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DEVELOPMENT AT A GLANCE

TGFβ family signaling and development

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ABSTRACT

The transforming growth factor β (TGFβ) signaling family is evolutionarily conserved in metazoans. The signal transduction mechanisms of TGFβ family members have been expansively investigated and are well understood. During development and homeostasis, numerous TGFβ family members are expressed in various cell types with temporally changing levels, playing diverse roles in embryonic development, adult tissue homeostasis and human diseases by regulating cell proliferation, differentiation, adhesion, migration and apoptosis. Here, we discuss the molecular mechanisms underlying signal transduction and regulation of the TGFβ subfamily pathways, and then highlight their key functions in mesendoderm induction, dorsoventral patterning and laterality development, as well as in the formation of several representative tissues/organs.

KEY WORDS: BMP, Development, Embryo, Nodal, TGFβ

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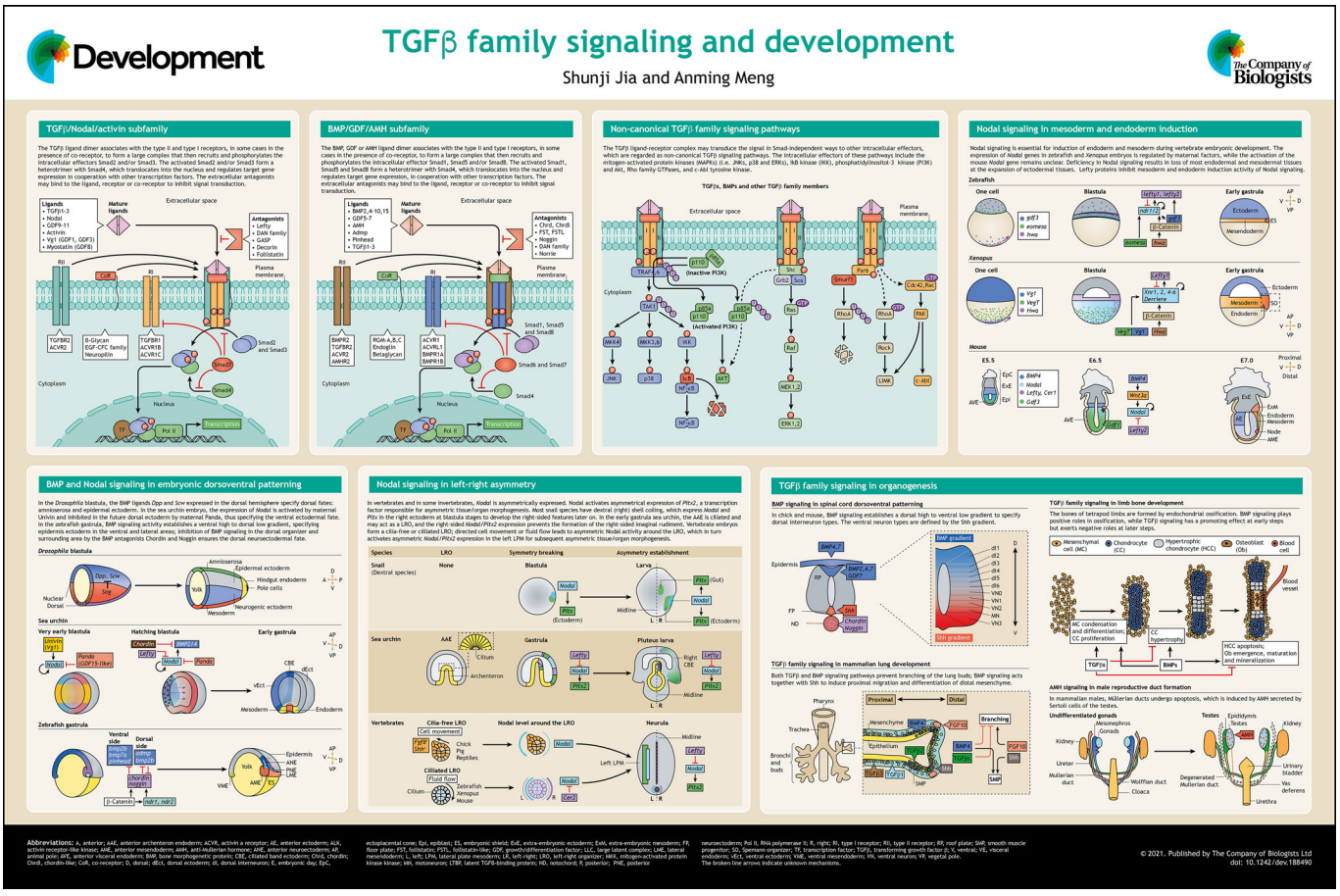
Introduction

Transforming growth factor β (TGFβ) was first discovered in the late 1970s as a secreted polypeptide growth factor with the ability to transform mammalian fibroblasts (Moses et al., 2016). Since then, a growing number of TGFβ family members have been identified, not only in vertebrates but also in invertebrates, including nematodes and flies (Huminiecki et al., 2009). It is now known that TGFβ family members are expressed in germ cells, embryos and various tissues/organs, and regulate versatile cell behaviors, including proliferation, differentiation, transdifferentiation, extracellular matrix (ECM) modeling and remodeling, migration, senescence, and apoptosis. Accordingly, TGFβ signaling pathways play important roles in various developmental processes, including embryonic development and regeneration (Tian and Meng, 2006; Mullen and Wrana, 2017; Zinski et al., 2017), and in pathological processes such as



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tumorigenesis and hereditary disorders (Wu and Hill, 2009; Akhurst and Hata, 2012; Yu and Feng, 2019). Given the large number of family members and multiple functions, TGFβ ligands transduce the signal intracellularly via several mechanisms that can be regulated at distinct modules. Based on differences in signaling mediators and effectors, the TGFβ family is categorized into distinct subfamilies – the TGFβ/Nodal/Activin subfamily and the bone morphogenetic protein (BMP)/growth differentiation factor (GDF) subfamily – or into different pathways: canonical pathway and non-canonical (detailed below). Here, we provide an overview of TGFβ family signaling pathways, and highlight important functions of these pathways in development. We largely refer the reader to review articles due to limited space.

TGFβ ligand-receptor interaction

Mammalian genomes each encode up to 33 TGFβ family ligands, whereas *Drosophila* and *C. elegans* have seven and five TGFβ family ligand genes, respectively (Huminiecki et al., 2009). These ligands have similar structures and share the basic machinery for signal transduction. TGFβ family precursor polypeptides consist of the N-terminal signal peptide, the large prodomain (PD) and the C-terminal mature growth factor (GF). Newly synthesized polypeptides are usually dimerized (e.g. a homodimer or heterodimer such as Bmp2-Bmp7 and Bmp4-Bmp7) in the endoplasmic reticulum. The signal peptides are cleaved, and the dimerized GFs are also cleaved from the PDs, but each PD remains noncovalently associated with its cognate GF for most of TGFβ family proteins (ten Dijke and Arthur, 2007; Derynck and Budi, 2019; Wisotzkey and Newfeld, 2020). The PD-GF complex, which covalently associates with a latent TGFβ-binding protein (LTBP) in the case of TGFβ ligands (TGFβ1, TGFβ2 and TGFβ3), is secreted into the ECM. Like TGFβ proteins, MSTN/myostatin (GDF8) and GDF11 (a PD-bound GF dimer) are inactive and only bind to receptors after removal of PDs (and LTBP for TGFβs) through distinct mechanisms (Derynck and Budi, 2019). In contrast, for some TGFβ family proteins, such as BMP4, BMP5, BMP7 and BMP9, PD-bound GF dimer is active for signaling (Sengle et al., 2011). Furthermore, PD-associated BMP10 is active in endothelial cells but inactive in C2C12 cells, suggesting cell-type dependence (Jiang et al., 2016).

The ligand dimer of the TGFβ family binds to TGFβ receptors on the plasma membrane to initiate signal transduction. Two classes of TGFβ receptors are recognized, type I and type II receptors, both of which are transmembrane dual-specificity kinases. These receptors may exist as monomers, homodimers or/and heterodimers at the cell surface in the absence of ligands (Derynck and Budi, 2019). Seven type I (ACVRL1/ALK1, ACVR1/ALK2, BMPR1A/ALK3, ACVR1B/ALK4, TGFBR1/ALK5, BMPR1B/ALK6 and ACVR1C/ALK7) and five type II (TGFBR2, ACVR2A, ACVR2B, BMPR2 and AMHR2) receptors are known in mammals (Huminiecki et al., 2009). The ligand dimer binds to two type II and two type I receptors, forming a large complex for intracellular signal transduction (Ehrlich et al., 2012; Derynck and Budi, 2019). Combinations of TGFβ type I and type II receptors allow diversity and selectivity for ligands and signaling (Feng and Derynck, 2005; Derynck and Budi, 2019). For example, upon TGFβs stimulation, TGFBR2 may form complexes, directly or indirectly, with TGFBRI, ACVR1, ACVRL1 or BMPR1 to execute different cell functions. ACVR2A/ACVR2B may interact with ACVR1, ACVR1B or ACVR1C to transduce activin signals, or in some cases with ACVR2, BMPR1A or BMPR1B to transduce BMP signals. In response to BMP signals, BMPR2A/BMPR2B may interact with ACVR1, ACVRL1, BMPR1A or BMPR1B. AMHR2 may interact with ACVR1, BMPR1A or BMPR1B to transduce AMH signal. In most cases, type II and type I receptors form homodimers; however, ACVRL1-TGFBR1, ACVR1-TGFBR1 or BMPR1A-TGFBR1 heterodimers interact with TGFBR2 to mediate TGFβ signaling in some cell lines and endothelial cells (Ehrlich et al., 2012; Heldin and Moustakas, 2016). BAMBI, a truncated type I receptor lacking cytoplasmic kinase activity, interacts with TGFβ proteins, activins, BMP proteins or type I receptors to inhibit formation of functional receptor complexes (Nickel et al., 2018).

In some cases, co-receptors are needed to facilitate the ligand-receptor interactions (Nickel et al., 2018; Derynck and Budi, 2019). For example, β-glycan, also regarded as the TGFβ type III receptor, acts as a membrane-anchored proteoglycan to enhance TGFβ association with the TGFBR2-TGFBR1 complex, but its soluble form may associate with TGFβs, activins or BMPs to inhibit signal transduction. The mammalian EGF-CFC family members cripto, cryptic and FRL-1, as well as the zebrafish Oep, associate with Nodal ligands to facilitate their interaction with ACVR1B; neuropilin associates with TGFBR1 to promote downstream signaling; endoglin may bind to BMP ligands or to BMPR2 receptors to facilitate signaling, and may also associate with TGFβ1 or TGFβ3 for signaling through ACVRL1; and the repulsive guidance molecules (RGMs) may function as co-receptors for BMP signaling by directly interacting with ligands and receptors.

The ligand-receptor interaction is highly cooperative, and this process can be regulated by a variety of proteins competing for ligands or receptors (Chang, 2016). For example, lefty (antivin) proteins, divergent TGFβ family members, bind directly to Nodal, GDF1 or GDF3, and/or their co-receptors to block functional ligand-receptor complex formation. Growth and differentiation factor-associated serum protein 1 (GASP1) and GASP2 can bind to and inhibit signaling of MSTN and GDF11; decorin, a small ECM proteoglycan, binds to and sequesters TGFβ1, TGFβ2 or TGFβ3 in the ECM. Secreted proteins chordin (Chrd), chordin-like (Chrdl), follistatin (FST), follistatin-like (FSTL) and noggin mainly bind to BMP subfamily members to prevent signal transduction. DAN family members, including cerberus (Cer or DAND), gremlin and cerberus-like (Cerl), are able to interact with BMP, activin and Nodal ligands, thus interfering with their signaling. Norrie, a secreted cysteine-rich protein, can associate with BMP and Nodal ligands to inhibit their signaling, but can also act as a ligand to activate canonical Wnt signaling. In some cases, the formation of heterodimers between members of different TGFβ subfamilies, such as Nodal-BMP7 heterodimers in *Xenopus* embryos (Yeo and Whitman, 2001), results in mutual antagonism.

Canonical (Smad-dependent) TGFβ signal transduction

TGFβ family signaling was originally found to use the intracellular Smad proteins to exert its biological effect. Therefore, Smad-dependent TGFβ signaling is regarded as the canonical pathway. Mammalian genomes each encode eight Smad proteins: five receptor-activated Smads (R-Smads; Smad1, Smad2, Smad3, Smad5 and Smad8), one co-Smad (Smad4) and two inhibitory Smads (I-Smads; Smad6 and Smad7). Upon the formation of TGFβ ligand-receptor complex, the phosphorylated type I receptors recruit and phosphorylate R-Smads at their C-terminal serines in an S-M/V-S motif (Massague et al., 2005). Depending on which R-Smads act as essential intracellular effectors, canonical TGFβ signaling has traditionally been classified into two major branches: the TGFβ/Nodal/activin signaling pathway and the BMP/GDF/AMH signaling pathway. The former transduces the signal through the type I receptors ACVR1B, ACVR1C or TGFBR1 to Smad2 and/or Smad3; the latter through the type I receptors ACVR1, ACVRL1, BMPR1A

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or BMPR1B to Smad1, Smad5 and/or Smad8. However, in certain circumstances, TGFβs and activin may activate Smad1, Smad5 and Smad8, and some GDFs may transduce the signal to Smad2/Smad3 (Schmierer and Hill, 2007; Xing et al., 2015; Ramachandran et al., 2018). The binding and activation of Smad2 and/or Smad3 by type I receptors can be facilitated by the membrane-anchored cytoplasmic protein SARA or Hgs, or by the rasGAP-binding protein Dok1 (Derynck and Budi, 2019).

Activated R-Smads are released from the type I receptors and usually form a heterotrimer consisting of two R-Smad and one Smad4 molecules. The complex is then translocated into the nucleus to regulate target gene expression (Massague et al., 2005; Derynck and Budi, 2019). With the exception of Smad2, the other R-Smads and Smad4 can directly bind to the specific promoter regions of target genes with weak affinity and limited specificity. The effective and specific DNA binding of R-Smads and Smad4 to target genes requires other transcription factors (TFs), such as the Forkhead family members (FoxH1, FoxO1, FoxO3 and FoxO4), the AP1 family members (FOS and JUN), the Mix homeobox family members (Mixer and Milk) and the E2F family members (E2F4 and E2F5), the RUNX family members (Runx1, Runx2 and Runx3), and the transcription factor SP1 can cooperate with Smad2, Smad3 and Smad4. The TFs Runx2, OAZ, HOXC8, NKX3-2, YY1, β-catenin/Lef1 complex, GATA4/GATA5/GATA6, Tcsit and *Drosophila* Schnurri can serve as Smad1/Smad5-Smad4 complex partners in response to BMP signaling (Feng and Derynck, 2005; Massague et al., 2005; Hill, 2016; Derynck and Budi, 2019). R-Smads may activate transcription of some target genes in the absence of Smad4 (Hill, 2016). In addition, Smad complexes in the nucleus may participate in RNA splicing, miRNA processing and DNA epigenetic modifications (Hill, 2016; Derynck and Budi, 2019).

I-Smads inhibit the receptor-mediated activation of R-Smads through various mechanisms, including associating with type I receptors to interfere in the recruitment and phosphorylation of R-Smads, combining with Smurf proteins and related ubiquitin ligases of the Nedd4 family to induce type I receptor degradation via proteasomal pathway in the absence of ligands, interacting with the GADD34-PP1c complex to dephosphorylate the activated type I receptors, and acting with the Toll-interacting protein Tollip to facilitate endosomal localization of type I receptors for lysosomal degradation (Miyazawa and Miyazono, 2016). In addition, Smad6 inhibits the BMP-induced association between Smad1 and Smad4. Notably, Smad6 prefers to inhibit BMP-induced signaling, whereas Smad7 inhibits both TGFβ and BMP signaling. Smad6 and Smad7 shuttle between the cytosol and the nucleus, and may play a role in transcriptional repression of some target genes (Miyazawa and Miyazono, 2016; Derynck and Budi, 2019).

Non-canonical (Smad-independent) TGFβ signal transduction

The TGFβ ligand-receptor complex may transduce the signal independently of Smads via other intracellular effectors, which is regarded as non-canonical TGFβ signaling. The intracellular effectors of non-canonical TGFβ signaling include: the mitogen-activated protein kinases, i.e. JNK proteins, p38 and ERK proteins; IkB kinase (IKK); phosphatidylinositol-3 kinase (PI3K); Akt, Rho family GTPases; and c-Abl tyrosine kinase. The identity of the effector depends on cell type and physiological/pathological conditions (Moustakas and Heldin, 2005; Vander Ark et al., 2018; Derynck and Budi, 2019).

Upon TGFβ ligand stimulation, TGFβ receptors associate with TRAF6 (or TRAF4) to promote the K63-linked polyubiquitylation of TRAF6, which activates TAK1 and, subsequently, the downstream MAP kinase kinases (MKKs) MKK4, MKK3 and MKK6. MKK4, MKK3 and MKK6 then directly activate the JNK and p38 MAP kinases, respectively, which phosphorylate their target transcription factors in the nucleus to regulate cell behaviors. TGFβ signaling-induced TAK1 activation also causes the phosphorylation of IκB, which is subsequently ubiquitylated and degraded by the proteasome, resulting in the nuclear translocation and DNA binding of NF-κB. TGFβ-induced polyubiquitylated TRAF6 may polyubiquitylate p85a, a regulatory subunit of the PI3K complex, activating PI3K and thus AKT. TGFβ signaling also activates PI3K/AKT pathway by stimulating p85a association with TGFBR1 receptors. The tyrosine-phosphorylated TGFBR1 receptors may recruit and phosphorylate ShcA on serine/tyrosine residues, resulting in the formation of the ShcA-Grb2-Sos complex. The resulting complex activates Ras and then the downstream Raf-MEK-ERK cascade, and may also activate AKT signaling.

TGFβ and BMP may activate the Rho family of small GTPases, including RhoA, Rho-associated protein kinase (ROCK), Cdc42 and Rac1, in a cell-type-dependent manner (Vander Ark et al., 2018). In epithelial cells, the TGFβ ligand-receptor complex activates RhoA; in mesenchymal cells, the BMP ligand-receptor complex induces RhoA and ROCK activation, and the TGFβ ligand-receptor complex recruits Rac1 and Cdc42, subsequently activating their downstream kinase PAK2. In addition, TGFBR2 may directly phosphorylate the polarity protein Par6 by recruiting Smurf1, which targets RhoA for degradation, dissolving tight junctions in epithelial cells and axons (Ozdamar et al., 2005).

Nodal signaling in mesoderm and endoderm induction

The formation of germ layers (endoderm, mesoderm and ectoderm) is a central event in the early development of bilaterians. It appears that TGFβ signaling is nonessential for induction of mesoderm/endoderm (mesendoderm) in invertebrates. In contrast, TGFβ signaling, particularly Nodal signaling, is indispensable for mesoderm and endoderm induction in vertebrate embryos, which is known to be mediated by R-Smads (Schier, 2003; Tian and Meng, 2006; Shen, 2007).

In the zebrafish, two Nodal-related genes, *ndr1*/*squint* and *ndr2*/*cyclops*, are expressed in the blastodermal margin in the late blastula. Nodal signaling activates the expression of its antagonists *lefty1* and *lefty2* in the blastoderm margin. As Lefty proteins diffuse further than Nodal proteins (Muller et al., 2012), they may prevent distant cells from receiving Nodal signal. Maternal Gdf3, a zebrafish ortholog of Vg1, forms heterodimers with Ndr1/Ndr2 to induce mesendoderm (Montague and Schier, 2017; Pelliccia et al., 2017). Recent studies indicate that zygotic expression of *ndr1* and *ndr2* is activated by maternal transcription factor Eomesa (an equivalent of *Xenopus* VegT) and maternal, Hwa-activated β-catenin signaling (Xu et al., 2014; Yan et al., 2018). The expression of *ndr2* also requires maternal *gdf3* (Pelliccia et al., 2017).

In *Xenopus* embryos, mesendoderm induction event resembles that of zebrafish: maternal VegT, likely in cooperation with maternal Vg1, induces zygotic expression of the Nodal-related genes *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5* and *Xnr6*, and another TGFβ family member, *Derriere*, in the vegetal-pole hemisphere. Maternal Hwa-activated β-catenin signaling activates/enhances the expression of these genes in the dorsal side. The Nodal proteins may form high-to-low gradients along the vegetal-animal and dorsoventral axes, thus inducing and patterning endoderm and mesoderm. *Lefty1* expression occurs in the Xnr gene-expressing territories and exerts an inhibitory effect (Smith, 2009; Yan et al., 2018). Mesodermal cells in the zebrafish embryonic

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shield (ES) and the *Xenopus* Spemann organizer (SO) have the highest Nodal activity and acquire axial mesodermal fate to form the prechordal plate and the notochord (Zinski et al., 2017).

The very first *Nodal* gene was originally identified in a mouse mutant line that lacks the node and mesoderm (Zhou et al., 1993). *Nodal* is expressed in the whole epiblast and overlying visceral endoderm (VE) in the pre-gastrula. Nodal signaling induces *Bmp4* expression in adjoining extra-embryonic ectoderm, which activates *Wnt3a* expression in the proximal epiblast and overlying VE. Upregulated *Nodal* expression by Wnt3a and antagonism of *Lefty1* and *Cerl* in the anterior visceral endoderm (AVE) gradually confine Nodal signaling to the proximal region, and then to the proximal-posterior region of the epiblast at the onset of gastrulation (Robertson, 2014). The high Nodal activity in the proximal-posterior epiblast initiates gastrulation, and induces mesoderm and definitive endoderm locally. Ubiquitously expressed GDF1 and GDF3, two Vg1 orthologs, in the epiblast may also play a positive role in mesoderm and endoderm induction (Shen, 2007).

In vertebrates, Nodal signaling can feed back to positively regulate *Nodal* gene expression; and Nodal deficiency results in loss of most mesodermal and endodermal tissues with the expansion of ectodermal tissues (Schier, 2003; Tian and Meng, 2006; Shen, 2007). It remains unclear if and how maternal factors directly regulate *Nodal* gene expression in mammalian embryos.

Bmp and Nodal signaling in embryonic dorsoventral patterning

The cell fates of animal embryos are patterned along the dorsoventral (DV) axis. Increasing evidence suggests that R-Smad-dependent BMP signaling plays a central role in DV patterning from protostomes (e.g. *Drosophila*) to deuterostomes (e.g. sea urchin and mammals). In relation to the position of the central nervous system, the DV axis in invertebrates is inverted in vertebrates (Mizutani and Bier, 2008). In *Drosophila*, the DV axis of an embryo is inherited from the oocyte in which components of the ventral-determining Toll signaling pathway are deposited in the prospective ventral side (Schüpbach and Roth, 1994). Upon cellularization of the syncytial blastoderm, maternal Dorsal protein is released from the protective Dorsal complex via local activation of Toll signaling and translocates into nearby nuclei, resulting in a ventral (high) to dorsal (low) gradient of nuclear Dorsal protein with specification of mesoderm and ventral (neurogenic) ectoderm in the ventral hemisphere. The *Drosophila* Bmp ligands Dpp and Scw are expressed in the dorsal hemisphere and throughout the blastoderm, respectively, while their antagonist Sog (ortholog of the vertebrate Chordin) is expressed in ventral ectoderm. In one way, Sog and the co-inhibitor Tsg complex with Dpp or Scw homodimers or Dpp/Scw heterodimer to prevent the ligands from binding to receptors; in another way, Dpp/Scw heterodimer associated with Sog and Tsg may be transported to and released in the dorsalmost region, greatly enhancing signaling in the midline. As a combinatory effect, Bmp signaling activity forms a dorsal (high) to ventral (low) gradient, which sequentially specifies amnioserosa (for initiating gastrulation) and dorsal (epidermis) ectoderm (O'Connor et al., 2006).

DV patterning of sea urchin embryos is regulated by Nodal and Bmp signaling pathways (Molina et al., 2013). In very early blastulas, *Nodal* expression is activated by maternal *Univin* (*Vg1*) in cooperation with other maternal factors, but repressed by maternal *Panda* (GDF15-like) enriched in the aboral (dorsal) territory, which results in a high (oral/ventral) to low (aboral/dorsal) gradient of *Nodal* transcripts (Range et al., 2007; Haillot et al., 2015). Subsequently, Nodal activates *Bmp2 Bmp4*, *Lefty* and *Chordin* in the oral territory; inhibition of Nodal signaling by Lefty may restrict Nodal expression to the oral territory that is specified as the ventral ectoderm. Bmp2 and Bmp4 may not function in the oral territory due to the presence of Chordin, and are assumed to diffuse to the aboral side to specify the dorsal ectoderm. Low levels of Nodal and Bmp activity in a narrow region between the ventral and dorsal ectoderm permit commitment of a ciliated band ectodermal fate that will give rise to neurons.

DV patterning of vertebrate embryos is best studied in zebrafish and *Xenopus*. The underlying mechanisms in these two species are similar, so only that in the zebrafish embryo is highlighted here (reviewed by Schier and Talbot, 2005; Tuazon and Mullins, 2015; Zinski et al., 2017). In the zebrafish blastula, the Bmp subfamily members *bmp2b*, *bmp4*, *bmp7a* and *pinhead* are expressed in the ventral side. *bmp4* may not be required for ventral development, whereas the other three Bmp members are essential for correct DV patterning. β-Catenin signaling and Nodal (Ndr1/Ndr2) signaling act together to activate the expression of the Bmp antagonists *chordin* and *noggin* in the dorsal margin, which prevents Bmp ligands from signaling in the dorsal side. Fgf signaling represses Bmp gene expression and positively regulates *chordin* and *noggin* expression. The Bmp subfamily members *admp* and *bmp2b* are also expressed in the dorsal margin, thus counteracting local Chordin and Noggin (Xue et al., 2014; Yan et al., 2019). At the onset of gastrulation, Bmp activity (pSmad1,5,8) establishes a ventral high to dorsal low gradient, and low Bmp activity in the dorsal side allows nearby ectodermal cells to acquire neuroectodermal fate, while the other ectodermal cells take the epidermis fate. As gastrulation starts, involution and convergence of mesendodermal precursors in the lateral and ventral blastoderm margins leads to the formation of the embryonic shield (ES) in the dorsal margin and the emergence of the anteroposterior body axis later on. The involuted mesendodermal cells within the ES express TGFβ/Wnt antagonists and move towards the animal pole, so that the overlying neuroectoderm (with low TGFβ/Wnt signaling activities) is specified as the anterior neuroectoderm, and the remaining neuroectoderm (with higher TGFβ/Wnt activities) as the posterior neuroectoderm.

Nodal signaling in left-right asymmetric development

The left-right (LR) asymmetric development of bilaterian embryos, which leads to asymmetric distribution of some tissues and organs in adults, starts with generation of asymmetric cues by various mechanisms. These cues will activate the Nodal-Pitx2 cascade in the right ectoderm of some invertebrate species, but in the left lateral plate mesoderm (LPM) via function of a LR organizer (LRO) in all studied vertebrate species; the transcription factor Pitx2 regulates target genes expression for asymmetric tissue/organ development (Hamada and Tam, 2020). In snails, which do not have a LRO, asymmetric cleavage at the 3rd division and subsequent chiral rotation of micromeres in the animal-pole tier determine the direction of the shell coiling at adulthood. Most of snail species have the dextral (right) chirality, and their blastulas express *Nodal* and *Pitx* in the right ectoderm; and Nodal signaling activates *Pitx* expression through R-Smads in the right ectoderm and gut at larva stages, eventually leading to dextral shell formation (Grande and Patel, 2009).

The anterior archenteron endoderm (AAE) cells of sea urchin midgastrula-stage embryos have a motile monocilium, and *Nodal*, *Pitx2* and *Lefty* are co-expressed in the right AAE and the right ectoderm. At pleutus larva stages, these genes are expressed in the right coelomic pouches and right ectoderm, which prevents the formation of the imaginal rudiment in the right side (Molina et al., 2013; Tisler et al., 2016). The AAE may be regarded as the ancestral form of vertebrate LROs (Tisler et al., 2016).

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In vertebrates, two types of LRO have been found: cilia-free LROs, such as Hensen's node in chick, the pig node and the reptilian blastopore; and ciliated LROs, such as the mouse node, the zebrafish Kuppfer's vesicle (KV) and the *Xenopus* gastrocoel roof plate (Hamada and Tam, 2020; Kajikawa et al., 2020). In chick embryos, leftward movement of Shh^{+};$Fgf8^{+}$ cells around the Hensen's node results in a higher expression level of *Nodal* in the left side of the node, which then activates *Nodal* and *Pitx2* expression in the left LPM. Lefty protein expressed in the midline of the body acts to prevent the Nodal signal in the left side from crossing the midline (Hamada and Tam, 2020). In embryos with a ciliated LRO, cilia movement generates a leftward fluid flow, essentially resulting in asymmetric expression of *Nodal* and the Nodal antagonist *Cer2* (*Dand5*) around the LRO, with higher Nodal levels in the left and higher *Cer2* in the right (Grimes and Burdine, 2017). *Lefty1* in mice, *lefty1* and *lefty2* in zebrafish, and *Lefty* in *Xenopus* are expressed in the midline, inhibiting Nodal diffusion from the left LPM to the right LPM. The zebrafish embryo uses a third nodal-related gene, *ndr3* (*spaw*), to regulate LR asymmetric development (Long et al., 2003).

TGFβ family signaling in organogenesis

TGFβ family signaling plays important roles not only in embryonic development but in the formation and patterning of many tissues and organs. Here, we briefly describe some examples.

Bmp signaling in spinal cord patterning

Following the closure of the neural tube, different zones of the spinal cord along the DV axis generate different neurons, which is mainly regulated by Bmp and Shh gradients (Meyers and Kessler, 2017). As summarized from chick studies, Bmp4 and Bmp7 signaling derived from the overlying epidermal ectoderm activates the expression of TGFβ family members (including Bmp2, Bmp4, Bmp7 and GDF7) in the roof plate, resulting in a Bmp-activity gradient that is antagonized by Chordin and Noggin expressed in the floor plate. The Bmp-activity gradient, in cooperation with Wnt signaling, specifies different types of interneurons in the dorsal region of the spinal cord. Conversely, Shh expressed in the notochord and floor plate forms a reverse gradient that specifies different types of neurons in the ventral region of the spinal cord.

TGFβ family signaling in limb bone formation

Vertebrate long limb bones are formed through endochondral ossification involving multiple processes: mesenchymal condensation and differentiation; chondrocyte proliferation, hypertrophy and apoptosis; osteoblast progenitor differentiation; and osteoblast mineralization. Bmp ligands, including Bmp2, Bmp4 and Bmp7, play positive roles in all these processes. TGFβ ligands, including TGFβ1, TGFβ2 and TGFβ3, appear to promote mesenchymal cells condensation and differentiation into chondrocytes and osteoblasts, but inhibit chondrocyte hypertrophy and osteoblast maturation/mineralization (Salazar et al., 2016; Wu et al., 2016). However, distinct roles for individual and for combinations of Bmp/TGFβ members in different processes remain elusive.

TGFβ family signaling in lung development

The development of mammalian lungs starts from the budding of the anterior ventral foregut to form the trachea, which is partitioned to generate two lung buds. Each lung bud then undergoes branching morphogenesis and alveologenesis, which require TGFβ and Bmp signaling for interaction between endodermal epithelium and overlying mesodermal mesenchymal cells, to form an airway network with thousands of alveoli (Herriges and Morrisey, 2014; Saito et al., 2018). During branching of a lung bud in mouse, *Bmp4* is expressed in the distal epithelium in a non-budding region to prevent Fgf10-induced bud branching. Bmp4 and Shh in the distal epithelium transduce the signal to the distal mesenchymal cells, inducing them to migrate proximately and to differentiate into airway smooth muscle progenitors. *Tgfb1* is expressed throughout the mesenchyme, *Tgfb2* mainly in the distal epithelium and *Tgfb3* in the proximal mesenchyme and the distal epithelium. TGFβ1 protein is enriched in the stalk region and assumed to inhibit budding locally. During alveologenesis, the lung epithelium will differentiate into distinct types of cells, which are likely subjected to regulation by TGFβ ligands (Saito et al., 2018). It has been reported that *Tgfb2* knockout mice have postnatal defective lungs with collapsed conducting airways (Sanford et al., 1997).

AMH signaling in development of the male reproductive organs

During early development of mammalian sexual organs, each bipotential gonad forms adjacent to mesonephros with a Wolffian duct, next to which is a Müllerian duct. In females, the Müllerian ducts will give rise to oviduct, uterus, cervix and the upper region of vagina, while the Wolffian ducts will degenerate; in males, the Müllerian ducts degenerate and the Wolffian ducts differentiate into vas deferens, epididymis and seminal vesicle. The degeneration of the Müllerian ducts in males are ascribed to apoptotic effect of anti-Müllerian hormone (AMH), a BMP subfamily member that is secreted by Sertoli cells in the developing testis (Klattig and Englert, 2007). Deficiency or insufficiency of AMH or AMHR2 in XY-male patients leads to persistent Müllerian duct syndrome, which is characterized by the existence of Müllerian duct derivatives, as wells as a uterus and fallopian tubes (Imbeaud et al., 1994, 1996).

Perspectives

As highlighted above, TGFβ family signaling plays crucial roles in various developmental processes of animals. A multi-cellular organism is a sophisticated system, making investigation of *in vivo* TGFβ signaling mechanisms and functions challenging. For example, as secreted proteins, TGFβ family members presumably form gradients from the source along multiple axes in an organism. Arising questions thus include: what do the gradients look like? How are the gradients and effects regulated? How are the gradients precisely interpreted and transformed to control cell behavior and organismal development? How is signaling activity timely terminated? Complicating this task, is the fact that several TGFβ family members are co-expressed in space and time. Much efforts is needed to dissect roles of individual members or their combinations in development. Accumulating data have indicated that TGFβ family members may transduce signal through non-canonical pathways, revealed mostly in cell cultures. So far, the developmental functions of TGFβ family members are exerted mostly through canonical pathways; it will be interesting to investigate to what extent non-canonical TGFβ signaling activity contributes to developmental processes that are known to be regulated by canonical TGFβ activity.

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Competing interests

The authors declare no competing or financial interests.

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Development at a Glance

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