the Alk5 degradation complex that also includes Smurf1. Moreover, SNF1LK kinase activity is required for Alk5 proteasomal degradation, although its substrate in this context is not yet clear. From these and other findings, Moustakas proposed a model in which TGFβ receptor signaling leads to the activation of TAK1 (MAP3K7), which is upstream of the LKB1 kinase (STK11), which, in turn, activates SNF1LK. Thus, SNF1LK would be a key component in a negative-feedback loop that terminates TGFβ signaling by regulating Alk5 receptor turnover. Ed Leof (Mayo Clinic, Rochester, MN) presented new information regarding the structural features of TGFβ receptors that determine membrane localization. By generating different truncation mutants of the TβR-II, he demonstrated that the basolateral surface targeting of the receptor requires a 10 amino acid region within the extreme C-terminal tail of the cytoplasmic domain. As this region is a hot spot for mutations in cancer and certain genetic disorders, this finding raises the important question of whether the pathogenic role of these mutants might be related to the mislocalization of receptors in cancer cells (Mitchell et al., 2004; Murphy et al., 2004).

New insights into TGFβ roles in disease

Lalage Wakefield (National Cancer Institute, Bethesda, MD) presented new work investigating the role of TGFβ in tumor-host cell interactions in breast cancer. Using tumor cells and fibroblasts marked with TGFβ-responsive GFP or RFP reporter constructs, she demonstrated that endogenous TGFβ signaling becomes activated in both cellular tumor components in co-culture in vitro, as well as in xenografts in vivo. Moreover, this activation becomes progressively stronger as the tumor cells acquire more invasive and metastatic properties, providing new support for the hypothesis that constitutive activation of TGFβ signaling plays an important role in breast cancer metastasis. Consistent with this idea, Wakefield showed that the pan-TGFβ neutralizing antibody 1D11 (Genzyme) inhibits primary tumor growth and metastasis in the syngeneic 4T1 spontaneous breast cancer metastasis model. However, surprisingly, in $CD8^+$ T-cell-depleted animals, 4T1 tumor growth was suppressed independently of 1D11 treatment. Further studies identified IL17 as one of the cytokines secreted by $CD8^+$ T-cells from tumor-bearing animals that acts as a survival factor for the tumor cells, but only in the presence of TGFβ. Moreover, induction of IL17 is driven by IL6, which, in turn, is produced by the tumor cells in response to TGFβ. Based on these and other results, Wakefield proposed a model in which tumor-associated TGFβ not only suppresses tumor-specific cytotoxic T-cell activity, but induces IL6 production by tumor cells, which, in turn, induces the differentiation of a subset of T-cells that secrete the anti-apoptotic cytokine IL17. Thus, these results suggest yet another mechanism whereby breast cancer co-opts a physiological inflammatory response to support its progression.

Kohei Miyazono (University of Tokyo, Tokyo, Japan) presented new studies using the JygMC(A) murine mammary carcinoma model. These cells efficiently metastasize to lungs and liver following establishment of subcutaneous xenografts in Balb/C nu/nu mice (Azuma et al., 2005). Miyazono reported that various murine mammary epithelial cell lines, including JygMC(A), undergo apoptosis under conditions of serum deprivation. Moreover, in this context, TGFβ acts as a prosurvival factor as the Alk5/4/7 kinase (Tgfβr1/Acvr1b/Acvr1c) inhibitor, A-44-03, effectively blocks TGFβ's anti-apoptotic activity. Under these serum-starved conditions, one of the major genes induced by TGFβ is the basic helix-loop-helix transcription factor Dec1 (Bhlhb). Dominant-negative Dec1 expression failed to affect the growth of subcutaneous xenografts, but was associated with a significant reduction in pulmonary and hepatic metastatic burden. From these and other results, Dec1 appears to represent a bona fide metastasis gene, and it could be that some of the anti-metastatic properties of TGFβ pathway antagonists seen in various models of mammary cancer might be mediated, in part, by downregulation of Dec1.

Alain Mauviel (INSERM, Paris, France) described an exciting new interaction between the sonic hedgehog (Shh) and TGFβ/Smad3 pathways in cancer (Dennler et al., 2007). Many cancers overexpress the Shh targets Gli1 and Gli2, and can be growth inhibited using the smoothened inhibitor, cyclopamine. In addition to Shh, *Gli1* and *Gli2* mRNA expression is also induced by TGFβ in an Alk5-dependent manner. A survey of pancreatic cancer cell lines revealed that, even though all overexpressed Gli1, some were not growth inhibited by cyclopamine. Interestingly, it is precisely this subset of cyclopamine-resistant pancreatic cancer cells that is growth inhibited by the Alk5 inhibitor SB431542, indicating that Gli1 expression is aberrantly driven in this context by TGFβ rather than by Shh. The transcriptional regulators Ski and SnoN represent important negative regulators of TGFβ signaling by physically interfering with the Smad4/R-Smad interaction within transcriptional complexes. In order to gain a better understanding of Ski and SnoN in cancer development, Kunxin Luo (University of California, Berkeley, CA) has successfully generated transgenic mice that overexpress a ubiquitination-resistant form of SnoN driven by a MMTV promoter. As expected, mammary epithelial cells from these mice are relatively insensitive to TGFβ-mediated growth arrest and they do not spontaneously manifest an increased cancer predisposition. However, when crossed with MMTV-polyoma virus middle T antigen (PyVmT) transgenic mice, mammary tumor development is significantly accelerated compared with MMTV-PyVmT control mice. Ski also showed an anti-metastatic effect when knocked down in vitro, which is paradoxical in light of its high expression in human cancers. However, Luo reported that TGFβ treatment rapidly downregulates Ski in metastatic breast cancer cells, apparently by inducing its proteasomal degradation. Thus, within a microenvironment rich in active TGFβ (such as exists, for example, in the metastatic niche), Ski expressed by carcinoma cells might be rapidly degraded, thereby relieving its anti-metastatic effect. Several examples of the role of BMP signaling in cancer development were presented. Hideyuki Beppu (Massachusetts General Hospital, Charlestown, MA) demonstrated that conditional inactivation of the BMP receptor Bmpr2 in mice caused colorectal epithelial hyperplasia and polyp formation, associated with

decreased apoptosis of both the epithelial and stromal cell compartment. Along the same lines, Stephanie Pangas (Baylor College of Medicine, Houston, TX) reported on the phenotypes of mice that had undergone conditional knockout of BMP *Smad1*, *Smad5* and *Smad8*, either singly or in combination, in gonadal tissue. Whereas single BMP Smad loss was not associated with a detectable phenotype, double *Smad1 Smad5* and triple knockout animals invariably developed malignant gonadal tumors at a young age. Thus, both of these studies indicate that the BMP pathway can function as a potent tumor suppressor.

Mohamad Azhar (BIOS Institute, University of Arizona, Tucson, AZ) has generated *Tgfb2*- and *Tgfb3*-null mice. He reported that approximately 30% of *Tgfb2*-null mice phenocopy the cardiovascular manifestations of Marfan-related disorders. Moreover, double *Tgfb2* and *Tgfb3* knockout mice developed myxomatous heart valves and aortic dilatation with 100% penetrance. Interestingly, this syndrome was associated not only with loss of Smad2 and Smad3 phosphorylation, but with a dramatic activation of the BMP Smads, Smad1 and Smad5. These findings suggest that Marfan-like syndromes might be caused by downregulation of canonical TGFβ Smad2 and Smad3 signaling, and/or constitutive activation of the BMP Smad pathway.

Given the fact that the BMP signaling pathway clearly plays a role in various genetic and acquired disease states, the development of pathway antagonists offers the potential for novel and effective treatments. Paul Yu (Harvard Medical School, Boston, MA) described the use of a high-throughput biological screen in zebrafish embryos, undertaken in collaboration with Charles Hong, Ken Bloch and Randy Peterson, to identify agents that antagonize BMP's dorsoventral development effects. From a 7500 compound library screen, a small molecule, nicknamed dorsomorphin, was identified that preferentially inhibits BMP signaling versus TGFβ signaling in vitro and in vivo. Dorsomorphin represents a first lead compound that selectively inhibits Alk2-, Alk3- and Alk6-mediated activation of Smad1, Smad5 and Smad8.

Conclusion

This meeting covered the full gamut of topics ranging from the role of the TGFβ superfamily in development, to the intricacies and complexities of the signaling pathway, to its role in human disease and possible therapeutic implications. Much excitement was generated by novel and paradigm-shifting insights into these important cellular pathways. We can all look forward to the next instalment in 2009.

We thank speakers and Caroline Hill and Michael O'Connor for a stimulating meeting that touched on many new aspects of TGFβ signaling. We apologize for omitting certain talks and posters owing to space constraints. We dedicate this review to Anita Roberts, who passed away this past year, and to whom we are grateful not only for her many seminal contributions to this field but also for her generosity, collegiality and integrity. R.W.P. is supported by grants from the NIH and DOD and M.R. by grants from the NIH.

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