REVIEW

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Regulation of spatial distribution of BMP ligands for pattern formation

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Abstract

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-ß (TGF-ß) family, have been shown to contribute to embryogenesis and organogenesis during animal development. Relevant studies provide support for the following concepts: (a) BMP signals are evolutionarily highly conserved as a genetic toolkit; (b) spatiotemporal distributions of BMP signals are precisely controlled at the post-translational level; and (c) the BMP signaling network has been co-opted to adapt to diversified animal development. These concepts originated from the historical findings of the Spemann-Mangold organizer and the subsequent studies about how this organizer functions at the molecular level. In this Commentary, we focus on two topics. First, we review how the BMP morphogen gradient is formed to sustain larval wing imaginal disc and early embryo growth and patterning in *Drosophila*. Second, we discuss how BMP signal is tightly controlled in a context-dependent manner, and how the signal and tissue dynamics are coupled to facilitate complex tissue structure formation. Finally, we argue how these concepts might be developed in the future for further understanding the significance of BMP signaling in animal development.

KEYWORDS

dpp, embryogenesis, morphogenesis, organogenesis

1 | INTRODUCTION

In 1924, Hans Spemann and Hilde Mangold proposed the organizing principles underlying the genesis of a body plan in the animal kingdom.[1] They demonstrated that tissue explants excised from the blastopore lip of salamander embryos had the capacity to induce the formation of a second body axis when transplanted into another embryo, resulting in a conjoined twin individual. The

inducing blastopore lip tissue, which invaginates into the blastocyst embryo during gastrulation, ultimately inducing genesis of endodermal, mesodermal, and ectodermal embryonic germ layers in the host recipient, came to be known as the Spemann-Mangold organizer after its discoverers.[2,3] Although it was believed that the organizer contains chemicals that play critical roles in dorsal induction, it took over half a century to unveil molecular mechanisms underlying embryogenesis regulated by the organizer. In 1994, De Robertis and his colleagues found that Chordin is a key molecule of the Spemann organizer

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in the *Xenopus laevis* embryo.[4] Although Chordin mRNA injection into the ventral part of the embryo is sufficient to induce secondary axes in the frog embryo, Chordin itself does not induce a cellular signal. Instead, Chordin was shown to inhibit BMP signal, and inhibition of BMP signal in the dorsal embryo leads to neural induction.[3,5,6]

Similar mechanisms were independently found in the fruit fly *Drosophila melanogaster*. In the 1980s, Christine Nüsslein-Volhard and Eric Wieschaus performed systematic forward genetics screens, and identified seven zygotic mutant alleles that are required for dorsal cell fate determination in the early embryo.[7] These include *decapentaplegic (dpp), screw (scw), short gastrulation (sog), shrew (srw), tolloid (tld), twisted gastrulation (tsg), and zerknüllt (zen).*[8,9] Interestingly, *sog* was identified as a Chordin ortholog that binds BMPs, and *dpp* and *scw* encode BMP-type ligands.[6,10-13]

Considering that Chordin/Sog and BMP signaling play key roles in dorsoventral axis formation in the early embryo, why Chordin and Sog are required for dorsal and ventral neuroectoderm in frog and fly embryos, respectively, was puzzling. Saint-Hilaire first attempted to address this question in the 19th century (reviewed by Louryan and Vanmuylder[14]). It was proposed that the position of the nervous system was inverted in the evolution of different animals, and therefore, the nervous system forms dorsally in vertebrates and ventrally in invertebrates. This hypothesis handily explained the inverted BMP and BMP antagonist axis observed between *Drosophila* and *Xenopus*.[15] The inverted axis notwithstanding, the evolutionary conservation of the BMP signaling network indicates its ancient origin in dorsoventral axis determination in bilaterians.[16]

2 | BMP MORPHOGEN GRADIENT FORMATION IN THE LARVAL WING IMAGINAL DISC AND EARLY EMBRYO OF *DROSOPHILA*

BMP signal is spatiotemporally controlled in various developmental contexts. We mainly focus on *Drosophila* BMP-type ligand Dpp, and how Dpp signal is precisely controlled in a context-dependent manner. One interesting feature of Dpp is that signal distribution is tightly controlled at the extracellular level to form an activity gradient as a morphogen. Several ways of ligand distribution have been proposed, including free diffusion, restricted diffusion, transcytosis, filopodia/cytoneme-mediated, and instructive/permissive models.[16-20] Here, we summarize two distinct models of Dpp morphogen signal in the larval wing imaginal disc and the early embryo.

2.1 | Long-range Dpp signaling in the larval wing imaginal disc

Lewis Wolpert's French flag model of morphogen signaling described the paradigm of developmental fate induction as function of concentration and space: a high local concentration of morphogen emanates from a source and forms a gradient, and cells in a developmental field receive decreasing amounts of morphogen as a function of distance from the source.[21] Cells then interpret their relative position based on threshold amounts of morphogen that reach them, and assume distinct developmental fates by expressing different target genes.

The *Drosophila* wing imaginal disc serves as an excellent model to understand how growth and pattern formation of developing tissues are regulated. Multiple conserved growth factor signaling pathways, including BMP/Dpp, Hedgehog (Hh) and Wnt/Wingless, are all involved as morphogens to sustain wing imaginal disc growth and patterning.[22,23] In particular, Dpp signaling exemplifies the French flag model. Establishing and stabilizing the Dpp morphogen gradient in the wing imaginal disc involves various molecules (listed in Table 1, Figure 1A).

Short-range Hh signaling induces *dpp* expression in the wing imaginal disc as a stripe at the anterior part of the anteroposterior boundary. Dpp ligand is subsequently produced and secreted at the stripe region and forms a concentration gradient by spreading across the disc to exert its effects (Figure 1B),[22,28] which include transcriptional regulation of downstream targets via phosphorylated Mothers against Dpp (pMad). Anti-pMad antibody staining of wild type wing discs reveals a characteristic pMad localization pattern, perturbation of which is indicative of aberrant Dpp signaling.[29]

Dpp signaling drives both patterning and growth in the wing disc. In patterning, Dpp forms a spatially opposed gradient with the transcriptional repressor Brinker (Brk): Brk expression is highest at the anterior and posterior edges of the disc, and tapers off medially.[30] The opposing Dpp and Brk gradients result in different relative levels of Dpp and Brk activity across the disc, which trigger spatially delimited, nested expression patterns of the downstream effector genes *spalt major* (*salm*) and *optomotor-blind* (*omb*) when threshold levels of signaling are met (Figure 1A,B). The wing longitudinal vein L2 forms in the anterior compartment at the boundary of Salm expression, whereas the longitudinal vein L5 is determined at the border of the Omb and Brk expression regions. Thus, Dpp and Brk gradients translate into spatially controlled patterning outputs.[22,31]

Concurrently with its patterning function, Dpp also drives disc growth: mutations that reduce Dpp levels

TABLE 1 Core components of BMP signaling pathway in *Drosophila* and human

	Drosophila melanogaster	Human
Ligand	Dpp (Decapentaplegic) Gbb (Glass bottom boat) Scw (Screw)	Bpm2/4 (bone morphogenetic protein 2/4) Bmp5/6/7/8a/8b Bmp5/6/7/8a/8b
Type I receptors	Tkv (Thickveins) Sax (Saxophone)	BMPR1B (bone morphogenetic protein receptor type 1B) ACVRL1 (activin A receptor like type 1)
Type II receptors	Put (Punt) Wit (Wishful thinking)	ACVR2B (activin A receptor type 2B) BMPR2 (bone morphogenetic protein receptor type 2)
R-SMADs	Mad (Mothers against dpp)	SMAD1/5/8 (SMAD family member 1/5/8)
Co-SMADs	Med (Medea)	SMAD4 (SMAD family member 4)
I-SMADs	Dad (Daughters against dpp)	SMAD6/7 (SMAD family member 6/7)
BMP antagonists	Sog (Short gastrulation) Tsg (Twsited gastrulation) Cv (Crossveinless)	CHRD (chordin) TWSG1 (Twisted gastrulation BMP signaling modulator 1)
Protease	Tld (Tolloid) Tok (Tolkin)	TLL1 (tolloid like 1) BMP1

Note: The functions of Wishful thinking, Medea, and Dad are outside the scope of this commentary and are discussed elsewhere.[24-27]

compromise wing disc growth,[32] while individual cell clones in which Dpp signaling is ablated are eliminated.[33,34] On the other hand, ectopic expression of Dpp can induce overgrowth of the disc.[35] Various experimental evidence points at different models of wing disc growth control by Dpp.[17] An approach employing morphotrapping, in which spread of GFP:Dpp is essentially abolished by spatially overlapping expression of an anti-GFP membrane-tethered antibody (the morphotrap), favors a model in which Dpp is essential only for medial wing disc growth.[36] Further studies suggest that down-

regulation of Brk by Dpp in the medial disc is sufficient for maintaining Dpp-dependent tissue growth, indicating the Dpp/Brk signaling axis coordinates pattern formation and tissue growth via distinct mechanisms.[37,38]

A pioneering study demonstrated the importance of the BMP I type receptor Tkv on the gradient profile. The pattern of Tkv distribution across the wing disc is the inverse of the Dpp pattern, with little Tkv detected at the medial Dpp stripe, and increasing Tkv in lateral disc regions (Figure 1B).[29,39] Tkv is mainly responsible for limiting the diffusion range of Dpp.[40,41] Tkv is expressed at low levels in the central region, allowing Dpp to be secreted and diffused easily. In contrast, high lateral Tkv transcriptional expression reduces lateral Dpp concentration. Thus, Tkv has the role of fine-tuning the long-range signal region and the concentration distribution band of the Dpp. Relatedly, a medial stripe of Dpp also forms in the wing-like haltere disc.[42] However, Tkv is expressed at high levels at the medial stripe, limiting Dpp diffusion and thus shrinking the tissue size compared to the wing.

Heparan sulfate proteoglycans (HSPGs), a.k.a. glypicans, are modulators of signaling molecule activity that exert their effects by nonspecifically interacting with ligands, stabilizing their location and retaining them to form a ligand pool, thus prolonging the ability of ligand to interact with its specific receptor. In the wing imaginal disc, the glypican Division abnormally delayed (Dally) contributes to the shape of the BMP gradient in this manner (Figure 1B).[43] Dally overexpression at the Dpp source stripe increases pMad at the stripe, but results in severely reduced pMad accumulation in cells immediately flanking the stripe, indicating that an excess of Dally ties up the Dpp pool available for forming the gradient by preventing binding to Tkv, thus abrogating downstream signaling.

The protein Pentagone (Pent) was uncovered as a modulator of BMP activity in the lateral wing disc in the context of studies characterizing genes for which expression is suppressed by BMP signaling.[44] Pent is expressed and secreted laterally at the anterior and posterior edges of the disc (Figure 1B). *pent* mutant adult wings show L5 loss distally to the PCV, and reduced wing size overall. In *pent* mutant wing discs, lateral Dpp diffusion from the stripe is severely compromised, and lateral Dpp signaling is correspondingly reduced, suggesting Dpp is "consumed" locally, thus losing its long-range signal. These aspects of the *pent* mutant phenotype are partially rescued by reducing Tkv receptor levels across the disc, which compromises Tkv receptor ability to serve as a ligand sink, and suggests that Pent functions by preventing medial Dpp retention and facilitating its lateral spread.
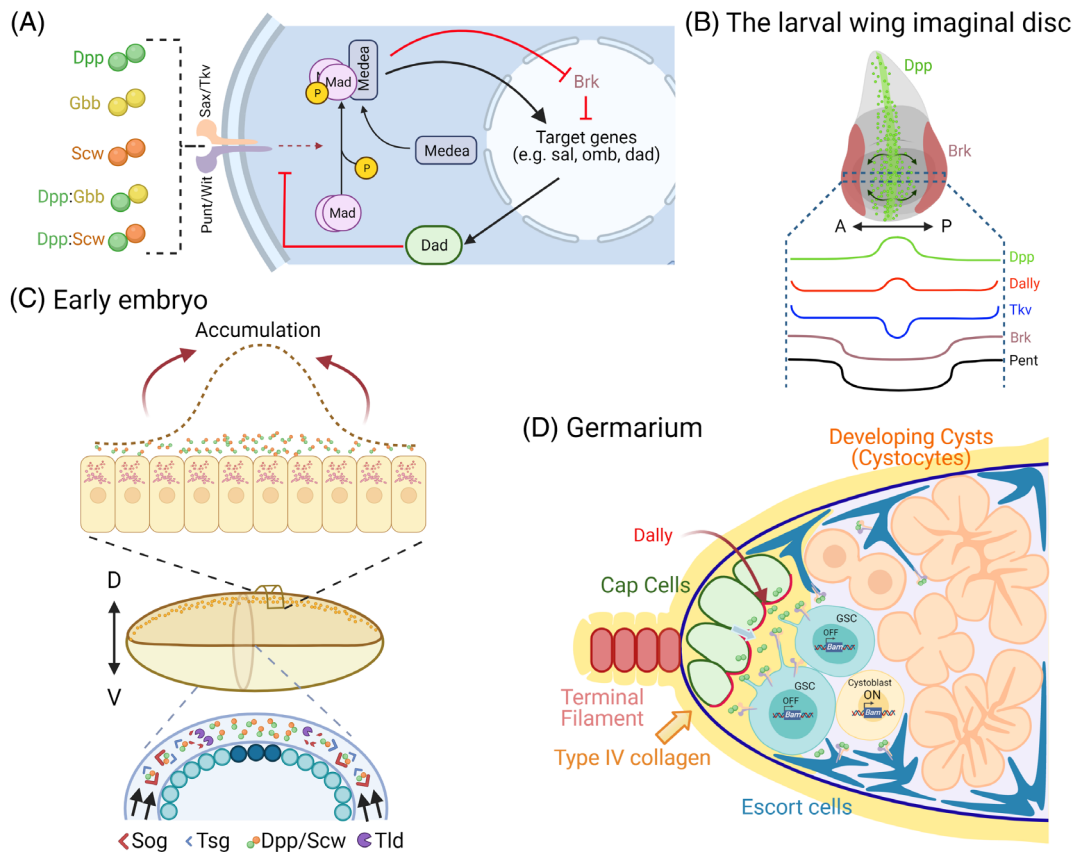
FIGURE 1 Overview of BMP signal and tissue development. (A) BMP signal transduction in *Drosophila*. The signal initiates when Dpp homodimer (or in heterodimers with one of two other *Drosophila* BMP ligands, Gbb or Scw) associates with tetrameric receptor complexes, comprising combinations of Type I receptors Tkv/Sax and Type II receptors Wit/Punt, at the plasma membrane. The activated receptors phosphorylate Mad, which associates with Med and accumulates in the nucleus to modulate gene expression. (B) Long-range Dpp morphogen gradient in the *Drosophila* wing imaginal disc. Schematic view of Dpp (green), Tkv (blue), Dally (red), Brk (brown), and Pent distribution (black) across the anteroposterior axis in the wing pouch. A, anterior; P, posterior. (C) Dpp morphogen gradient in the *Drosophila* early embryo. Top: schematic illustration of Dpp accumulation to form the morphogen gradient. Bottom: Schematic of the molecular events that facilitate the formation of the embryonic Dpp activity gradient. Scw, Tld, Tsg, and Sog are secreted, and physically interact to form a stably transient complex with ligands, resulting in a net flow of the Dpp:Scw heterodimer to the dorsal midline. Dorsal (D) is up and ventral (V) is down. (D) Short-range Dpp signal in the *Drosophila* germarium in the ovary. The cap cells, a key part of the GSC niche, are located at the anterior tip of the germarium. A *Drosophila* germline stem cell (GSC) identity is maintained by Dpp secreted by cap cells. Dally, secreted by and presented on cap cells, promotes short-range signaling possibly as a ligand co-receptor. Vkg is deposited in between cap cells and GSCs, which binds to extracellular Dpp near GSCs and prevents its posterior spread. Expression of the Tkv receptor on escort cells (ECs) acts as a ligand sink. Ligand binding to receptors leads to phosphorylation of Mad, which, together with Med, represses *bam* transcription. (Created with BioRender.com)

At the level of adult wing L5 vein loss and wing size reduction, *pent* and *dally* single and double mutant phenotypes resemble each other. Additionally, Pent and Dally proteins interact biochemically with each other, and Pent fails to accumulate in *dally* mutant clones, suggesting that Pent exerts its Dpp gradient-spreading effects by removing Dally from the cell surface.[45] Thus, Pent misexpression anteriorly or posteriorly results in corresponding Dally removal, less anterior or posterior Dpp retention, and more Dpp diffusion into, and reciprocal enlargement of, posterior and anterior compartments, whereas medially misexpressed Pent enlarges both compartments. Pent therefore functions by removing Dpp co-receptor Dally, scaling, and shaping the Dpp gradient by freeing Dally-bound Dpp to spread and exert its growth and patterning effects in lateral regions of the disc. Moreover, given that Dpp signaling controls *pent* expression, and Pent in turn controls Dpp spread, Dpp and Pent form a feedback loop that can fine-tune Pent levels across the disc to enable Dpp gradient shaping.[46] Dpp and Pent interactions thus apparently constitute an expansion-repression (ER) feedback mechanism, in which a morphogen negatively regulates the expression of a diffusible molecule that expands morphogen spread

as a tissue increases in size.[47] A prerequisite for an ER mechanism of morphogen gradient scaling to apply is the ability of the expander to spread in a uniform manner across the morphogen field.[47] Conversely, recent work illustrates that the mobility range of Pent is tightly spatially constrained, and thus that Pent fulfills the expander concentration requirement invoked in the ER model only when the wing disc is sufficiently small.[48] Therefore, during the third larval instar, as the wing disc reaches its maximal size, alternative mechanisms of gradient shaping and scaling must be in play.

The experimental evidence for the roles of BMP ligands, receptors, co-receptors and gradient-scaling factors in generating the morphogen gradient that drives growth and patterning of the wing disc is considerable. Nonetheless, how precisely these molecules interact to shape the gradient, resulting in the highly reproducible developmental outcome of correctly patterned adult wings, remains an object of intense investigation. Recent experimental approaches involving engineered ligands and receptors elucidate plausible mechanisms of morphogen gradient shaping: Stapornwongkul et al. describe a synthetic system in which GFP replaces Dpp as the morphogen, high-affinity GFP nanobody-fused Tkv and Punt serve as GFP receptors that transduce signal upon GFP docking, and low-affinity GFP nanobody-fused Dally functions as a nonsignaling GFP receptor that slows GFP ligand spread.[20,49] Co-expressing GFP ligand and fused synthetic high-affinity GFP receptors in a wing-specific knockout *dpp* background partially rescues both wing disc vein prepatterning, and normal adult wing growth and patterning. In contrast, expressing a low-affinity nonsignaling GFP receptor along with GFP ligand and high-affinity signaling GFP receptors results in improved wing disc and adult wing rescue outcomes. This synthetic approach thus recapitulates wild-type Dpp signaling developmental outcomes to a significant extent, and provides a paradigm for further experimental dissection of the roles of ligands, receptors and co-receptors in morphogen gradient shaping, scaling, and subsequent organ growth and patterning.

2.2 | Morphogen gradient formation in the early embryo

Among the aforementioned seven zygotic mutant alleles that are required for dorsal cell fate determination in the early embryo, *dpp*, *scw*, *sog*, *tsg*, and *tld* were eventually shown to encode BMP signaling pathway ligands and ligand interactors.[8,9] BMP signaling plays its essential roles in patterning the early *Drosophila* embryo by forming a BMP morphogen gradient. Genesis of the BMP activity gradient in this developmental context is unique: in lieu of high local production of BMP ligand that subsequently spreads across a developmental field, the ligand is instead initially expressed more widely and at low levels across the field, and then concentrates locally by transport mechanisms to form a gradient. Therefore, the molecular mechanisms that shape the BMP gradient in this developmental context are distinct from those in the wing imaginal disc. A description of the molecules involved in early embryonic BMP gradient formation follows.

Ligands Dpp and Scw

Early embryonic expression of *dpp* is regulated by the dorsoventral body axis determinant Dorsal (Dl), which localizes uniformly throughout the embryo but forms a nuclear concentration gradient in a manner dependent on Toll receptor signaling, with peak nuclear Dl concentration ventrally.[50,51] This Dl gradient provides positional information that subdivides the dorsoventral axis into distinct regions, each in which differential gene activation occurs. *dpp* expression is initially uniform across roughly the dorsal half of the embryo; accordingly, Dpp protein is distributed in the dorsal half of the embryo.[52,53] At the onset of cellularization, Dpp accumulates at the dorsal midline of the embryo and forms a sharp gradient, leading to a peak level there that drives amnioserosa genesis, and lower signaling levels laterally for dorsal ectoderm formation (Figure 1C).[8,54]

scw encodes a BMP5/6/7/8-type ligand.[11] Its expression occurs widely throughout the embryo periphery during early embryogenesis before shutting down prior to gastrulation. In *scw* mutant embryos, Dpp fails to accumulate at the dorsal midline, suggesting Dpp functions in a complex with Scw. Experimental evidence points at Dpp and Scw forming a heterodimer (Figure 1C).[53] Additional studies revealed that Dpp:Scw heterodimers show higher signaling activity than Dpp homodimers, and that heterodimers are thought to buffer perturbations in ligand availability.[53]

Ligand transporters Sog and Tsg

Whereas *dpp* expression is activated farthest from the Dl source, *sog* is expressed immediately ventrolaterally to *dpp*.[55] Sog, a Chordin ortholog, encodes a BMP-binding protein to generate a Dpp activity gradient within the dorsal region by preventing Dpp from interacting with its receptor dorsolaterally. Consistent with a role as a BMP antagonist, *sog* null mutant embryos have an expanded dorsal ectoderm.[8,55,56] However, *sog* null mutant embryos do not show an expansion of the amnioserosa, a transient field of cells functioning as a signaling center that directs germ band retraction and dorsal closure, which arises dorsally and requires peak-level BMP signaling in the dorsal midline stripe for its specification. Instead, only a

few amnioserosa cells differentiate in *sog* null embryos, suggesting that Sog is required to promote high-level BMP signaling at the dorsal midline. Another extracellular modulator of BMP signaling, *tsg*, is expressed in the dorsal 40% of the embryo and encodes a secreted protein.[57] *tsg* mutants also display a loss of amnioserosa, suggesting that Tsg as well is required for peak-level BMP signaling at the dorsal midline. Tsg physically interacts with Sog, the Sog/Tsg complex binds to Dpp:Scw dimers to inhibit BMP signaling, and Dpp:Scw heterodimers have a higher affinity for Sog and Tsg than either homodimer.[52,53] The apparent paradox of a requirement for BMP inhibitors that nonetheless facilitate amnioserosa-specifying BMP activity at the dorsal midline is explained by the Dpp shuttling function of Sog/Tsg, which results from ventrolaterally produced Sog diffusing dorsally down its concentration gradient, thus facilitating dorsalward Dpp transport. In the absence of the Sog/Tsg shuttling complex, extracellular BMP levels remain low and uniform throughout the entire dorsal domain instead of concentrating at the dorsal-midline cells.

Proteolytic release of Dpp:Scw from Sog/Tsg through Tld

tld encodes a metalloprotease orthologous to vertebrate TLL1 and is expressed in a manner overlapping with *dpp*.[58] Accordingly, a *tld* allelic series comprising successively stronger mutant alleles shows increasing ventralization toward the dorsal midline, and Dpp overexpression can partially rescue these ventralized *tld* mutant phenotypes. Upon Dpp:Scw transport by Sog/Tsg to the dorsal midline, Dpp binding to its receptor requires proteolytic activity of Tld, which cleaves and inactivates Sog in a Dpp-dose-dependent manner.[53,59] Although *tld* is broadly expressed dorsally, biochemical cell-based assays revealed that the dynamic range over which Dpp functions in signaling is the same over which Dpp stimulates Sog proteolysis by Tld,[59] hinting at a mechanism that can spatially limit Tld activity to the dorsal Dpp stripe.

BMP receptors Tkv, Sax, and Punt

Upon Tld-mediated release of Dpp:Scw from Sog/Tsg, receptor docking occurs to receptor complexes comprising Tkv, Sax, and Punt. Although in a cell-based assay, Dpp:Scw was shown to signal synergistically through both Tkv and Sax,[53] under normal signaling conditions, downstream pMad activation and subsequent gene activation occurs only via receptor complexes comprising Tkv and Punt. In the context of early embryonic development, while receptor complexes with Sax can bind to ligand, it is normally prevented from activating downstream signaling by O-GlcNAcylation via the O-linked glycosyltransferase Super sex combs (Sxc).[60] In the absence of Sxc activity, or under limited maternal access

to sugar, Sxc fails to glycosylate Sax, resulting in Sax interactions with Dpp:Scw, overactivation of downstream Dpp signaling, failures in dorsal closure and embryonic death. Sax' role in the BMP signaling axis in embryogenesis under normal conditions may thus be modulation of BMP activity in the dorsal embryo by titrating ligand.

Viking (Vkg), a Type IV collagen

The fact that Dpp in a complex with Sog is widely diffusible in the dorsal embryo, but free Dpp is tightly localized, suggested a separate mechanism that facilitates Dpp retention for generating the dorsal stripe.[61] Vkg was characterized as an extracellular matrix component that interacts specifically with Dpp, immobilizing it and facilitating the stepwise formation of the Dpp/Sog/Tsg shuttling complex.[62,63] In *vkg* heterozygous mutant embryos, Dpp and pMad are found in a narrower stripe at the dorsal midline than in wild type, suggesting that reducing Vkg levels results in retention of less Dpp at the dorsal midline stripe, with concomitant lower pMad activation there. Additionally, expression of other downstream Dpp target genes in the amnioserosa is largely abrogated.[62] Therefore, Vkg facilitates extracellular immobilization of ligand, thus enhancing its availability to bind receptor.

In summary, in wing imaginal discs and in embryos, the BMP signaling toolkit fulfills key roles in patterning and growth, and functions via BMP ligand gradients that operate over a long range. Although the core signaling pathway components and proteins in the extracellular environment function in similar ways, divergent mechanisms bring about gradient formation in different developmental contexts, dependent on whether the gradient is generated by ligand production in a limited area with subsequent spreading, or by widespread ligand production and its subsequent spatial accumulation. Irrespective of the mechanism that forms the gradient, however, the gradient itself is essential in achieving the correct patterning effects, and its formation is therefore tightly controlled, using multiple mechanisms to generate and stabilize its shape.

3 | SPATIAL REGULATION OF BMP SIGNAL AND COUPLING BETWEEN BMP SIGNAL AND MORPHOGENESIS

When BMP signal transduction takes place in developing tissue, spatial distribution of BMP signal is differentially and robustly regulated in a context-dependent manner. BMP signal not only induces a gene regulatory network which is involved in pattern formation, but often directly impact cell shape dynamics, which are crucial

mechanisms of tissue morphogenesis. Importantly, the dynamics of cell and tissue shaping caused by BMP signaling may feed back into the signaling itself. Therefore, understanding how developmental signal and dynamic morphogenesis are coupled has become a focus of investigation. Here, we review several examples of spatial regulation of BMP signal and coupling mechanisms between signal and morphogenesis.

3.1 | The *Drosophila* female germline stem cell niche: short range BMP signaling in tissue homeostasis

Tissue homeostasis in adult metazoan organs is often maintained by a pool of adult stem cells that divide asymmetrically to generate new stem cells and differentiating daughter cells. The stem cells reside in spaces termed niches, which themselves comprise cells from which signals emanate over a short range that preserve the stem cell identity of the cells at the niche. Imbalances in stem cell division can result either in a surplus of stem cells, which can give rise to harmful neoplasms, or in overdifferentiation into daughter cells, which depletes the stem cell pool and leads to tissue degeneration.

A *Drosophila* ovary comprises several ovarioles. Each ovariole is a blind tube in which 2 to 3 germline stem cells (GSCs) reside at the closed end, termed the germarium. Terminal filament, cap, and escort cells comprise the niche (Figure 1D). A pioneering study by Xie and Spradling demonstrated that Dpp signal from the cap cells plays an instructive role in GSC maintenance.[64] Without Dpp signal, GSCs are not maintained in the germarium; in contrast, ectopic expression of *dpp* leads to a significant increase in the number of GSCs. The Dpp signal maintains GSC identity by silencing expression of *bag-of-marbles* (*bam*).[65] Daughter cells lose contact with the niche and move away from it to start the oocyte differentiation program, first becoming cystoblasts. In moving away and losing contact with the niche, Dpp signaling in daughter cells falls below the threshold required to suppress *bam* expression.

In the germarium, control of the Dpp ligand range in the localized space is achieved through several mechanisms that aid in intercellular Dpp signal interpretation, and provide robustness to the extracellular Dpp gradient itself. Indeed, interaction with ECM components helps to localize Dpp ligands. For example, Dally is expressed in cap cells to reinforce specific Dpp signals at the GSCs by either concentrating the Dpp ligand, or by sensitizing the GSCs to the ligand.[66] Elimination of Dally from cap cells reduces Dpp signaling, leading to loss of GSCs due to differentiation, while misexpressing Dally in escort cells

increases the number of GSCs. Vkg is also localized in the GSC niche, where it limits Dpp diffusion range. Vkg functions by binding to extracellular Dpp around the GSCs, restraining its spread, and potentially boosting signaling by promoting Dpp-receptor interactions.[62,67] Consistent with a role for Vkg in concentrating and retaining Dpp in the immediate vicinity of GSCs, *vkg* mutants result in Dpp spread away from the niche region, with a concomitant increase in GSC number. Dpp overexpression in the niche saturates the ability of Dally and Vkg to spatially limit Dpp signaling to the niche, giving rise to far-ranging Dpp spread, continued suppression of *bam* expression in daughter cells, and a tumorigenic phenotype. On the other hand, Tkv expression in escort cells can partially rescue Dpp overspreading phenotypes by functioning as a ligand sink.[68]

Recent studies further reveal that cell shape changes may also contribute to sustain the short-range Dpp signal.[69] Membrane protrusions called cytosensors are often induced by Dpp signal and attenuate Dpp signals in GSCs adjacent to cap cells. Interestingly, importance of membrane protrusion formation has been proposed in male GSC-niche interactions as well.[70]

In sum, the BMP signaling toolkit and extracellular accessory proteins have been adapted to the context of the physically constrained space of the germarium to produce a short-range BMP signal that facilitates stem cell identity maintenance and resulting tissue homeostasis.

3.2 | Coupling between BMP signal and epithelial morphogenesis

In *Drosophila* development, metamorphosis occurs during pupariation. During this period, the wing imaginal disc, a single layered epithelial sheet, undergoes disc eversion to become a pupal wing composed of two epithelial cell layers (Figure 3A).[71,72]

Dpp signaling plays a critical role in pupal wing development. One group of *dpp* mutant alleles called *shortvein (shv)* contains mutations in the enhancer region responsible for *dpp* transcription in the pupal wing.[13,73] Phenotypes of *shv* alleles indicate that Dpp functions as a wing vein determinant during pupariation.[73] Although *dpp* is expressed in the longitudinal veins but not in crossveins of the pupal wing, BMP signal is positive in all wing vein primordial cells, including crossveins (Figure 2).[74] To sustain BMP signal in the posterior crossvein (PCV) cells, two BMP ligands, Dpp and Gbb, two BMP binding proteins, Sog and Tsg-related Crossveinless, and Tld-like protease Tolkin are required.[74-77] Further studies demonstrated that ligand trafficking mechanisms similar to those in the early
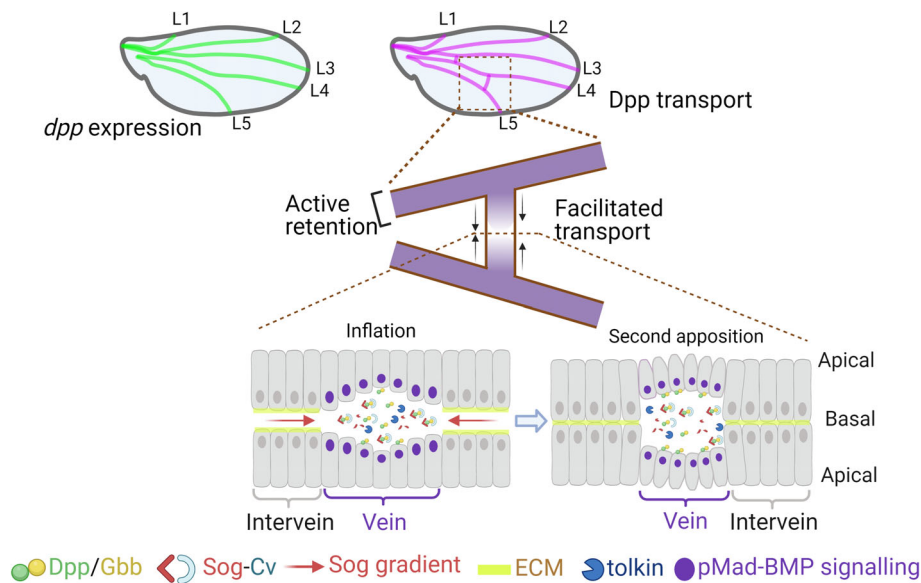
FIGURE 2 Schematic diagram of wing vein development in the pupal wing. Top: *dpp* mRNA is expressed in longitudinal veins but not in crossveins during early pupal stages (left). However, Dpp signal distribution is detected at all wing vein primordia, including longitudinal veins and crossveins (right). Bottom: The facilitated transport of Dpp ligands from the longitudinal veins into the posterior crossvein (PCV). Dpp or Dpp:Gbb, produced and retained by L4 and L5 cells, form a complex with transporter BMP-binding proteins such as Sog and Cv, and move by a facilitated transport mechanism. Proteolytic cleavage of Sog by Tolkin releases the Dpp:Gbb heterodimer from the ligand-transporter complex, thus freeing Dpp:Gbb to signal in the PCV. (Created with BioRender.com)

embryo are utilized, suggesting that molecular mechanisms underlying BMP signal in the PCV appear to be co-opted.[74]

Since BMP-induced PCV development involves wing morphogenesis, and BMP is the only growth factor needed for the initial stage of PCV development, PCV development serves as a model to address how BMP signaling and morphogenesis are coupled. Besides the core BMP pathway components involved in signal induction in the PCV, several co-factors have been identified. Analysis of those factors provide hints at how the coupling mechanisms can be interpreted.

One of the classical crossveinless alleles, *crossveinless-c (cv-c)*, originally identified in 1934, was subsequently identified as a RhoGAP.[78] Cv-c is induced in the PCV primordial cells by Dpp signaling, where it cell-autonomously inactivates signaling from several Rho-type small GTPases.[79] This leads to the cell-autonomous downregulation of Rho GTPase targets such as integrins. The downregulation of integrins in the basal compartment of PCV primordial cells provides an optimal extracellular environment for Dpp trafficking. Therefore, these cellular mechanisms mediate a feed-forward loop to sustain PCV development.

Another study indicates that coupling between BMP signal and apico-basal polarity determinant Scribble (Scrib) is required for PCV development.[80] Although Scrib expression is generally crucial for homeostasis of epithelial cells,[81] BMP signal boosts Scrib levels for further polarization, which then facilitates basal accumulation of BMP type I receptor Tkv to capture extracellular Dpp.[80] These coupling mechanisms illustrate how BMP signal and cellular mechanisms mutually interact in PCV development.

3.3 | Instructive role of tissue dynamics for spatial regulation of BMP signal

Although Dpp serves as a wing vein determinant in the pupal wing, recent studies suggest that Dpp signaling coordinates both growth and patterning/differentiation at this stage.[82] Conditionally ablating *dpp* in the pupal wing reduces tissue size, and veins largely fail to form. How Dpp signaling coordinates both growth (involving broader tissue-level control) and wing vein development (which is wing vein progenitor-specific) is not immediately obvious. However, these phenomena can be explained by the evidence that Dpp forms a long-range signal during the early pupal stage (proliferation stage), then later operates over a short-range during differentiation. Interestingly, the change of Dpp signaling spatial distribution coincides with changes in 3D tissue architecture. When the two epithelial layers are separated during inflation, Dpp sustains cell proliferation by lateral long-range trafficking. As the two epithelia appose, the Dpp

signaling range becomes refined laterally, with active vertical Dpp trafficking between the two epithelial sheets for wing vein patterning/differentiation (Figure 3B). The idea that 3D tissue architecture itself has an instructive role for signal distribution is further confirmed by the experiments that artificially change 3D tissue architecture, which sufficiently changes spatial distribution of the BMP signal (Figure 3C).
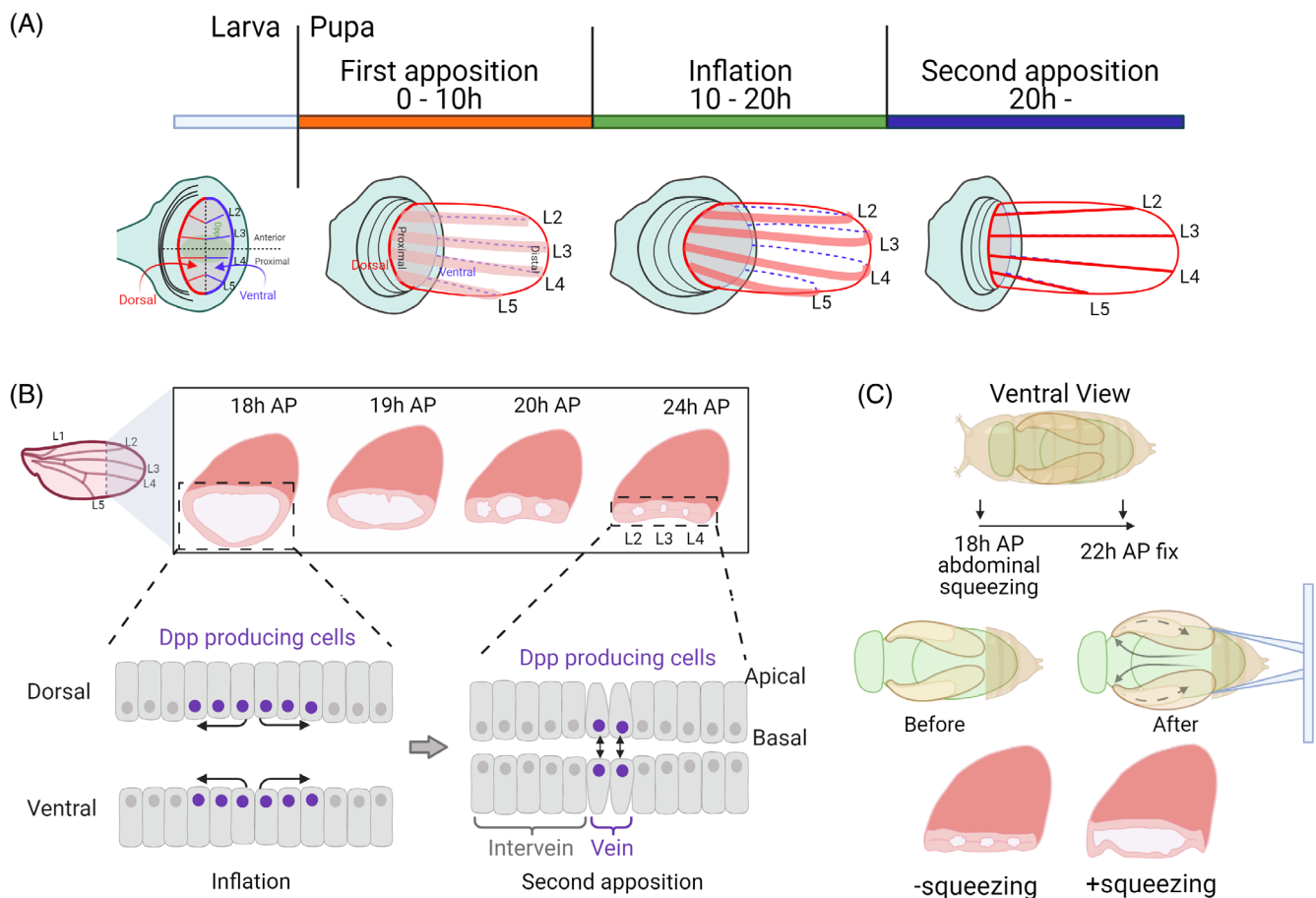
3.4 | BMP signal and tissue dynamics

Mutual interactions between BMP signal and tissue morphogenesis described in the previous *Drosophila* wing examples are likely common during organogenesis across species, summarized as follows:

Formation of the mammalian neural tube

In vertebrate embryo development, there are instances in which two apposed tissues approach one another and fuse to form a continuous tissue. During formation of the neural tube, which gives rise to the central nervous system, the neuroepithelium forms hinge points and bends on both sides in a U-shape to form the neural folds. The apposed folds approach one another and come into contact to undergo a tissue fusion event that results in neural tube closure (NTC) and formation of the neural tube.[83]

A host of cell signaling pathways affects the cell shape changes, cell movements and genesis and positioning of the cell-cell connections during neural tube closure. In the early mouse embryo, a cross section of the nascent neural tube resembles an inverted acute isosceles triangle

FIGURE 3 Coupling between BMP signal and tissue dynamics. (A) Overview of timing of wing development during the first 24 hours after pupariation (AP) at 25°C. Pupal wing development is divided into three phases; first apposition (0-10 hours AP), inflation (10-20 hours AP), and second apposition (from 20 hours AP onward). A schematic of each pupal stage is shown below. Size and tissue shape are not proportional to actual wings. (B) Schematics of coupling between 3D tissue architecture and Dpp signaling. Dpp expressed in longitudinal vein primordial cells diffuses laterally to regulate tissue proliferation during the inflation stage. Dpp signaling then actively takes place between dorsal and ventral cells to refine the signaling range for vein patterning/differentiation. (C) Modulation of tissue architecture of the pupal wings through abdominal squeezing. Abdominal squeezing before second apposition prolongs pupal wing inflation, therefore, lateral diffusion of Dpp is still observed at 22 hours AP. In contrast, without squeezing, second apposition takes place normally, and accordingly vertical signal refinement occurs between the two epithelia.[82] (Created with BioRender.com)

with rounded angles. The triangle base is located dorsally, and the vertex, where the notochord forms, is ventral. Dorsolateral hinge points are found roughly at the base angles, and the median hinge point (MHP) at the ventral vertex.[83]

BMP signaling plays a crucial role in bending the neuroepithelium into the neural tube by inducing cell shape changes at the DHLPs and MHP.[84] In the DHLP case, the BMP antagonist Noggin is expressed dorsally in the neural folds where DHLPs will form. Abrogation of *noggin* expression or exogenous BMP2 both result in failure of DHLP formation, indicating that BMP2 signaling (normally originating from the overlying non-neural ectoderm) negatively regulates downstream gene expression that induces the cell shape changes in the neuroepithelium where DHLPs form. On the other hand, at the MHP, a complementary feedback loop between BMP and TGFβ signaling within individual cells exerts its effects via biochemical interactions between active SMADs and junctional proteins along the apicobasal axis in a manner dependent upon the cell cycle.[85] These signaling events are accompanied by dynamic remodeling of cell-cell junctions, which facilitates the bending of neuroepithelium at the MHP via cell shape changes. Thus, BMP and TGFβ signaling result in an intracellular spatiotemporal morphogen gradient output that contributes to bending the neuroepithelium into the neural tube while maintaining epithelial integrity and permitting mitotic growth.

Controlling size of the zebrafish pectoral fin

Fish pectoral fins are homologous to forelimbs in tetrapod vertebrates. In the developing zebrafish larva, the pectoral fin develops as fin primordium from a mesenchymal bud surrounded by ectoderm, immediately dorsally to the yolk sac. As the pectoral fin develops, two BMP signaling gradients form, one anterior and the other posterior, extending proximodistally from the trunk of the animal and observable 48 to 78 hours postfertilization.[86] During this time period, fin growth is mostly anisotropic along the anteroposterior and proximodistal axes, and the BMP signaling gradients scale linearly as the fin increases in size. When BMP activity is compromised, the fin is reduced in size, indicating the importance of BMP signaling in fin growth. As the BMP gradient scales along with fin growth in a manner related to the *Drosophila* wing imaginal disc, a gradient-expanding mechanism related to Pentagone seems plausible. Two zebrafish Pentagone orthologs, Smoc1 and Smoc2, are likely candidates for gradient-expanding activity.[87] Smoc1 localizes along the distal fin between the two BMP gradients, consistent with the idea that BMP signaling suppresses Smoc1 expression in its own anterior and posterior expression domains; Smoc1 is expressed more widely across the fin in a dominant-

negative BMP receptor background; and reduced fin growth is an aspect of the Smoc1 mutant phenotype. Smoc1 in the fin, similarly to Pentagone in the wing imaginal disc, is therefore a BMP signaling gradient expander, and indicates that the principle of morphogen gradient expansion in organ growth, and the molecules that support this process, are evolutionarily conserved.

Tissue self-organization in *in vitro organogenesis*

In vitro organogenesis from stem cell precursors constitutes a promising approach to investigate the function of cell signaling toolkits in development when studies are technically and/or ethically not feasible, and to establish reproducible protocols for in vitro generation of medically relevant replacement organs. Organs generally comprise several cell types that must be properly arranged spatially for correct function, and this spatial arrangement arises from a process of self-assembly and organization. Therefore, whether an organ develops in vivo or is cultured in vitro, understanding the signaling pathways that guide organ self-assembly and organization is essential.

The endodermal, ectodermal, and mesodermal germ layers of the mammalian embryo arise upon gastrulation from an apparently homogenous layer of epiblast cells. To pattern the developing embryo, these germ layers exchange both inductive and inhibitory signals. Understanding how this signaling is linked to embryo geometry, how signaling information is exchanged between developing tissues, and how signals are translated into outputs is therefore critical.

Warmflash and colleagues describe a technique to generate gastruloids from human embryonic stem cells (hESCs) in which the genesis of germ layers is recapitulated in vitro.[88] When cultivated in medium containing BMP4, monolayer colonies of hESC disks differentiate into a radially symmetric self-organized concentric micropattern of trophectodermal, endodermal, mesodermal, and ectodermal cells (from colony edge to center) that orient with their apical aspect facing the culture medium. Etoc et al use these gastruloids to determine the mechanism of BMP4-induced germ layer positioning.[89] Using pSMAD1 expression as a readout, hESC colony responsiveness to BMP4 is a function of cell density in the colony, with colonies with more densely packed cells responding to BMP4 only at the edges. Only cells at the edge were shown to display BMP receptor to the medium environment, whereas cells at the colony center displayed BMP receptor laterally. This in turn results in a differential response of edge vs center cells to BMP4 signaling. Noggin is a downstream target of BMP4 signaling, and NOGGIN protein inhibits BMP4. Thus, NOGGIN is produced in edge cells, from where it diffuses centrally, resulting in a BMP4 activity gradient with high activity at

the edge, and low activity centrally. Therefore, the 3D architecture of the colony influences how cells within it respond to BMP4 signaling.

Inner ear hair cells are mechanosensitive receptors that detect motion, gravity and sound, and are essential in hearing and balance. These cells, if lost due to environmental or genetic causes, do not regenerate, resulting in both hearing loss and reduced sense of balance. IEHC genesis is therefore a topic of intense investigation, and in vitro approaches of generating such cells from stem cells show potential for drug screens, functional studies and therapeutic replacement. Existing developmental studies implicate the involvement of the BMP, Wnt, and fibroblast growth factor (FGF) signaling toolkits in IEHC genesis. In vivo, the initial step involves BMP signaling in the otic-epibranchial progenitor domain of the definitive ectoderm that results in the genesis of non-neural ectoderm (NNE).[90] Subsequently, BMP signaling is inhibited and FGF signaling activated, driving formation of the preplacodal region from the NNE. Wnt signaling in this region then induces formation of the otic placode, which then invaginates to form the otic vesicle, from which most inner ear cell types, including IEHCs, are derived.

In vitro, recapitulating these steps involves generation of definitive ectoderm spheroids from murine pluripotent stems cells under conditions that facilitate production of basement membrane.[90] Serial culturing in medium with BMP4 and a TGFβ signaling inhibitor, then medium with FGF-2 and BMP signaling inhibitor, result in formation of a preplacodal region that subsequently differentiates into an otic placode. Upon genesis of this placode, apparently normal sensory epithelia form in culture, which include numerous IEHCs with the structural and functional properties of their native counterparts. A similar protocol using hESCs proved successful in IEHC generation as well.[91] Notably, after BMP- and FGF signaling-induced formation of the preplacode in culture, the unfolding of the IEHC differentiation program occurs without additional exogenous BMP or FGF signaling inputs, indicating that preplacodal precursors generate their own signaling molecules to direct self-organization and differentiation into recognizable inner ear sensory epithelial cells in culture.

In summary, whether in native or in in vitro organogenesis, BMP signaling and cell and tissue morphogenesis are inextricably linked, with BMP signaling directing morphogenesis at the cell and epithelial levels, which in turn results in spatial changes that feed back into how BMP signaling operates. The reciprocal influence of signaling and morphogenesis on each other likely applies to many signaling toolkits in various developmental contexts across species, and is therefore a likely general developmental principle.

4 | SUMMARY AND FUTURE PERSPECTIVES

Here, we review studies that illustrate how BMP signaling is spatiotemporally controlled in various contexts during tissue development, and how BMP signaling and dynamic morphogenesis are coupled. To enhance further our mechanistic understanding of the significance of BMP signaling in animal development, two broad themes merit further development.

First, the investigation of molecular mechanisms involved in directing and controlling evolutionarily conserved signaling toolkits should reveal the extent to which coupling of signaling and morphogenesis are generalizable across species. Given the diversity of body plans in metazoan development, it seems reasonable to conjecture that BMP signaling gives rise to dynamic morphogenesis in myriad ways, even when the conserved BMP signaling toolkit is in play.[16]

Second, cell shape changes are dynamic processes, yet most extant studies employ approaches that analyze these dynamic changes in fixed tissues. Therefore, while many excellent studies reveal how BMP signal is important in various contexts and how BMP signal is regulated in a context-dependent manner, our knowledge is still very limited about how dynamic morphogenesis feeds back into signaling. To address this, experimental approaches that harness the power of live imaging have the potential to revolutionize investigation of the links between signaling and morphogenesis. Recent progress in biological imaging techniques include the development of lattice light sheet and spinning disk confocal microscopy.[92,93] In current technical application, not all model organisms are suitable for this approach. Instead, suitable systems such as *Drosophila* and zebrafish, as well as in vitro organoids formed from stem cells, could be actively employed to address this question. Additionally, the development of novel fluorescent proteins,[94] along with the ability to introduce relevant transgenes that encode them with nucleotide-level precision into the genomes of an increasingly diverse range of organisms using CRISPR-Cas9 gene editing,[95] should enable live imaging analysis of signaling molecule action and resulting morphogenetic effects in a near-native context. Such advances will be extremely important in advancing our understanding of fundamental developmental phenomena, both in vivo and in organoid formation from stem cells. These will result in enhanced understanding of implications in human health and disease.

AUTHOR CONTRIBUTIONS

Martti Montanari: Writing - original draft; writing-review & editing. **Ngan Vi Tran:** Visualization; writing - original draft. **Osamu Shimmi:** Writing - original draft; writing - review & editing.

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