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Spatial Regulation of BMP Signaling by Patterned Receptor Expression

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ABSTRACT

Local delivery of TGF-β/BMP ligands is commonly used as a tissue engineering strategy for the spatial regulation of cell growth and differentiation. While the location and the dose of ligand are the only parameters that influence the spatial distribution and biological effects of the ligand *in vitro*, *in vivo* genetic studies of development reveal that spatial control of TGF-β/BMP signaling can be accomplished at multiple levels, from ligand release to signal interpretation. Here we focus on spatial control of BMP signaling by patterned receptor expression. Motivated by our recent experimental analysis of the two-dimensional BMP signaling patterns in the developing *Drosophila* egg, we formulate one- and two-dimensional models of ligand diffusion and internalization in the presence of patterned receptor expression. Our analysis of these models shows that they can capture the quantitative features of the experimentally observed pattern of phosphorylated SMAD in *Drosophila* oogenesis and shows that patterned receptor expression provides versatile control of BMP signaling in developing tissues. Quantitative understanding of the mechanisms of spatiotemporal control of signaling pathways in development is essential for successful harnessing of these pathways in tissue engineering.

INTRODUCTION

THE TGF-β/BMP SIGNALING PATHWAYS are critical regulators of adult and developing tissues in animals ranging from worms to humans.[1–4] The TGF-β/BMP system usually operates in a paracrine mode, whereby locally secreted ligands act through receptors on the neighboring cells. Activated receptors phosphorylate the receptor-regulated SMADs (R-SMADs), which are the intracellular mediators of TGF-β/BMP signaling homologous to the *Drosophila* protein MAD (Mothers Against Decapentaplegic) and *C. elegans* protein SMA.[5] Complexes formed by the phosphorylated R-SMADs and their appropriate cofactors translocate to the nucleus where they regulate gene expression by direct binding to other transcription factors, chromatin, and DNA (Fig. 1).[2] Transcriptional changes induced by these events control a variety of cellular processes involved in tissue regulation.[6]

TGF-β/BMP signaling *in vivo* can be monitored using antibodies that recognize the phosphorylated SMADs. In a remarkable demonstration of the highly conserved nature of this pathway, the same antibodies can be used to explore the dynamics of TGF-β and BMP signaling across species.[7] At the same time, this assay provides essentially the only tool for monitoring the dynamics of a complex system that is regulated at multiple levels, from ligand release to nuclear import of SMADs.[6] As a result, visualizing the phospho-SMAD pattern is just the first step in understanding the mechanism that controls this pattern in any given system. Genetic approaches are invaluable in dissecting the regulatory mechanisms of signaling, but they quickly run into technical problems in systems where multiple redundant mechanisms control the same signaling outcome and/or in problems with complex dynamics. At this level of complexity, computational models can be useful in testing the proposed mechanisms and guiding future experiments.[8]

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Over the past few years, several models have been proposed for BMP signaling in development. The closest connection between modeling and experiments was achieved for two systems in *Drosophila*, where a BMP-like ligand Decapentaplegic (Dpp) acts as a morphogen at multiple stages of development.[3,9] In the wing imaginal disk, Dpp forms a long-range signaling gradient that defines the expression thresholds of multiple target genes.[10] Computational models of Dpp diffusion, binding, and internalization were used to explore the dynamics of this gradient.[11,12] Dpp signaling has also been modeled in the early embryo, where Dpp is secreted from a wide spatial source and, initially, forms a broad signaling gradient.[13] Over time, however, this gradient is sharpened by a network of molecules that bind Dpp into extracellular complexes, shuttle these complexes in the direction opposite to the gradient of the free ligand, and proteolytically process these complexes, releasing Dpp in a narrow region of space.[14–19] Several models were used to explore the robustness of this patterning system.[20–22]

In both of these systems, locally secreted ligand acts on uniformly expressed receptors.[13] Here we focus on a different mode of regulation, where signaling is controlled by patterned expression of cell surface receptors. This work is motivated by our recent analysis of the Dpp pathway in *Drosophila melanogaster* egg development (oogenesis).[7]

In oogenesis, an eggshell is formed that houses the developing embryo, providing protection and mediating its interaction with the environment. The eggshell is derived from the follicular epithelium that covers the growing oocyte (Fig. 2A).[23] Eggshell morphogenesis relies on extensive patterning of the follicular epithelium by two inductive signals from neighboring tissues. The first inductive signal is mediated by the epidermal growth factor receptor (EGFR) pathway. In early stages of eggshell patterning, EGFR is activated by Gurken, a TGF-α–like ligand secreted from the dorsal cortex of the oocyte (Fig. 2A).[24] The pattern of Gurken-mediated EGFR activation has been recently quantified using an imaging and modeling approach.[25,26] The second inductive signal is Dpp. Genetic approaches have shown that Dpp is essential for multiple roles in eggshell morphogenesis, but the quantitative picture of Dpp signaling in oogenesis has yet to be developed.[27–32] Dpp is secreted along the anterior border of the follicular epithelium and initially forms a one-dimensional signaling gradient (Fig. 2B, C).[28,29,33] Over time, however, this simple pattern splits into three disconnected domains: a ventral anterior band and two symmetrically positioned dorsolateral "eyebrows."[7] A genetic approach was used to establish that these changes in the Dpp signaling pattern reflect the corresponding changes in the expression pattern of Thick-veins (Tkv), a type I Dpp receptor (Fig. 2C).[7] *tkv* is initially expressed uniformly throughout the follicular epithelium. In later stages of oogenesis, however, *tkv* expression is repressed in the dorsal anterior and midline areas as well as in most of the ventral cells. Its expression is refined to a ventral anterior band and two dorsolateral patches (Fig. 2C).[7,34] We recently discovered that this expression pattern of *tkv* reflects its regulation by the EGFR pathway.[7] Here we propose a simple model that describes this effect of dynamic patterned receptor expression and provides the first step toward a quantitative description of Dpp signaling in oogenesis.

MATERIALS AND METHODS

Fly and media

Wild-type OreR flies were used. Flies were grown on agar cornmeal medium at 23°C. Baker's yeast was added to the fly medium 24 h prior to ovary harvesting.

In situ *hybridization*

In situ hybridization was carried out as previously described by Yakoby *et al.*[7]

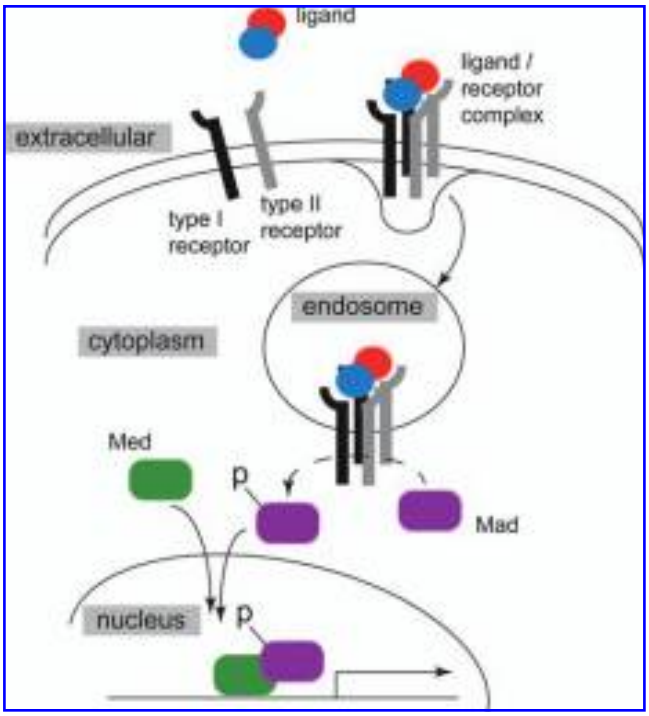

FIG. 1. Schematic of the Dpp signaling pathway in *Drosophila*, showing major components of the pathway. The ligand (known to form dimers) binds to the extracellular binding domains of the transmembrane type I receptor dimer, which then recruits the type II receptors to phosphorylate an intracellular domain of the type I receptors. The activated ligand–receptor complex is then internalized to the endosome where it is able to phosphorylate Mad. Phosphorylated Mad then binds to its co-SMAD, Med, and translocates to the nucleus to regulate gene transcription.

Immunofluorescence and microscopy

Dissection and fixation of ovaries was done as described elsewhere.[35] The primary antibody of rabbit anti-P-SMAD1/5/8 (1:3000) was a generous gift from D. Vasiliauskas, S. Morton, T. Jessell, and E. Laufer. Secondary antibodies: Alexa Fluor and Oregon Green (1:2000; Molecular Probes, Carlsbad, CA). Images were taken with a PerkinElmer RS3 Spinning Disk Confocal microscope and the Nikon Eclipse E800 compound microscope. Images were processed and quantitated with ImageJ (Rasband, 1997–2006) and Photoshop (Adobe Systems, San Jose, CA).

Computational methods

The system of equations was solved numerically using finite difference methods in MATLAB (The MathWorks, Natick, MA). The two-dimensional model was solved on a 640×160 prolate spheroidal grid.

RESULTS

One-dimensional model of signal regulation by patterned receptor expression

As a first step toward analyzing the spatial control of signaling by patterned receptor expression, we analyzed a one-dimensional model of morphogen diffusion and receptor-mediated degradation (Fig. 3A). Morphogen (whose concentration is denoted by M in our model) enters the tissue at position $x = 0$ with a constant flux of Q. The ligand is free to diffuse, with effective diffusivity D in a tissue of size L. Binding to cell surface receptors (R) is characterized by the rate constants k_{on} and k_{off}. The morphogen–receptor complex, C, undergoes endocytosis with rate constant k_e. We are assuming that only the internalized morphogen–receptor complex (S) is capable of signaling; this signaling complex is degraded in a first-order process with rate constant k_d. We only consider one type of receptor because our experimental data showed that type II receptor *punt* is uniformly expressed

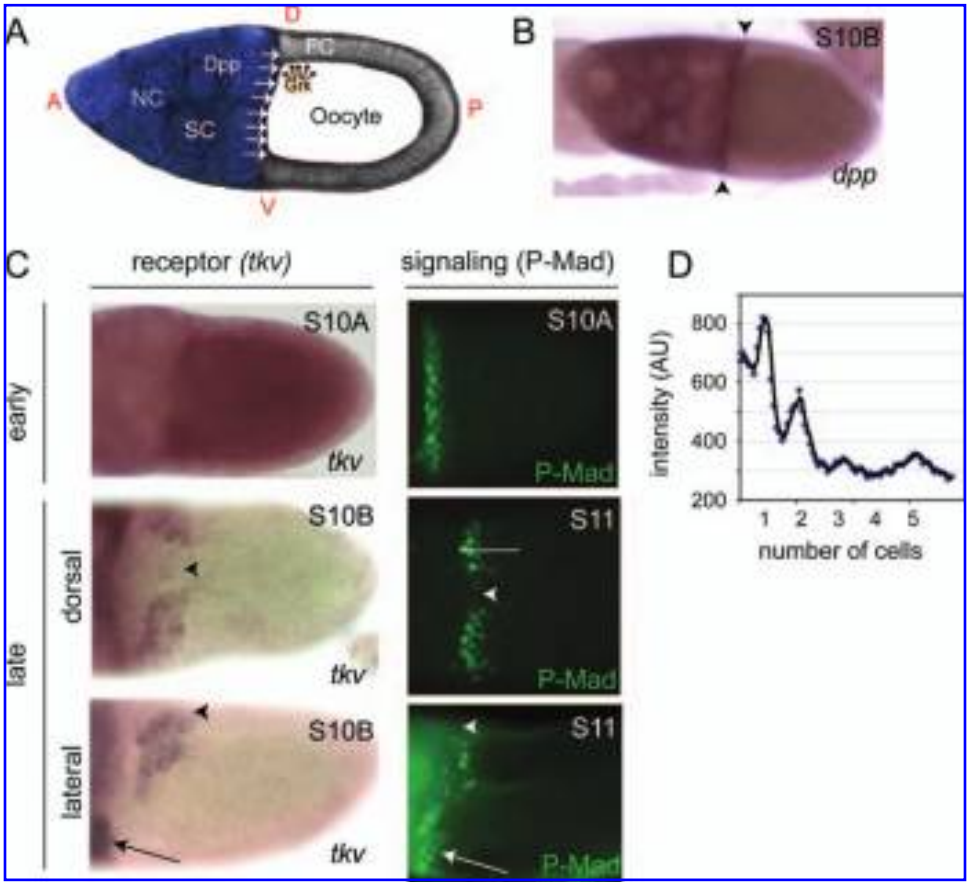

FIG. 2. Expression patterns of Dpp receptor and signaling in the follicular epithelium are dynamic. (**A**) Phase contrast image of the artificially colored stage-10 egg chamber, showing the follicular epithelium (FC, gray), the oocyte (white), the stretch follicle cells (SC, blue), and the nurse cells (NC, blue). SC are follicle cells stretched over the nurse cells. The BMP ligand Dpp is secreted from the stretch cells and the most anterior columnar follicle cells, called the centripetally migrating follicle cells (CMFC). The EGFR ligand Gurken (Grk) is secreted from the dorsal anterior cortex of the oocyte. A, anterior; P, posterior; D, dorsal; V, ventral. (**B**) *In situ* hybridization image of *dpp* at stage 10B. Dpp is expressed in the stretch cells and the few most anterior follicle cells covering the oocyte, called the CMFC. Black arrowheads show the expression of *dpp* in the CMFC. (**C**) Left: *In situ* hybridization images of type I receptor *tkv* in the egg chamber for both early (until stage 10A) and late oogenesis. Right: The corresponding Dpp signaling activity, monitored by phosphorylated Mad (P-Mad), shown in the immunofluorescence images. The top row shows expression patterns in early oogenesis, and the middle (dorsal view) and bottom (lateral view) rows show expression patterns in late oogenesis. The arrowheads mark the dorsal midline, and the arrows mark the ventral anterior expression of *tkv* (bottom, left panel) and P-Mad (bottom, right panel) patterns. Anterior is to the left. (**D**) Quantitation of the P-Mad immunofluorescence image was performed along the white line shown in the late P-Mad image in (**C**). AU = arbitrary units. Figure (**A**) and images of the *tkv* pattern in late oogenesis in (**C**) are reproduced from Yakoby *et al.*[7]

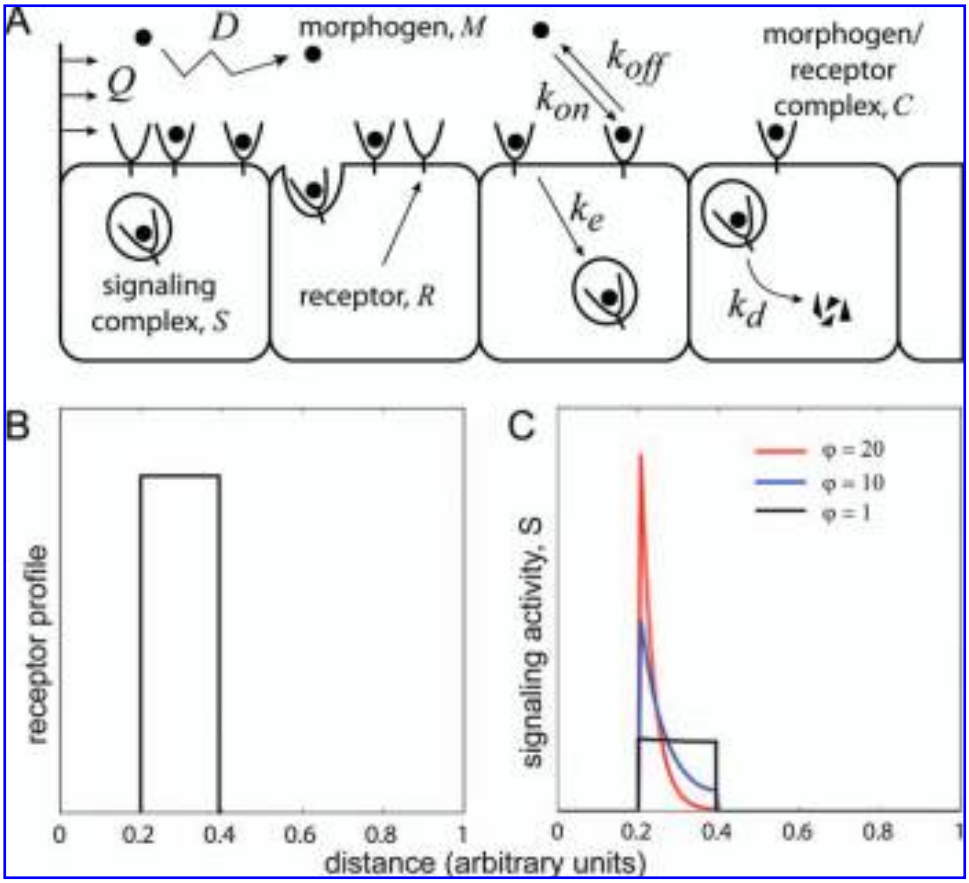

FIG. 3. One-dimensional model of Dpp diffusion and internalization shows that Dpp signaling profile depends on the ligand diffusion length. (**A**) Schematic of the processes involved in the simple, one-dimensional model of Dpp production, diffusion, and receptor-mediated signaling and degradation. See the "One-dimensional model of signal regulation by patterned receptor expression" section in the text for details. (**B**) Schematic representation of the nonuniform pattern of receptor expression. Receptor level is constant within a "band" of the tissue and zero otherwise. (**C**) Signaling patterns across the receptor-expressing patch computed for different values of the ratio of the size of the patterned tissue and the length scale of the patterning ligand (φ).

throughout the follicular epithelium (data not shown) and thus will have no effect in the signaling distribution. This set of processes is consistent with a recent set of observations, showing that Dpp transport in tissues can be modeled as a diffusive process, that ligand internalization is essential for signaling, and that the signaling level reflects receptor occupancy.[11,12,36–40]

We assume that patterning operates in the ligand-limited regime, where the levels of free receptors are not affected significantly by the presence of the ligand. The slowest process in the model is the internalization of ligand–receptor complex, which happens in the order of minutes[41,42] while the time scale of tissue patterning is ~ 10 h, in the case of stage 10–11 of oogenesis.[23] Thus, since the processes modeled can be considered at steady state on the time scale of tissue patterning, we consider only the steady state solution, which satisfies the following equations:

$$D\frac{d^2M}{dx^2} - k_{on}RM + k_{off}C = 0 \quad D\frac{dM}{dx}\bigg|_{x=0} = -Q, \quad D\frac{dM}{dx}\bigg|_{x=L} = 0$$

$$k_{on}RM - k_{off}C - k_eC = 0, \quad k_eC - k_dS = 0 \tag{1}$$

At steady state, the signaling level is proportional to the concentration of a free morphogen: $S = \frac{k_eC(x)}{k_d} = \frac{k_ek_{on}RM(x)}{(k_d(k_e+k_{off}))}$, and $M(x)$ is given by:

$$D\frac{d^2M}{dx^2} - \frac{k_ek_{on}R}{k_e+k_{off}}M = 0 \quad D\frac{dM}{dx}\bigg|_{x=o} = -Q, \quad D\frac{dM}{dx}\bigg|_{x=L} = 0 \tag{2}$$

Nondimensionalizing the problem using $z \equiv x/L$ and $m = M/(QL/D)$, we obtain:

$$\frac{d^2m}{dz^2} - \varphi^2 m = 0 \quad \frac{dm}{dz}\bigg|_{z=0} = -1, \quad \frac{dm}{dz}\bigg|_{z=1} = 0 \tag{3}$$

Thus, the shape of the spatial distribution of the free ligand depends on a single parameter, φ, which can be viewed as a ratio of the length of the tissue (L) to the length scale of a diffusing morphogen, λ (Reeves *et al.*[8]):

$$\varphi^2 = (L/\lambda)^2 \quad \text{where } \lambda \equiv \sqrt{\frac{D(k_e+k_{off})}{k_ek_{on}R}} \tag{4}$$

The signaling profile, rescaled by its maximal value, $Q/(k_dL)$, is given by $s(z) = \varphi^2 m(z)$. Solving Eq. 3, we obtain the following expression for the signaling pattern:

$$s(z) = \frac{\varphi \cosh(\varphi(z-1))}{\sinh(\varphi)} \tag{5}$$

This solution corresponds to the case of uniform receptor expression. When receptor is expressed only in a part of tissue, for example, when $R(x) = R$, for $x_1 \le x \le x_2$ and zero otherwise (Fig. 3B), the signaling profile is given by:

$$s(z) = \frac{\varphi \cosh(\varphi[z-z_2])}{\sinh(\varphi[z_2-z_1])} h(z) \tag{6}$$

where z_1 and z_2 are the nondimensionalized form of x_1 and x_2, and $h(z) = 1$ over the receptor-expressing part of the tissue, and zero otherwise.

One can show that the average signaling level across the receptor-expressing patch, $\langle s \rangle$,

$$\langle s \rangle \equiv w^{-1} \int_{z_1}^{z_2} s(z) dz = w^{-1}$$

does not depend on the value of φ, and hence on the level of receptor expression. Here $w = z_2 - z_1$. As a result, signaling is "redistributed" in response to changing the level of receptor expression. For low values of φ, signaling is uniform across the receptor-expressing patch of cells. For higher values of φ, signaling increases in the front of the patch (the side facing the source of the ligand) and decreases in the back of the patch (Fig. 3C).

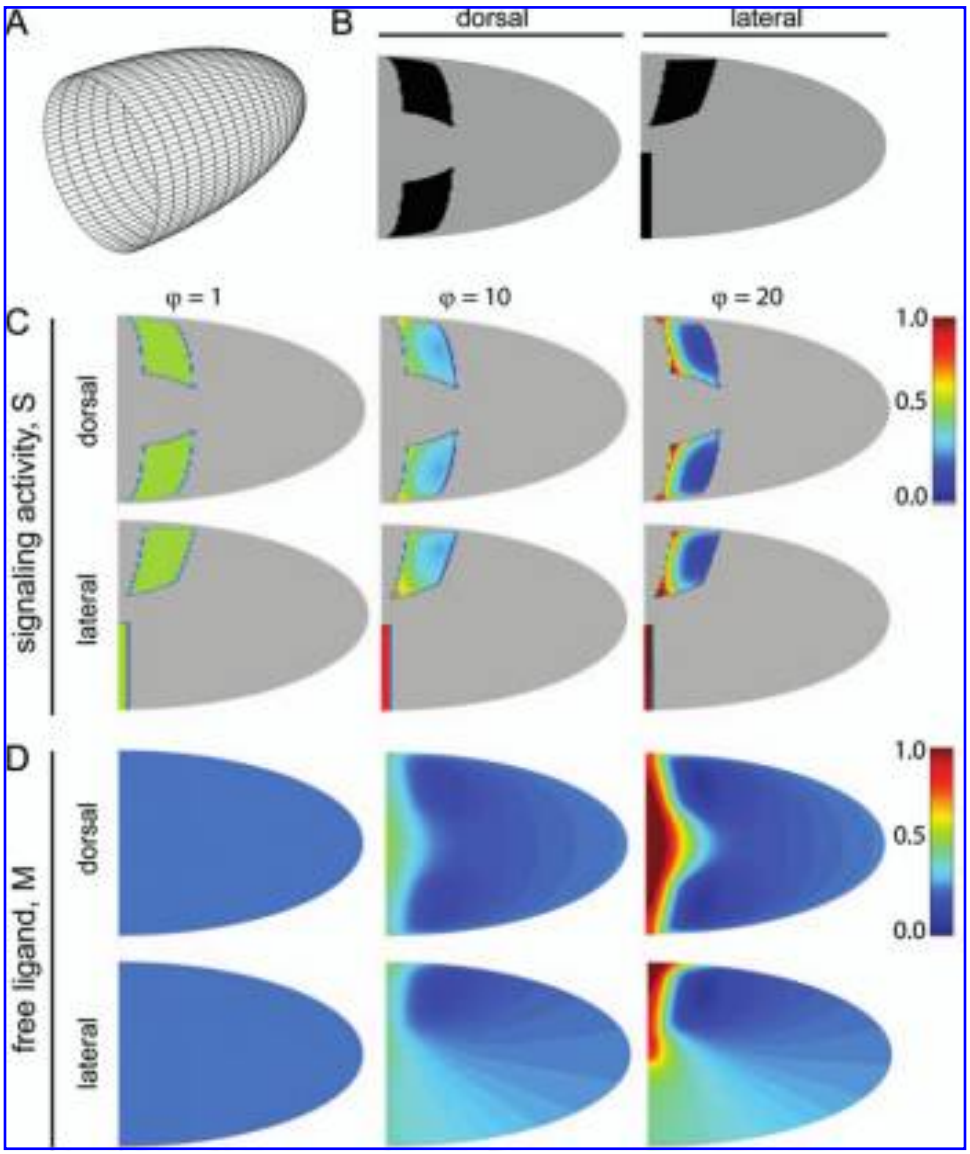

FIG. 4. Two-dimensional model of Dpp diffusion and internalization determines ligand diffusion length. Ligand is secreted at the anterior border of the follicular epithelium and interacts with receptors in the follicle cells. Information about the ligand and receptor expression is based on the *in situ* hybridization analyses of *dpp* and *tkv* expression (Fig. 2B, C). The model accounts for the processes described in Figure 3A and is solved numerically on a prolate spheroidal finite difference grid (**A**). The *tkv* expression pattern is placed on the grid as shown in (**B**). Computed solutions are shown as two-dimensional projections. The patterns of signaling (**C**) and free ligand concentration (**D**) for different values of the ratio of the size of the patterned tissue and the length scale of the patterning ligand (φ). Each of the patterns is shown in both dorsal (top) and lateral (bottom) views. Signaling profiles obtained experimentally can be recapitulated with simulations using $\varphi = 20$, suggesting that Dpp is a short-ranged ligand (see the "Two-dimensional model can estimate the spatial range of Dpp in the follicular epithelium" section in the text for details). For all values of φ, both the signaling activity and the free ligand concentration are normalized to the maximum value of each for $\varphi = 20$.

Two-dimensional model can estimate the spatial range of Dpp in the follicular epithelium

While the one-dimensional model is able to predict the signaling trend as a function of the ligand diffusion range, the complexity of the Tkv and P-Mad (phosphorylated form of Mad, a fly R-SMAD homolog) expression in the follicular epithelium calls for a two-dimensional model for a better understanding of the structure and regulation of the experimentally observed signaling pattern. The same effect as observed in the one-dimensional model is clearly seen in the signaling patterns computed with the two-dimensional model of the follicular epithelium (Fig. 4). In the model, the ligand is secreted along the anterior border of the follicular epithelium and diffuses along the layer of cells with a nonuniform pattern of receptor expression (Fig. 4B).

The model was analyzed in the prolate spheroidal coordinate system and solved numerically as described elsewhere (Fig. 4A).[25] We have used this coordinate system in our earlier analysis of the Gurken morphogen gradient, which is responsible for the early phase of the EGFR signaling in patterning of the follicle cells. As predicted by the one-dimensional model analyzed above, the nonuniformity of the signaling profile across the receptor-expressing patch progressively increases as the diffusion length of the ligand is decreased (Fig. 4C).

Quantitation of the late phase of the P-Mad pattern shows that the signaling intensity drops to $\sim 40\%$ of its value at the anterior of the receptor-expressing patch by the third row of cells within the patch (Fig. 2D). To be consistent with this rate of spatial decay, the value of φ in the model must be ~ 20. Simulations with long-ranged ligands ($\varphi < 20$) predict signaling patterns that are more uniform over the patches of Tkv-expressing cells (Fig. 4C), contrary to the experimentally observed P-Mad pattern at late stages of oogenesis (Fig. 2C). Also, only when φ is ~ 20, are the signaling intensities in the dorsolateral patches and in the ventral stripe comparable, in agreement with experimental results (Figs. 2C and 4C). Thus, a combination of a quantitative analysis of the experimentally observed signaling pattern and a simple biophysical model can be used to quantify the signaling range of Dpp in patterning of the follicle cells.

Since φ is the ratio of the size of the patterned tissue to the diffusion length scale of the ligand, $\varphi \sim 20$ means that, in the presence of receptors, the ligand is only able to diffuse for a short distance (one to two cells) before it is captured and degraded by receptors. This property of Dpp is different from that of the EGFR ligand Gurken, which is known to be a long-ranged ligand ($\varphi \sim 3$).[25] Further, our model predicts a higher concentration of free Dpp on the dorsal side of the egg chamber, as a consequence of the decreased rate of receptor-mediated Dpp degradation in the dorsal anterior region of the follicular epithelium, where Tkv is repressed (Fig. 4D). This is consistent with a recently published observation that Dpp is concentrated on the dorsal side of the follicular epithelium.[43]

Using the estimated value of φ, which is the only free parameter in our model, we can explore the roles of different domains within the two-dimensional pattern of receptor expression. We found that removal of Tkv from the anterior dorsal follicle cells is essential for allowing the anteriorly secreted Dpp to reach the dorsolateral patches of Tkv-expressing cells. To show this, we have "filled in" the dorsal anterior part of the follicular epithelium with receptors and observed an almost complete loss of the signaling within the dorsolateral patches, but an ectopic signaling in the dorsal anterior area (Fig. 5A). Using a similar approach, we found that signaling within the dorsolateral patches and within the ventral anterior band of the follicle cells are dependent on each other. When the ventral domain of Tkv was removed, although most of the signaling on the patches is barely affected, the signaling level on the posterior side of the patches increases almost threefold (Fig. 5B). In simulations where Tkv in the dorsolateral patches was removed, the signaling intensity within the ventral anterior band increased with the largest increase experienced at the most dorsal tips of the ventral band. These effects can only be studied using a two-dimensional model. One-dimensional modeling of anteriorly secreted ligand over a patch of receptors will not completely capture the complex consequences of patterned receptor expression in the real system.

DISCUSSION

Spatiotemporal control of TGF-β/BMP signaling is essential for proper tissue patterning and morphogenesis across species.[1–4] Our genetic and imaging studies of pattern formation in *Drosophila* oogenesis revealed that spatial regulation of Dpp signaling in this system can be attributed to the patterned expression of type I Dpp receptors.[7] Here we have shown that a simple biophysical model can describe many of the quantitative characteristics of the two-dimensional pattern of Dpp signaling. The model provides the first estimate of the spatial range of Dpp in *Drosophila* oogenesis and can be used to systematically explore how spatial regulation of receptor expression affects the spatial pattern of signaling. We found that Dpp acts as a short-ranged ligand and that the effects of patterned receptor expression are non-cell-autonomous, since removal of receptors in one part of the follicular epithelium leads to both local and nonlocal effects. This conclusion is supported by our previous results with mosaic epithelial layers that contain clones of cells lacking Tkv and through the analysis of the model presented in this paper.[7]

Previous genetic and imaging studies have established that the dorsolateral cells that express Tkv form the roof of the future dorsal appendages, tubular respiratory structures on the dorsal side of the eggshell.[23,27–32] The formation of dorsal appendages requires highly coordinated shape changes and movements of cells in this region of the follicular epithelium. Our modeling and imaging results reveal a

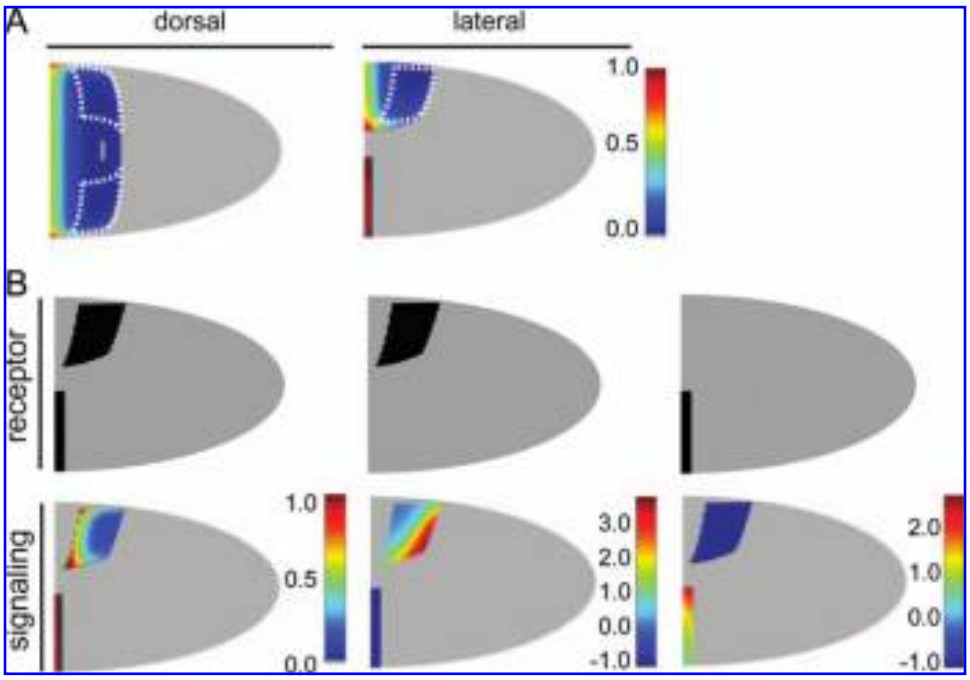

FIG. 5. Patterned receptor expression has non-cell-autonomous effects. (**A**) Simulations where ectopic dorsal anterior Tkv was added resulted in a very low level of Dpp signaling in the dorsolateral patches. The wild-type location of the patches is outlined by the dashed white line. (**B**) Simulations where the ventral anterior band of Tkv or the dorsolateral Tkv patches was removed show the effects that signaling on the dorsolateral patches have on that within the ventral band and *vice versa*. Top: Simulated receptor pattern. Bottom: the signaling activity for the wild-type simulation (left), and the differential signaling when compared to the wild-type case for the simulations where the ventral band of receptor was removed (middle) and where the dorsolateral receptor patches were removed (right). The differential signaling was calculated as follows: $(S - S_{WT})/S_{WT}$ where S is the signaling profile resulting from the simulation where either the ventral band or the dorsolateral patches of receptor was removed and S_{WT} is the wild-type signaling profile. Simulations in (B) are shown as lateral views with dorsal on top. The part of the follicular epithelium that does not express Tkv in the wild type is colored gray. All computed solutions are shown as two-dimensional projections.

hitherto unappreciated gradient of Dpp signaling that can potentially act as a spatial coordinator of these events. One function of the gradient of Dpp signaling might be to establish the graded expression of genes responsible for cell adhesion and migration. Our preliminary results support this mode of regulation. On the other hand, there have been no studies of the potential role of the ventral anterior Tkv band in the eggshell patterning. Since our model showed that the removal of the ventral Tkv band resulted in small changes in the signaling activity in the dorsolateral patches, this ventral band of Tkv must at least play a role in regulating Dpp signaling in the dorsolateral patches. However, besides this non-cell-autonomous function, the ventral Tkv might also be required cell-autonomously for local signaling.

The functional significance of the Dpp signaling control by patterned receptor expression is underscored by the fact that the dynamics of Dpp signaling seen in *Drosophila melanogaster* are conserved in other fruit fly species. The early pattern of P-Mad is uniform along the dorsal-ventral (DV) axis in egg chambers of various species of fruit flies that have two, three, and four dorsal appendages. The later pattern of P-Mad, however, acquires a clear DV polarity in each of these species, just as it does in *D. melanogaster*. In at least one of these species (*Drosophila phalerata*), we established that the change in the pattern of Dpp signaling is correlated with the change in the spatial expression of Dpp receptor.[7] Future work is required to understand the mechanisms that control the expression of Dpp receptors in *D. melanogaster* and related fly species. Moving beyond *Drosophila* oogenesis, we note that spatial patterns of Dpp receptor expression have been documented at other stages of fruit fly development, including wing patterning.[10,44] We argue that our model can be applied to these patterning contexts as well.

Local delivery of TGF-β/BMP ligands is often used as part of tissue engineering strategies for bone regeneration,[45] stem cell renewal and differentiation,[46] and skin development.[47] Generally, ligand is added in variable dosage and the tissue response to the ligand addition is then observed. Thus, the spatial pattern of signaling is controlled at the level of ligand production. Genetic studies of development suggest that different modes of regulation, for example, patterned receptor expression, also must be taken into account. Depending on the particular context, they can either present an obstacle to a successful ligand delivery, or be exploited in more sophisticated tissue engineering designs to provide a more precise control over the spatial distribution of ligand and signaling. The more we understand how signaling pathways are regulated in relatively simpler systems, such as the *Drosophila* eggshell, the more chances we stand to harness them for the design of man-made tissues.

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