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A taste of TGFβ in Tuscany

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Summary

The recent FASEB Summer Research Conference entitled 'The TGFβ Superfamily: Signaling in Development and Disease' was held in August, 2011 in the spectacular setting of Il Ciocco, Lucca, amidst the olive trees in Tuscany, Italy. The organizers assembled an amazing forum, which included 53 speakers and 67 poster presentations from laboratories around the world, to showcase recent advances made in our understanding of the transforming growth factor-β (TGFβ) signaling pathway.

Key words: TGFβ, FASEB, Conference

Introduction

The transforming growth factor-β (TGFβ) signaling pathway is evolutionarily conserved and underlies cell-cell communication in all metazoans examined so far. In August 2011, the Federation of American Societies for Experimental Biology (FASEB) Summer Research Conference on 'The TGFβ Superfamily: Signaling in Development and Disease', which was organized by Kunxin Luo (University of California, Berkeley, USA) and Peter ten Dijke (Leiden Medical Center, Netherlands), brought together researchers from across the world to discuss the recent advances and discoveries that have been made in the field of TGFβ signaling. Based on its breadth and quality of coverage, encompassing mechanisms, development, cancer, systems, structures, stem cells, and diseases, this conference was a wonderful success. In this meeting review, following a brief description of the current status of our understanding of the TGFβ signaling pathway, we highlight a very small number of individual presentations that we believe had a high impact, changing or adding to our knowledge of the pathway mechanism and its interface with developmental biology and human disease. We hope that this review provides a glimpse of the wonderful experience we had in the breathtaking Tuscan hilltops.

A summary of the TGFβ signaling pathway

The vertebrate genome carries more than thirty different TGFβ superfamily ligands [including bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activin, Nodal and TGFβs] that activate the TGFβ pathway, and a large number of secreted factors (including chordin, noggin and follistatin) that inhibit TGFβ signaling, all operating non-autonomously, outside of cells (Fig. 1). Upon secretion, homo- or hetero-dimers of TGFβ ligands are either: (1) sequestered in the extracellular matrix, ready for future deployment; (2) bound to secreted inhibitors that allow long and short range transport, thereby blocking interaction with receptors; or (3) bound to TGFβ receptors at the cell membrane.

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Acting as morphogens, TGFβ ligands exert diverse cellular responses based on the level and duration of their signaling. Dimeric TGFβ ligands bind type II receptors (TβRII) that recruit and phosphorylate, and thereby activate, type I receptors (TβRI or ALK5) in a hetero-tetrameric complex. Receptor activation, in turn, leads to the propagation of signaling (Fig. 1) by at least two seemingly independent pathways involving Smad (in the canonical pathway) or TRAF/TGFβ-activated-kinase-1 (TAK1; now known as MAP3K7 in mammals) (in the non-canonical pathway).

In the canonical pathway, a type I receptor propagates the signal by phosphorylating serine residues located at the carboxyl (C) terminus of proteins called receptor-activated Smads (R-Smads). There are two groups of R-Smads that transduce signals received from two groups of ligands: R-Smads 2 and 3 transduce signals from activins/Nodals and TGFβ1, 2 and 3; and R-Smads 1, 5 and 8 transduce signals from BMPs 2, 4 and 7 and a subset of GDFs. In the canonical pathway, R-Smads then form a trimeric complex with a common-mediator Smad, or co-Smad4, and translocate to the nucleus to bind and regulate transcription via subgroup-specific transcription factors. In the non-canonical pathway, activated TβRIIs signal through the TRAF and TAK1 proteins. TAK1, in turn, activates JNK, p38 and MEK kinases, as well as the NF-κb pathway. Interestingly, in addition to the TGFβ pathway, TAK1 can also be activated by a variety of cytokines, the Wnt pathway and the mitogen-activated protein kinase (MAPK) pathway, providing integration sites for crosstalk amongst different signaling pathways and contributing to regulation of gene expression (Fig. 1).

Input from TGFβ signaling is balanced by a series of inhibitory influences exerted at multiple levels that modulate the threshold levels and duration of signaling (Fig. 1). First, there are inhibitory influences that act on ligand-receptor interactions, regulated by secreted antagonists, such as noggin, chordin, follistatin and cerberus, and extracellular matrix-bound proteins, such as latent TGFβ binding proteins (LTBPs) and fibrillins. Second, co-receptors, such as Bambi, epidermal growth factor-cripto, FRL-1, cryptic family proteins (EGF-CFCs) and tomoregulins, regulate the activity and selectivity of TGFβ receptor transduction. Third, inhibitory influences can be exerted on R-Smads via phosphorylation by a variety of inputs, including signaling from MAPKs, glycogen synthase kinase-3β (Gsk3β), the TGFβ receptors themselves, and the cyclin-dependent kinases (CDKs). R-Smad linker-phosphorylation results in either recycling to the degradation machinery by the ubiquitylation pathway via Smurf1 and 2, or changes in their specificity in gene regulation. Fourth, two inhibitory Smads (Smad6 and Smad7) also inhibit TGFβ signals cell-autonomously: Smad6 acts in a BMP-dependent manner to compete with Smad4 binding and inhibit nuclear translocation of Smad1, 5 and 8; Smad7, however, seems to act in a ligand-independent manner to inhibit the pathway at multiple levels, including downstream of the activated type I receptor. Finally, phosphatases that dephosphorylate the C terminus of R-Smads, such as small C-terminal domain phosphatases (SCPs), have also been shown to downregulate the TGFβ signal.

Together, the multiplicity of secreted ligands and their inhibitors, the various receptor/co-receptor complexes, the canonical and non-canonical branches, and the input of cell-autonomous inhibitors and other signaling pathways leads to the refinement of signaling to

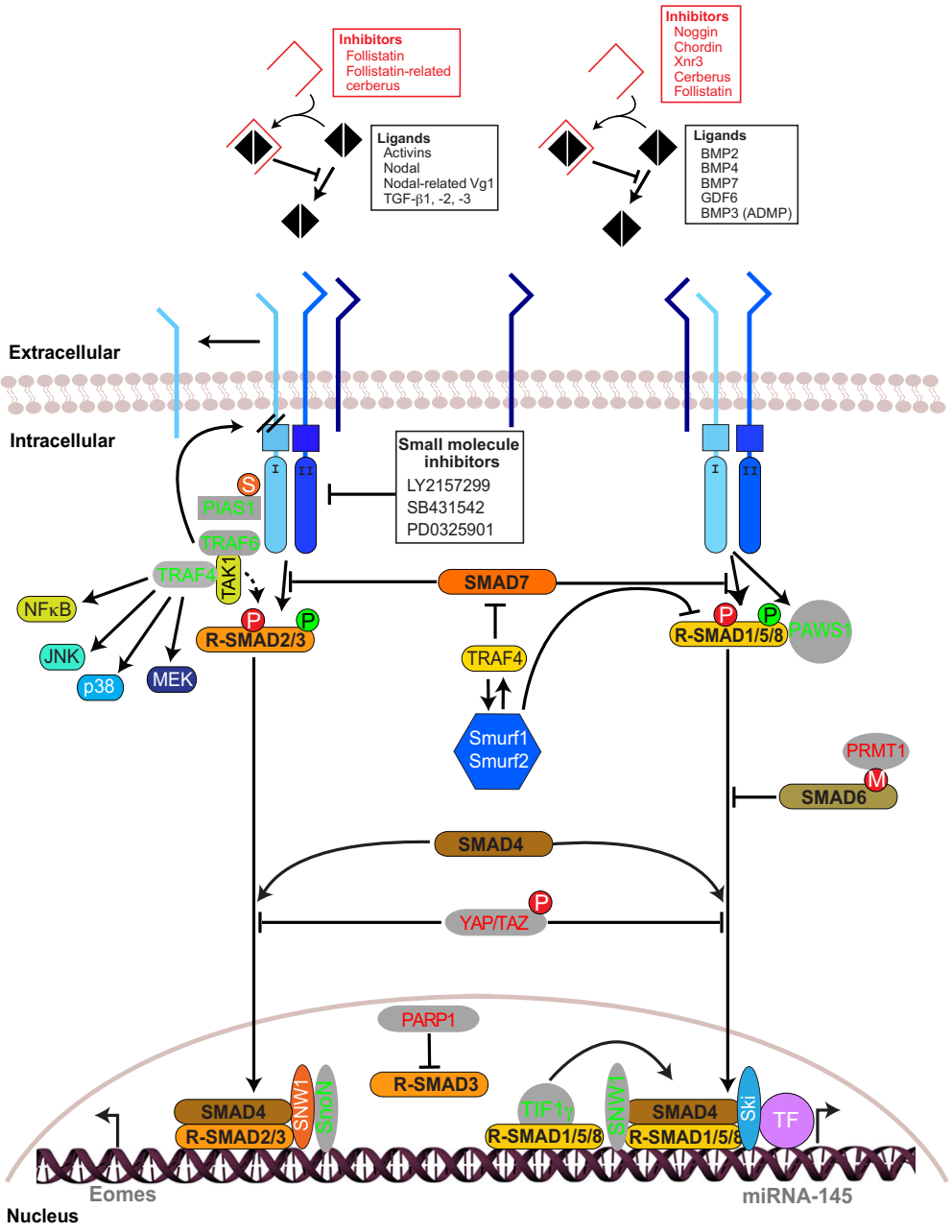

Fig. 1. An overview of the TGFβ signaling pathway. The components of the canonical and non-canonical TGFβ pathways are shown, with novel interactions discussed at the Tuscany meeting shown in gray (red text on gray refers to activation, green text on gray to inhibition). Upon secretion, dimeric TGFβ ligands bind type II receptors (dark blue) that recruit and phosphorylate, and thereby activate, type I receptors (light blue) in a hetero-tetrameric complex. Receptor activation leads to the propagation of signaling by at least two seemingly independent pathways: the Smad (canonical) pathway, and the Traf/TGFβ-activated-kinase-1 (TAK1) (non-canonical) pathway. R-Smads engage in a trimeric complex with a common-mediator Smad, or co-Smad4 (SMAD4), and translocate to the nucleus where they bind DNA and regulate transcription. In the non-canonical pathway, activated type II TGFβ receptors signal through Traf and TAK1 proteins, which activate the JNK, p38 and MEK kinases, as well as the NF-κb pathway. Input from TGFβ signaling is modulated by a series of inhibitory influences exerted at multiple levels to modulate threshold levels and duration of signaling. At the meeting, a number of newly identified molecules that contribute to the TGFβ signaling were discussed, including TRAF4/6, PAWS, PIAS1, YAP/TAZ, Parp1, PRMT1 and TIF1γ.

establish and maintain morphogen thresholds levels by converting extrinsic signaling to intrinsic, context-dependent cellular responses. TGFβ-mediated cellular responses are involved in most, if not all, biological activities, including (but not limited to) the development of an organism, maintenance of homeostasis, and pathogenesis. It is against this backdrop of our current imperfect understanding of this complex pathway that the meeting in Tuscany took place and provided greater elucidation of some aspects of this fascinating molecular circuitry. Below, we highlight some of the contributions with a rough attempt to follow the hierarchy of the pathway steps, moving from outside to inside, detailing cellular response and fate acquisition during embryonic development.

Novel insights into the components and mechanism of TGFβ signaling

Ligands and inhibitors: consequences for cancer and wing development

Several small molecule inhibitors of TβRI have been utilized to investigate the role of TGFβ signaling in vitro and in vivo; however, none of these inhibitors has been developed as drugs. Jonathan M. Yingling (Eli Lilly and Company, Indianapolis, USA) presented the characterization of a new inhibitor of TGFβ R1 (LY2157299), which binds the ATP-binding site of type I receptors ALK4 (ACVR1B) and TβRI, and inhibits TβRI kinase activity. His studies showed that TGFβ-dependent epithelial-to-mesenchymal transition (EMT) in NMuMg mouse mammary epithelial cells is inhibited by this compound. Furthermore, LY2157299 rapidly inhibits C-terminal phosphorylation of Smad2 and 3 in xenograft tumors and in a metastatic breast cancer model, thereby suppressing tumor growth. The compound is currently in Phase II clinical trials, having shown interesting activity in a glioblastoma multiforme (GBM)-enriched Phase I trial. Importantly, the pre-clinical pharmoacokinetic-pharmacodynamic modeling that was instrumental in defining the therapeutic window for optimal activity in patients was refined to support Phase II dose selection, and highlights the delicate nature of inhibiting the TGFβ pathway. Thus, LY2157299 is in an exciting phase of development as a first-in-class oncology therapeutic with broad anti-TGFβ signaling activity.

In a more developmental context, Michael O'Connor (University of Minnesota, USA) discussed the regulation of *Drosophila* tissue growth by opposing BMP and activin signals. In *Drosophila*, Dpp (the *Drosophila* ortholog of BMP) and three activin ligands (*Act88F*, *Dav* and *Myo*) are required for proper wing growth. Loss of the only activin type I receptor, Baboon (Babo), leads to a smaller wing. Surprisingly, loss of Smad2 (Smox – FlyBase) increases the size of the wing similarly to the *Dpp* gain-of-function mutant. In fact, whereas loss of *babo* has no effect on the expression of the Dpp antagonist Brinker (Brk), which is known to be downregulated by Dpp, loss of Smad2 eliminates Brk expression. *Babo-Smad2* double mutants exhibit the same phenotype as the *Babo* mutant, suggesting that Babo can still generate a signal that blocks Brk expression in the absence of Smad2. In *Drosophila* cell culture studies, Babo can phosphorylate both the Dpp R-Smad (Mad) and the activin R-Smad (Smad2). Reduction of Smad2, however, leads to increased phosphorylation of Mad. In *Drosophila* embryos, however, although the Smad2 phenotype is dependent on pMad, no increase of Mad phosphorylation was observed, indicating that the molecular mechanism underlying this phenotype remains unknown.

Receptors/co-receptors: cleavage, kinetics and modifications

Marene Landström (Umeå University, Sweden) extended her work on the regulation of TGFβ signaling by TRAF6 (Sorrentino et al., 2008), presenting an interesting finding that in the non-canonical TRAF and TAK1 branch, TβRI is cleaved extracellularly in a ligand-dependent manner. Upon association of TRAF6 with TβR1, TRAF6 causes activation of tumor necrosis factor α (TNF-α)-converting enzyme or TACE (ADAM17) to generate a liberated TβRI intracellular domain (TβRI-ICD), resulting in nuclear accumulation of TβRI-ICD, in a PKCζ-dependent manner (Mu et al., 2011). Nuclear TβRI-ICD then contributes to a transcriptional complex and regulates gene expression. Interestingly, this novel mechanism can be observed only in a subset of tumor cells and promotes invasion of cancer cells. It would be interesting to find out how and why tumor cells adopted such a unique signaling mechanism. Furthermore, it is intriguing to speculate that the N-terminal portion of the cleavage product, which includes the extracellular ligand-binding domain, might act as a sponge for TGFβ ligands and add an additional inhibitory input in the TGFβ pathway.

Rik Derynck (University of California, San Francisco, USA) noted that, compared with growth factor signaling through receptor tyrosine kinase receptors, TGFβ signaling is activated rather slowly; it takes 15-20 minutes from ligand activation of receptors to R-Smad phosphorylation, in contrast to MAPK phosphorylation, which occurs within a few minutes in response to growth factors. His data suggested that the slow TGFβ signaling kinetics might involve a requirement for ligand-induced post-translational modifications, such as sumoylation and methylation, at the receptor level. Indeed, TβRI is sumoylated in response to TGFβ, and this is mediated by phosphorylation of TβRI by TβRII, allowing the interaction of the E3 ligase with TβRI. These findings illustrate functional crosstalk between phosphorylation and sumoylation of the TGFβ receptors in response to TGFβ. For methylation, Derynck invoked arginine methylation of Smad6 as a requirement for initiation of BMP-induced Smad signaling. BMP-induced Smad6 methylation and dissociation from the receptor occurs before Smad1 is phosphorylated in response to BMP. These events might provide the basis for the delay and reveal novel early aspects of TGFβ signaling.

Peter ten Dijke (Leiden University Medical Center, Netherlands) addressed the mechanisms of TGFβ receptor signaling in breast cancer cell invasion and metastasis in a two-part presentation. Initially, he presented a unique in vivo screen that aimed to characterize genes that induce or modify invasiveness of breast carcinoma cells; injection of such cells into the embryonic blood circulation of zebrafish embryos leads to invasion and metastasis into vascular areas of the tail fin. Interestingly, in the case of Smad4 knockdown cells or embryos treated with TGFβ receptor kinase and MMP2/9 inhibitors, invasion and metastasis were attenuated. In the second part, he showed that TRAF4 mediates TGFβ-induced TAK1 activation. Knockdown of TRAF4 in breast carcinoma cells reduces TGFβ-mediated responses, such as cell migration and invasion. TRAF4 also influences the Smad-dependent pathway and, together with phosphorylated TAK1, leads to maximum cell invasion. Along with Landström's presentation on TRAF6, these studies further our understanding of how the TRAF family of proteins can influence TGFβ signaling.

Smads and transcription factors: post-translational modifications and dynamics

The studies presented by Rik Derynck highlighted the fact that TFGβ pathway receptors are subject to various post-translational modifications, but the intracellular mediators of TFGβ signaling are also modified. For example, Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden) discussed the regulation of Smad function by ADP-ribosylation and ubiquitylation. He presented evidence demonstrating that the nuclear Smad3-Smad4 complex associates with poly-ADP-ribose polymerase 1 (Parp1), resulting in poly-ADP ribosylation of Smad3, which negatively regulates DNA-binding activity. This is a novel post-translational modification of Smad activity, specifically in the nucleus, and provides another way of refining the intensity and duration of canonical TGFβ signaling (Lönn et al., 2010).

Gopal Sapkota (University of Dundee, UK) presented the discovery of a new player in the BMP signaling pathway: a novel Smad1-associating protein named protein associated with Smad1 (PAWS1), which contains an SSXS motif similar to that found in the C terminus of R-Smads. Indeed, this motif is phosphorylated in

response to BMP. PAWS1 translocates to the nucleus with BMP-Smads in response to BMP and plays a role in the regulation of Smad4-independent genes, raising the possibility that a subset of BMP signals are transduced by Smad1/5/8 in PAWS1-dependent manner.

Aryeh Warmflash (The Rockefeller University, New York, USA) presented one of a few talks dedicated to the dynamic nature of TGFβ signaling. Using a time-lapse imaging technique with single-cell resolution in a mammalian cell culture system, he followed the behavior of Smad2 and Smad4 in response to different types of ligand presentations. Surprisingly, in contrast to Smad2, which stably translocates to the nucleus and reports the ligand level upon stimulation, Smad4 nuclear localization is confined to short pulses that coincide with transcriptional activity, and is thus responsible for temporal control of the pathway. This also occurred in vivo, as Smad4 in *Xenopus* embryos shows stereotyped, uncorrelated bursts of nuclear localization whereas activated R-Smads exhibit a uniform nuclear localization. Thus, the current model in which R-Smad activation is synonymous with signaling activity should be revised such that it accounts for graded information that is integrated with intrinsic temporal control into the R-Smad-Smad4 signaling complex.

Pathway crosstalk with Hippo signaling

Jeff Wrana (Samuel Lunenfeld Institute, Toronto, Canada) addressed the crosstalk between the TGFβ pathway and the Hippo signaling pathway, which regulates cell growth and tissue size. The evolutionarily conserved Hippo pathway is regulated by two kinases, Mst and Lats, which target the transcriptional regulators TAZ and YAP (Halder and Johnson, 2011). Phosphorylation of TAZ and YAP blocks their nuclear translocation and leads to the sequestration of Smad complexes, thereby suppressing TGFβ signaling (Varelas et al., 2010). TAZ interacts with Smad2/3 in ligand-dependent manner and provides a convergence point for the two pathways. Consistently, subcellular localization of TAZ and YAP during mouse embryogenesis defines the embryonic territories in which Smad signaling is active. Furthermore, in polarized epithelial cells, the Crumbs polarity complex also interacts with and facilitates phosphorylation of TAZ and YAP, and elimination of the Crumbs complex increases TGFβ signaling as well as EMT (Varelas et al., 2010). Interestingly, TAZ is required for human embryonic stem cell (hESC) pluripotency, and regulates the expression of multiple pluripotency factors (such as Nanog and Lefty), as part of a transcriptional complex that includes Smads, Tead and Oct4. Thus, as highlighted by Wrana, various aspects of Smad signaling can be governed by cell density through the Hippo pathway.

Hippo-TGFβ crosstalk was also addressed by Alain Mauviel (Institut Curie, Orsay, France), who showed that in various lines of melanoma cells, YAP1 and YAP2 expression levels and Lats1/2 and Mst1/2 phosphorylation vary dramatically. Surprisingly, however, cell density did regulate the nuclear translocation of YAP and TAZ in these cell lines, yet did not lead to a significant inhibition of TGFβ signaling.

Novel insights into cell fate determination and embryonic development

Early mouse embryo development

Elizabeth J. Robertson (Oxford University, Oxford, UK) focused on how different thresholds of Nodal signaling are integrated in early mouse embryos as they develop (from ~110 cells at the epiblast stage to ~660 cells of the gastrula stage). Low levels of Nodal signaling produce mesoderm, whereas higher levels produce definitive endoderm (DE). The intensity and duration of signaling is regulated by a complex set of positive and negative feedback loops. The transcription factor eomesodermin (Eomes), a target of Nodal, is required for EMT, which in turn is required for proper mesoderm migration during the epiblast stage and for DE formation during gastrulation. Robertson showed that Eomes functions in cooperation with different doses of Nodal signaling to regulate discrete spatiotemporally dependent transcriptional cascades that lead to the sequential deployment of mesoderm posterior 1 (Mesp1) and to the generation of two independent sets of fates (Costello et al., 2011).

Kunxin Luo (University of California, Berkeley, USA) addressed the function and mechanism of ski-related novel (SnoN; Skil) regulation of embryonic development and tissue morphogenesis, and showed that showed that the expression of an SnoN mutant, which fails to interact with Smad2/3/4, leads to elevation of ALK5-mediated Smad2/3 activation and repression of ALK1-mediated Smad1/5 activation in endothelial cells. This observation demonstrates a unique role of SnoN in controlling the balance between the ALK5 pathway and the ALK1 pathway in endothelial cells.

Epigenetics, neural crest cells and muscle

Joan Massagué (Memorial Sloan-Kettering Center, New York, USA) argued for the presence of a Smad epigenetic switch in stem cell regulation. He presented a novel function for transcriptional intermediary factor 1γ (TIF1γ, also known as ectodermin or TRIM33) as an essential regulator of transcriptional activation of genes, such as goosecoid (*gsc*), that are regulated by Nodal signals. He showed that the TIF1γ-Smad2/3 complex is recruited to the region of the *gsc* promoter marked with a heterochromatin mark; a histone 3 trimethylation mark located upstream of the previously identified Smad2/Smad4-FoxH1 binding site. This study uncovers a novel role for the TIF1γ/R-Smad complex as a 'pioneer factor' which, upon binding, renders the *gsc* promoter competent for transcriptional activation by Nodal signals.

Caroline Hill (Cancer Research UK, London, UK) investigated how BMP activity is spatiotemporally regulated during *Xenopus* development. A functional screen aiming to isolate genes involved in neural crest specification identified an evolutionarily conserved nuclear protein Snw1 (also called SKIP for SKI-interacting protein). Surprisingly, both loss- and gain-of-function experiments of Snw1 led to the same phenotype: loss of neural crest specification at the border between the neural and non-neural ectoderm at the end of gastrulation and at the beginning of neurula stages. A combination of immunostaining and imaging of a BMP-dependent reporter transgenic zebrafish line established that Snw1 specifically eliminates BMP activity in the ectoderm at the neural/non-neural border. Furthermore, she showed that Snw1 regulates the activity of BMPs by the novel mechanism of participating in a splicing complex that controls expression of target genes that act upstream of the BMP receptor (Wu et al., 2011).

Akiko Hata (University of California, San Francisco, USA) discussed the TGFβ signaling-mediated transcriptional regulation of microRNA-145 (miR-145), which is crucial for differentiation of vascular smooth muscle cells (vSMCs). miR-145 expression is induced upon TGFβ or BMP4 stimulation in vSMCs, which results in downregulation of KLF4, an inhibitor of vSMC-specific gene expression. This finding adds another layer of control to regulation of the vSMC phenotype by TGFβ signaling that is crucial for understanding the pathogenesis of vascular diseases, such as pulmonary artery hypertension and hereditary hemorrhagic telangiectasia (Davis-Dusenbery et al., 2011).

Stem cells and reprogramming

Sheng Ding (The Gladstone Institute, San Francisco, USA) discussed a chemical approach to controlling cell fate, and demonstrated that the combination of three drugs SB431542 (an inhibitor of the Smad2 branch of the TGFβ pathway), PD0325901 (a MEK inhibitor) and Thiazovivin (Tzv) improves the efficiency of reprogramming mammalian somatic cells to induced pluripotent stem cells (iPSCs) when presented after a window of time following retroviral transduction of the four Yamanaka factors (Takahashi et al., 2007). In addition, he provided evidence that another small molecule, Tyrintegin (Ptn), and Tzv improve survival of hESCs when dissociated into single cells, and that Tzv is a novel inhibitor of Rho kinase (ROCK).

Ali Brivanlou (The Rockefeller University, New York, USA) also discussed the role of TGFβ signaling in pluripotency and differentiation of hESCs. The use of 5Z-7-Oxozeaenol, a specific inhibitor of TAK1, demonstrated that TAK1 inhibition results in the rapid loss of pluripotency and the induction of trophoblast differentiation in a BMP-dependent manner. Phospho-proteomic analysis suggested that TAK1 prevents differentiation by activation of MEK, p38 and JNK and by suppression of autocrine BMP signaling. Surprisingly, TAK1 also activates Smad 2/3, which is necessary for TβRI-dependent phosphorylation and the maintenance of pluripotency in hESCs. Thus, TAK1 acts as a central node that integrates signals from the TGFβ, MAPK and BMP pathways to maintain self-renewal and inhibit differentiation.

Conclusions

The FASEB meeting provided an illustration of the current state of our knowledge of the evolutionarily conserved TGFβ signaling pathway. It also indicated priorities for future studies. The dramatically escalating roster of molecular players involved in the pathway includes those that act in an organismal or cell type-specific manner, as well as those that accommodate cross-talk between TGFβ and other signaling pathways. In parallel, the linkage of the pathway to disease states, such as cancer, has had a determining influence on the discovery and development of small compound inhibitors that can be used for therapeutics as well as for basic research. Despite this gain of knowledge, however, some properties of the signaling pathway, namely its ability to act as a morphogen eliciting different outcomes based on different thresholds of activation, remain neglected. This is mostly due to technical complications associated with the measurement and interpretation of morphogen readouts. The elucidation of the dynamics underlying morphogen signaling via both the canonical and the non-canonical branch of the TGFβ signaling pathway also seem to lag behind the elegant biochemical dissections. The future, therefore, promises to provide better resolution to our understanding of morphogen readouts, dynamics of signaling and cross-talk with other pathways. This, in turn, will inevitably impact both our basic understanding of the pathway as well as the molecular circuitry underlying pathogenesis due to inadequately regulated TGFβ signaling. This provides fertile ground for major breakthroughs that will undoubtedly be put forth at the next exciting FASEB TGFβ meeting in 2013.

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Competing interests statement

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