Signaling interactions between squamous and columnar epithelia of the *Drosophila* wing disc

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Summary

Understanding the interactions between distinct epithelial cells would help us to understand the development of tissues. *Drosophila* imaginal discs, which are made up of two types of epithelial cells, provide good model systems for such studies. The disc proper or the columnar epithelial cells are apposed to a layer of squamous epithelial cells (the peripodial membrane). We have examined organization of peripodial and disc proper cells vis-à-vis their polarity since cell polarity plays an important role in the polarized transport of signaling molecules. With the help of polarity-specific cell markers, we have observed that apical surfaces of peripodial and disc proper cells face each other. This provides the cellular basis for the recently demonstrated signaling interactions between peripodial and disc proper cells during disc patterning. We also report significant similarities as well as differences between peripodial and disc proper cells in Engrailed-dependent wingdisc-patterning events, which make them an appropriate model system for studying the mechanism of diffusion of signal molecules, such as Hedgehog. Results with wild-type and two mutant forms of Hedgehog suggest that direct cell-cell contact is a requirement for the movement of wild-type Hedgehog signal and reconfirm that cholesterol-modification of Hedgehog makes it a short-range signaling molecule by restricting its movement.

Key words: Hedgehog, Cell polarity, Peripodial membrane, Notch, A/P axis

Introduction

All imaginal discs in *Drosophila* are made up of columnar epithelium on one side that grades into a squamous epithelium on the other (Fristrom and Fristrom, 1993). The columnar epithelium represents the disc proper (DP; Fig. 1A,B), which differentiates into wing, hinge and the notum. The squamous epithelium is also known as the peripodial membrane (PM; Fig. 1A,B) and contributes to integument cuticle (for example, to fuse the lateral sides of the two wing discs during thoracic closure) (Fristrom and Fristrom, 1993). While the developmental and molecular events in DP are well understood, the PM has gained attention only recently. The two epithelia are connected via long microtubular extensions, although in wing discs such translumenal extensions have been observed only in the notum (Cho et al., 2000; Gibson and Schubiger, 2000). Signaling from PM to DP, at least in the eye imaginal disc, has been shown to be dependent on these microtubule extensions (Gibson and Schubiger, 2000; Cho et al., 2000). It has been proposed that PM supplies inductive signals to DP through these cellular processes for disc patterning events.

As far as the molecular nature of PM-DP interactions is concerned, several known signal transduction pathways have been implicated. For example, downregulation of signaling molecules such as Hedgehog (HH) (Cho et al., 2000) and Serrate (Ser) (Gibson and Schubiger, 2000) in the PM alone is sufficient to affect eye development. Similarly, inhibition of Decapentaplegic (DPP) function in PM affects the growth of the entire wing disc (Gibson et al., 2002), and downregulation of EGFR/RAS pathway in the wing disc PM affects wing-notum/hinge decision in DP (Pallavi and Shashidhara, 2003). It has also been observed that *puckered* (*puc*), a negative regulator of the Jun N-terminal kinase (JNK) pathway, and *hemipterous* (*hep*), which encodes *Drosophila* JNK-kinase, are expressed in cells at the medial edge of the wing disc PM (Agnes et al., 1999). The *hep* mutants are defective in thorax closure suggesting a functional role for the JNK pathway in epithelial morphogenesis. These studies, therefore, have established signaling interactions between PM and DP and they further suggest that the PM has a significant role to play during disc patterning. With the demonstration of signaling interactions between PM and DP and the availability of powerful genetic techniques to manipulate one epithelial layer (PM or DP) at a time makes *Drosophila* imaginal discs good model systems for studying epithelial interactions.

Here we examine the morphology of PM cells in the context of their signaling capabilities. Cell polarity plays an important role in the polarized transport of signaling molecules. In the eye imaginal disc, HH and Ser are expressed in PM, yet they control the activation of corresponding pathways in DP (Cho et al., 2000; Gibson and Schubiger, 2000). Both HH and N pathway activation involves cell-cell interactions at the apical ends of epithelial cells. We made use of proteins that mark different spatial domains along the apico-basal axis of epithelial cells. For example, actin is an apical marker, Armadillo (ARM) marks the subapical region, and fasciclin III (FASIII) and Discs-large (DLG) mark basolateral sides of the cell (Woods et al., 1997). While cell polarity is an established
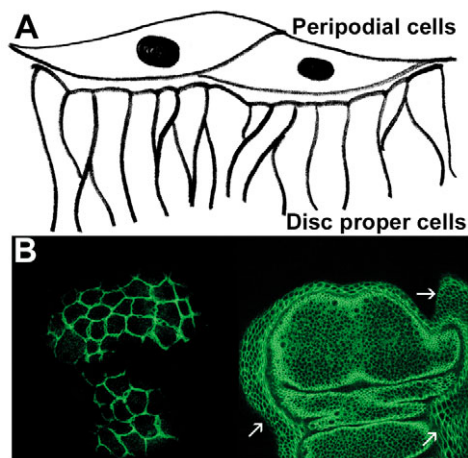
Fig. 1. Morphological features of peripodial and disc proper cells. (A) Thin squamous peripodial cells overlaying long columnar disc proper cells. (B) A top view of peripodial membrane of a wild-type wing disc stained for Armadillo expression. Left: large PM cells, which normally overlay the pouch and the notum. Right: an optical section at a lower focal plane showing medial edge (arrows) cells normally found on the lateral sides. These cells are more elongated than the PM cells overlaying the pouch and the notum.

feature of epithelial cells, no such characterization is reported for PM, probably because of the squamous nature of cells of this epithelial layer. PM and DP cells diverge from a common set of precursor cells at the first larval instar stage (Pallavi and Shashidhara, 2003). Thus, although PM and DP cells have distinct morphological differences, it is likely that they are similarly polarized. However, in the context of signaling interaction between the two membranes, the relevant question here is the orientation of PM and DP cells vis-à-vis their cell polarity. With the help of the above-mentioned polarity-specific cell markers, we have observed that apical surfaces of PM and DP cells face each other. Overexpression of Delta (Dl; a membrane-bound ligand of the Notch (N) receptor) in PM cells causes ectopic activation of Wingless (WG; a target of N pathway in the wing pouch) in DP cells, thus providing a functional validation of PM and DP organization with respect to their cell polarity.

Cell-cell interactions in the epithelial cells are best demonstrated in the *Drosophila* wing disc in the context of its patterning along the anterior-posterior (AP) and the dorsal-ventral (DV) axes. The HH pathway is required for patterning along the AP axis, whereas the Notch (N) pathway regulates patterning events along the D/V axis. However, elucidation of these cell-cell interactions is entirely related to events in the disc proper, whereas patterning events in PM are not well understood. Possible differences between PM and DP in these events are indicated in the reports that DPP diffused from the DP acts as a survival signal for PM cells (Gibson et al., 2002) and suppression of WG and epidermal growth factor receptor signaling at early stages of wing specification helps PM cells to acquire squamous morphology (Baena-Lopez et al., 2003). The complete absence of Apterous (AP) and WG expression in PM (Pallavi and Shashidhara, 2003) suggests ventral identity to all PM cells. In DP cells, A/P boundary is specified by the activity of HH, which diffuses from the posterior compartment

to the anterior compartment. In the presumptive A/P boundary, HH activates Cubitus-interruptus (CI) by stabilizing its full-length isoform, which in turn activates DPP expression. DPP is not activated in the posterior compartment because of direct inhibition of CI by Engrailed (EN) (reviewed by Aza-Blanc and Kornberg, 1999; Ingham and McMahon, 2001). Our results reported here suggest that, unlike DP cells, EN-expressing PM cells do not express HH. However, similar to DP cells, EN-expressing PM cells express DPP in response to ectopic expression of CI, but they do not respond to ectopic HH. Ectopic expression of HH in the PM, however, can activate DPP in the anterior compartment of DP.

Finally, we made use of PM-DP interactions to re-examine the movement of HH protein between epithelial cells. We expressed wild-type HH and two mutant forms of HH, which are not cholesterol modified (one of them being also a membrane-tethered form of HH) in PM cells and examined the effect on DP using DPP expression as the read-out. Our observations suggest that diffusion of wild-type HH requires direct cell-cell contact and confirms earlier observations that cholesterol modification of HH causes its restricted diffusion.

Materials and Methods
Genetics
Balancing mutations, making recombinant chromosomes and combinations of different mutations and/or markers were according to standard genetic techniques. *Ubx*-GAL4 and *EN426*-GAL4 are previously reported (Pallavi and Shashidhara, 2003). *Ubx*-GAL4 expression in wing discs (from as early as mid-second larval instar) is restricted to the peripodial membrane, whereas *EN426*-GAL4 is exclusive to the disc proper. UAS lines used are UAS-CI (Alexandre et al., 1996), UAS-Delta (Hepker et al., 1997), UAS-HH (Ingham and Fietz, 1995), UAS-DPP::GFP (Teleman and Cohen, 2000), UAS-HH::CD2 (Strigini and Cohen, 1997), UAS-HH-N (Gallet et al., 2003), UAS-nuclear lacZ (Brand and Perrimon, 1993) and UAS-PTC (Johnson et al., 1995). *hh*-lacZ (Ma et al., 1993) was used to examine the expression of HH at the transcriptional level. *dpp*-lacZ (Blackman et al., 1991) was used to monitor the expression pattern of DPP in different genetic backgrounds.

Histology
Immunohistochemical staining was essentially as described by Patel et al. (Patel et al., 1989). Rhodamine-labeled phalloidin (to visualize F-actin) was purchased from Molecular Probes, USA. The primary antibodies used are, anti-ARM (Riggleman et al., 1990), anti-β-galactosidase (Sigma, St Louis, USA), anti-CI (Motzny and Holmgren, 1995), anti-DLG (Parnas et al., 2001), anti-EN (Patel et al., 1989), anti-FASIII (Patel et al., 1987), anti-HH (gift from T. Tabata, Tokyo, Japan), anti-pMAD (Persson et al., 1998), anti-PTC (Capdevila et al., 1994), anti-UBX (White and Wilcox, 1984) and anti-WG (Brook and Cohen, 1996). Anti-ARM, anti-DLG, anti-EN, anti-FASIII and anti-WG antibodies were obtained from the Development Studies Hybridoma Bank, University of Iowa, USA. Confocal microscopy was on Zeiss LSM/Meta.

Results
Apical surfaces of peripodial and disc proper cells face each other
The polarity of cells is crucial for signal transduction events, and reported signaling interactions between PM and DP led us to examine the orientation of PM cells in relation to DP cells.

For this purpose, we examined the localization of proteins that mark different spatial domains of epithelial cells such as the apical (actin), subapical (ARM) and basolateral (FASIII and DLG) regions.

First, the distribution of DLG and ARM in peripodial cells was visualized by confocal microscopy. As one starts taking optical sections (with a step size of 0.2 µm; average thickness of PM cells is ~1.8 µm) in the region corresponding to PM from the uppermost surface of an unmounted wing disc and moves towards the DP, DLG staining is observed first followed by ARM (Fig. 2A1). Even when DLG staining fades off almost completely, ARM is still observed (Fig. 2A2), suggesting distinct cell-polarity of PM cells. Optical sections through the entire depth of an unmounted wing disc along its Z-axis showed that DLG, FASIII, ARM and actin are localized in that order in a PM cell as we move from the surface towards DP (Fig. 2B-E). All these markers further showed that PM and DP cells are oriented with their apical sides facing each other. Thus, a cross-section of the wing disc along its Z axis first shows DLG staining, then FASIII, ARM and actin in PM cells, which is followed by actin, ARM, FASIII and DLG in that order for DP cells (Fig. 2B-E). The same was evident in the optical sections of an eye imaginal disc (data not shown).

Overexpression of Delta in PM activates Wingless expression in the pouch but not in the notum

In the context of earlier reports that inductive signals from PM cells are necessary for the proper development and patterning of the disc proper, the above-mentioned orientation of the PM could be essential for the polarized transport of signals/morphogens. To further examine the same, we overexpressed Dl, a transmembrane ligand of the N receptor. Dl activates the N pathway by binding to N, which is also a transmembrane protein, and thus direct cell-cell contact is a prerequisite for this molecular interaction. Furthermore, Dl and N are both localized on apical sides of epithelial cells (Le-Borgne and Schweisguth, 2003). We used expression of WG as a readout of the activation of the N pathway. Overexpression of Dl exclusively in the peripodial membrane using the *Ubx*-GAL4 driver, a PM-specific driver (Fig. 3B) (Pallavi and Shashidhara, 2003), caused activation of WG in the entire

pouch region of the wing imaginal disc (Fig. 3C). As a consequence, there was considerable overgrowth in the pouch. There was no detectable ectopic WG expression or overgrowth in the notum. Also, there were no signs of any notum-to-wing transformation normally associated with ectopic WG in the notum. It is likely that the transmembrane contacts that exist between PM and DP in the notum region (Gibson and Schubiger, 2000) are not sufficient for Delta-mediated activation of the N pathway. It is possible that PM-DP are separated in the pouch by a narrow lumen and a secreted form of Dl may have activated N in the disc proper. However, secreted forms of Dl and Ser are known to function as
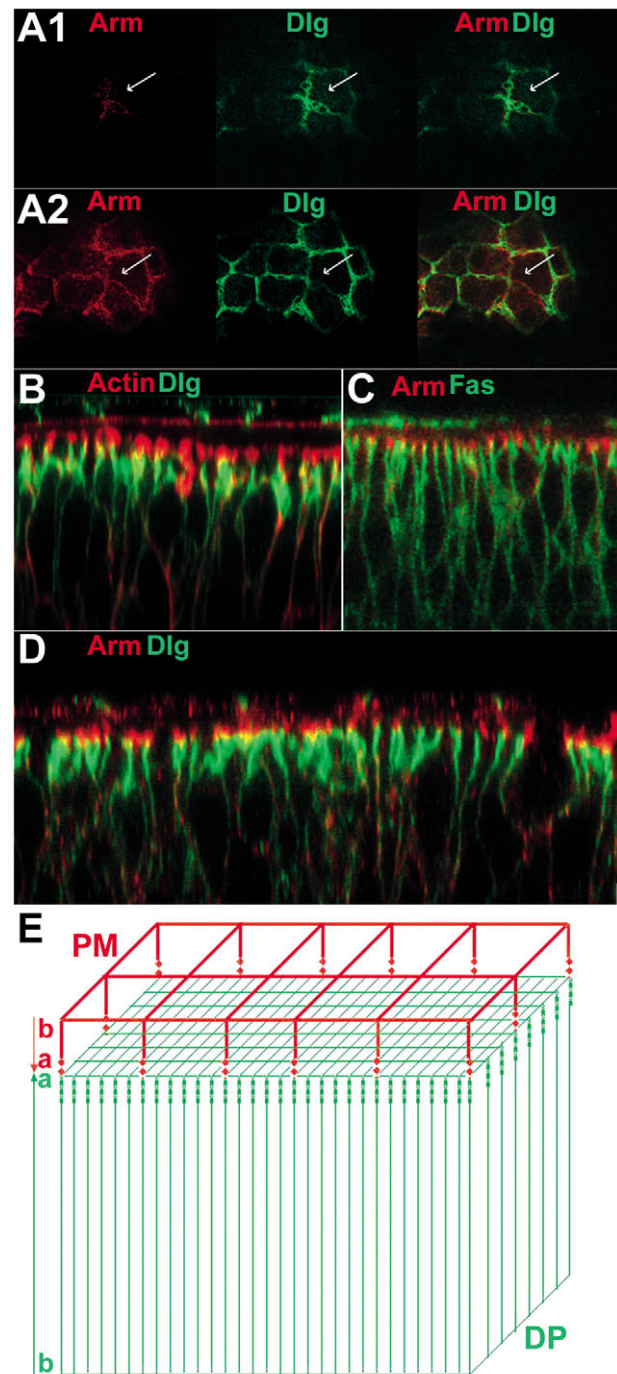

Fig. 2. Peripodial cells exhibit apical-basal polarity and their apical surfaces face the apical side of disc proper cells. (A1,A2) Two representative optical sections of peripodial cells of a wild-type wing disc stained for ARM (red) and DLG (green). The plane of focus in A1 is the top surface (away from the disc proper cells) and A2 is at a lower surface than the A1 (more towards the disc proper cells) of PM cells. Note staining for DLG is stronger in A1 (arrows) and that for ARM is stronger in A2 (arrows), suggesting that peripodial cells have their apical side towards the disc proper. (B-D) Cross-section along the Z-axis of wild-type wing discs stained for rhodamine conjugated phalloidin (which binds F-actin) and DLG (B), ARM and FAS (C) or ARM and DLG (D). Actin marks the apical side, ARM marks subapical side, and DLG and FAS mark lateral and basal sides. Note that regions of intense actin or ARM staining in PM cells face the corresponding regions of DP cells (B-D). (E) Proposed model of organization of peripodial and disc proper cells, with respect to their apical (a) -basal (b) polarities. Only a small section of wing disc is shown here. PM cells are shown in red and DP cells are in green. The apical sides of PM and DP cells are shown in patterned red and green lines, respectively.
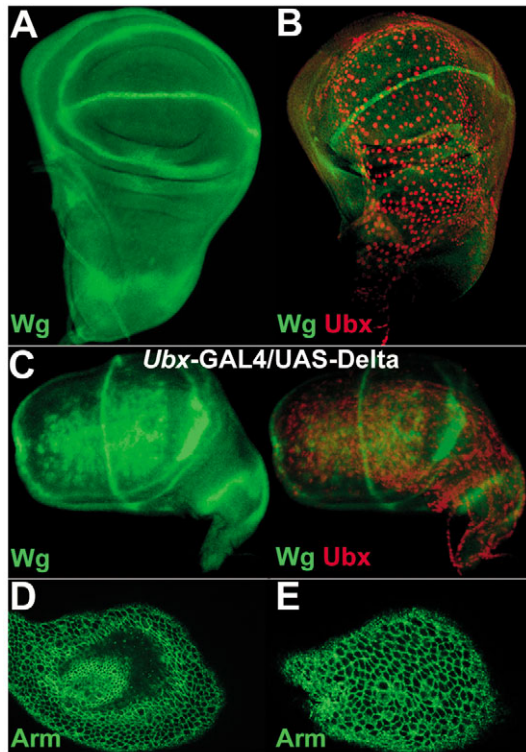
Fig. 3. Overexpression of Delta in the peripodial cells induces WG expression in the wing pouch. (A,B) Wild-type wing discs showing the expression pattern of WG (A). WG is expressed only in DP cells. (B) Wing disc of a *Ubx*-GAL4/UAS-lacZ larva stained for lacZ (red) and WG (green). Note that lacZ, and therefore *Ubx*-GAL4, is expressed only in peripodial cells. (C) Wing disc of a *Ubx*-GAL4/UAS-Delta larva stained for WG (green) and UBX (red). Note ectopic activation of WG all over the pouch and the resultant overgrowth phenotype. No such ectopic activation of WG is observed in the notum region of the disc proper, nor was there any notum-to-wing transformation normally associated with ectopic WG. Also note that peripodial cells (even the medial edge or margin peripodial cells) themselves do not express WG in response to ectopic Delta. (D,E) Wing disc of a *Ubx*-GAL4/UAS-Delta larva stained for ARM. The focal plane in D is at the level of wing pouch and medial edge cells of PM. Both DP and medial edge cells are normal. In E, only PM cells are shown at higher magnification. Note that PM cells are more elongated and densely arranged than in wild-type (Fig. 1B). It is possible that Dl-induced overgrowth in DP cells may have stretched PM and hence changes in cell morphology. Nevertheless, the identity of PM cells is still maintained as they continue to express UBX and do not express WG.

antagonists of Notch signaling (Sun and Artavanis-Tsakonas, 1997). Our observations, therefore, confirm that PM and DP cells are in direct contact in the pouch region and not in the notum. Activation of WG in DP in response to ectopic expression of Dl in PM also confirms that the two layers have their apical sides facing each other.

Interestingly, we did not observe any activation of WG in cells at the medial edge of the wing disc PM, which do not express *Ubx*-GAL4. It has been shown earlier that repression of WG early during development is a prerequisite to specify peripodial fate (Baena-Lopez et al., 2003). It is possible that the mechanism operating in PM cells to repress WG is downstream of the N signaling pathway.

Patterning of PM along the anterior-posterior axis

The majority of PM cells (with the exception of medial edge cells) express UBX and are derived from UBX-expressing parasegment 6, from which posterior T2 and anterior T3 develop (Pallavi and Shashidhara, 2003). In addition, all UBX-expressing PM cells also express EN (Pallavi and Shashidhara, 2003) (Fig. 4A-A′). These observations confirm posterior identity of UBX-expressing PM cells.

Unlike the posterior compartment of DP, staining for *hh*-lacZ reporter construct suggests the absence of HH transcripts in EN/UBX-expressing PM cells (Fig. 4B). At the protein level too, we did not detect any HH in PM cells (Fig. 4C). However, similar to the situation in the posterior compartment of DP, CI is not expressed in EN/UBX-expressing PM cells (Fig. 4D). Although much of the peripodial membrane is posterior in nature, regions comprising medial edge cells (on either sides) do not express UBX or EN (Fig. 4A,A′). Interestingly, the peripodial epithelium shows strong DPP expression in a narrow row of cells, which abut EN/UBX-expressing peripodial cells and the medial edge cells (Fig. 4E). The double staining for EN or UBX and *dpp*-lacZ shows that the EN and UBX are not expressed in those PM cells that express DPP. Assuming that PM cells are patterned in the same way as DP cells, EN/UBX-expressing cells may mark the posterior compartment, and medial edge cells, which do not express EN or UBX, may form the anterior compartment. The DPP-expressing domain would then mark the A/P boundary, a landmark similar to the DPP-expressing domain in DP. The phenomenon, however, is more complex because no PM cells including medial edge cells express CI (Fig. 4D).

CI is repressed by EN in the posterior cells of the disc proper and hence they do not respond to HH signaling. However, overexpression of CI in the posterior compartment causes ectopic DPP expression and thereby pattern duplications. Similar to these patterning events in DP cells, overexpression of HH using the PM-specific *Ubx*-GAL4 driver did not activate DPP in PM cells (see below), whereas overexpression of the full-length (activator) form of CI induced ectopic DPP expression in PM cells (Fig. 4F). These observations indicate that downstream of EN, both types of epithelial cells (PM and DP) have a similar hierarchy of gene regulation during patterning along the anterior-posterior axis.

The absence of HH and CI expression in PM cells, however, raises the question regarding the mechanism of DPP expression in those cells. Patched (PTC), the receptor of HH, is also a target of HH signaling. Antibody staining for PTC did not reveal any expression in PM cells (Fig. 4G), although a *ptc*-GAL4 driver showed expression in PM cells (data not shown). Moreover, overexpression of PTC, which antagonizes HH signaling (Johnson et al., 1995; Chen and Struhl, 1996), using *Ubx*-GAL4 driver did not affect DPP expression in PM (Fig. 4H). It is possible that the disc proper may influence DPP expression in the peripodial membrane. However, overexpression of HH only in DP using the *426*-GAL4 driver, a DP-specific GAL4 driver (Fig. 4I) (Pallavi and Shashidhara, 2003), did not induce ectopic DPP expression in PM cells (Fig. 4J,J′). Thus, the mechanism of activation of DPP expression in PM cells remains unclear and requires further investigation.

Nevertheless, our observations bring out significant similarities (the absence of any response to ectopic HH by
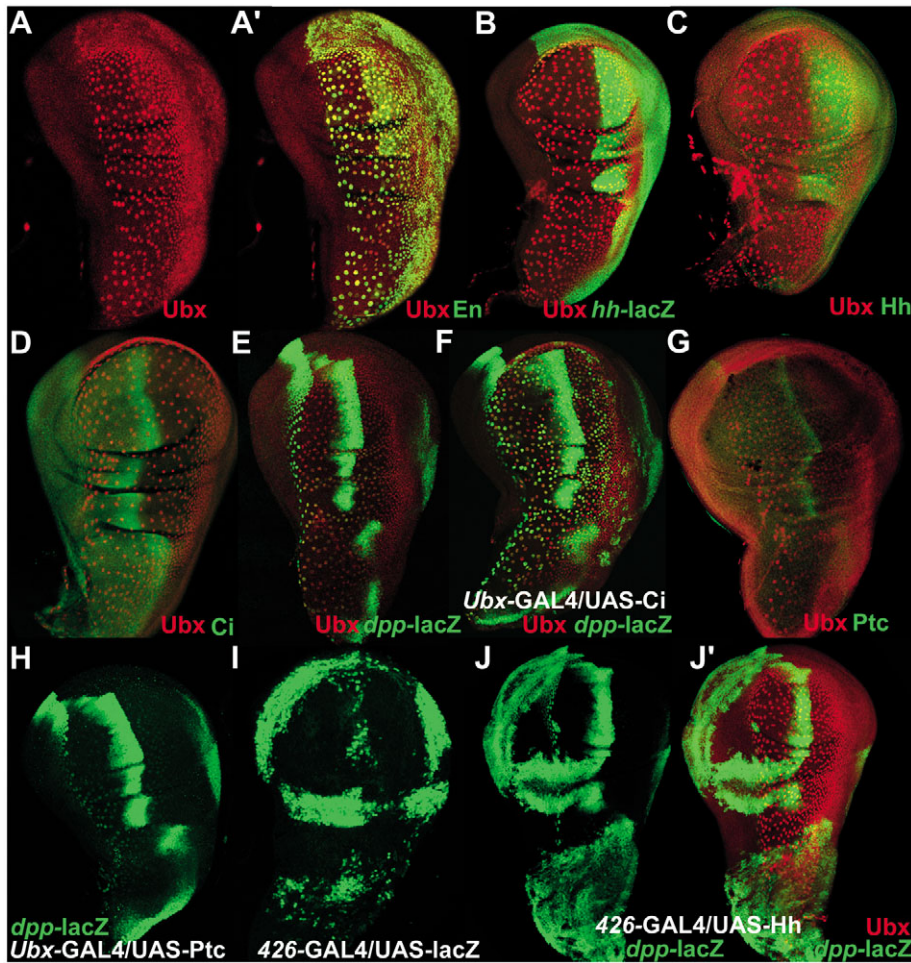
Fig. 4. Anterior-posterior patterning in the peripodial membrane. (A,A′) Wild-type wing disc stained for UBX (red) and EN (green) expression. All UBX-expressing PM cells express EN. (B,C) Wild-type wing discs stained for *hh*-lacZ (green) and UBX (red) expression (B) or for HH protein (green) and UBX (red) expression (C). HH expression is not detectable in PM cells. (D) Wild-type wing disc stained for CI (green) and UBX (red) expression. CI too is not expressed in PM cells. (E) *dpp*-lacZ wing disc stained for lacZ (green) and UBX (red). DPP is expressed in a subset of PM cells, overlaying the anterior compartment of DP. (F) Wing disc of the genotype *dpp*-lacZ; *Ubx*-GAL4/UAS-CI stained for lacZ (green) and UBX (red). Note ectopic activation of DPP in all cells overexpressing CI. (G) Wild-type wing disc stained for PTC (green) and UBX (red) expression. In DP, PTC is expressed in the anterior compartment with A/P boundary expressing the highest levels, whereas it is not detected in PM cells. (H) Wing disc of the genotype *dpp*-lacZ; *Ubx*-GAL4/UAS-PTC stained for lacZ. DPP expression in PM cells is not affected by ectopic PTC. (I) *426*-GAL4/UAS-nuclear lacZ wing disc showing expression pattern of *426*-GAL4 driver. It is not expressed in PM cells. (J,J′) Wing disc of the genotype *dpp*-lacZ; *426*-GAL4/UAS-HH stained for lacZ (green) and UBX (red). Note ectopic DPP in the anterior compartment of DP. No such ectopic DPP is observed in PM cells.

EN/UBX-expressing PM cells; activation of DPP expression in PM cells in response to ectopic CI) and differences (absence of HH expression in EN/UBX-expressing PM cells) between PM and DP of the wing disc. These observations, along with morphological differences in cell shapes, and the established signaling between the two membranes strengthens the utility of DP and PM as a unique model system to study epithelial cell interactions and molecular mechanism/s of related signal transduction pathways.

Overexpression of Hedgehog in PM activates DPP expression in the pouch but not in the notum

The Hedgehog family of proteins comprises short-range morphogens. *Drosophila* HH and vertebrate Sonic Hedgehog (SHH) are known to tether to the membrane with the help of covalently attached cholesterol (Porter et al., 1996), although small amounts of HH/SHH are known to be present in soluble fractions (Zeng et al., 2001). Furthermore, HH diffuses from the apical side of posterior cells and is received at the apical side of anterior cells (Stringini and Cohen, 2000; Gallet et al., 2003). Consistently, the localization of PTC receptor has been shown to be at the apical side of the cells (Capdevila et al., 1994; Denef et al., 2000). As both PM and DP are made up of epithelial cells with their apical ends facing each other and the two layers maintain cell-cell contacts in the pouch and connect

via long microtubular extensions in the notum, overexpression of HH in PM would be a good assay system to study morphological requirement for the diffusion of HH. Furthermore, the absence of endogenous HH signaling in PM cells would be an advantage as there would not be any interference to the ectopically expressed HH. We used the expression of DPP (monitored using *dpp*-lacZ) as a molecular readout of HH pathway. Overexpression of HH using UAS-HH (it expresses wild-type cholesterol-modified HH) (Ingham and Fietz, 1995) only in PM using *Ubx*-GAL4 driver induced overgrowth of DP, which was restricted only to the anterior wing pouch (Fig. 5B). We also observed strong ectopic expression of DPP in a large number of DP cells spread all over the anterior wing pouch (Fig. 5B) (as mentioned above, ectopic HH did not induce DPP expression in PM cells). The levels of pMAD were also significantly high in the entire anterior wing pouch (Fig. 5D). WG expression patterns in these discs were unaffected (Fig. 5E), suggesting that the overall patterning events in these discs were normal and there was no mixing of PM and DP cells (PM cells do not express WG). However, we did not observe such ectopic DPP or pMAD in the anterior notum (Fig. 5B,D). It is possible that notum cells are intrinsically not sensitive to overexpression of HH. However, overexpression of HH directly in the notum using a DP-specific GAL4 driver showed ectopic DPP staining in the notum (Fig. 4J). Therefore, the absence of ectopic DPP in the notum in the

above-mentioned experiment is probably due to the inability of HH to diffuse from PM to the notum.

The above observations suggest that wild-type HH can diffuse from PM to DP in the pouch and not in the notum. This is interesting considering the observation that cellular processes that arise from PM in the notum are membranous in nature. It is possible that these structures do not support cholesterol-aided diffusion of proteins or that the gap between the PM and DP in this part of the wing disc is too long for HH to show any effect. To test these possibilities we overexpressed two different mutant forms of HH. (1) Only the N-terminal domain of HH, which is not cholesterol-modified (HH-N) (Gallet et al., 2003) and (2) a membrane-tethered form of HH generated by fusing the N-terminal domain of HH with rat CD2 (HH::CD2) (Stringini and Cohen, 1997). Owing to the absence of the C-terminal domain, HH::CD2 is also not cholesterol-modified. We expressed these two mutant forms of HH only in PM using the *Ubx*-GAL4 driver. Overexpression of HH-N induced DPP expression and overgrowth phenotype both in the pouch as well as in the notum (Fig. 5F), whereas HH::CD2

induced DPP activation and overgrowth phenotype only in the pouch region (Fig. 5G). Similar to wild-type HH, overexpression of HH-N and HH::CD2 directly in the anterior notum activates DPP expression in notum cells (data not shown). These results suggest that cholesterol-modification of HH is a mechanism to ensure its restricted or short-range diffusion. PM-DP interactions in the wing disc, thus, provide us with a tool to examine the mechanism of such signal transduction pathways.

Discussion

Recent reports suggest that varied signals traversing between PM and DP help in patterning the wing imaginal disc (Gibson and Schubiger, 2000; Cho et al., 2000; Gibson et al., 2002; Pallavi and Shashidhara, 2003). These reports suggest a functional role for PM cells in disc patterning apart from their role in providing mechanical support during disc eversion. Considering that PM is made up of squamous epithelial cells and DP is made of columnar epithelial cells, they provide us with a model assay system for understanding signaling interactions between distinct types of epithelial cells. Such studies may provide us with clues to understand development of tissues and also pathological situations leading to metastasis. Here we have explored the possible utility of squamous peripodial epithelium and columnar disc epithelium of *Drosophila* wing disc as a model system to study the mechanism of signal transduction between distinct epithelial cell types.

Apical-basal polarity in PM cells

Many morphogens are present on the apical surfaces of the cells and are secreted to the adjoining, adjacent cells where they lead
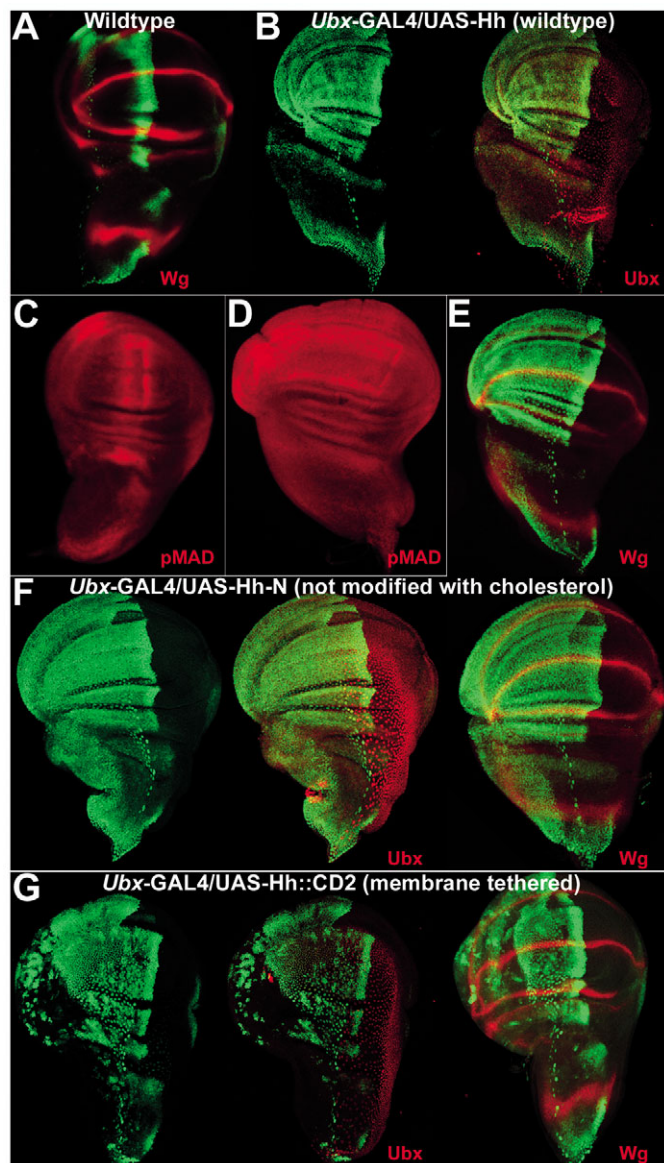

Fig. 5. Signaling by HH is dependent on cell-cell contacts, and HH does not diffuse through trans-membrane extensions that bridge peripodial and disc proper cells of the notum. Discs in A, B, E-G are stained for *dpp*-lacZ (green) along with either UBX or WG (red) as shown on individual images. Discs in C and D are stained for pMAD. (A,C) Expression pattern of *dpp*-lacZ (A) and pMAD (C) in wild-type background. (B,D-E) Wing disc (of the genotype *dpp*-lacZ; *Ubx*-GAL4/UAS-HH), wherein wild-type HH is overexpressed in the peripodial cells. Note ectopic DPP expression, which is restricted to the anterior compartment of the pouch region (B). As a consequence, these discs show increased levels of pMAD in the entire anterior wing pouch (D). No DPP and pMAD activation or much less DPP and pMAD activation is observed in the notum, which is connected to the peripodial membrane through long microtubule-membrane extensions. (F) Wing disc (of the genotype *dpp*-lacZ; *Ubx*-GAL4/UAS-HH-N), wherein the cholesterol-unmodified form of HH is overexpressed in the peripodial cells. This form of HH does not have cholesterol moiety and therefore diffuses freely in the extracellular space. Note activation of DPP in the anterior compartments of both the pouch and the notum. (G) Wing disc (of the genotype *dpp*-lacZ; *Ubx*-GAL4/UAS-CD2::HH), wherein the membrane-tethered form of HH is overexpressed in the peripodial cells. Note that similar to wild-type HH, membrane-tethered HH is capable of inducing the activation of DPP only in the anterior compartment of the pouch region. All the three forms of HH caused considerable overgrowth in the anterior pouch. In addition to the pouch, HH-N caused overgrowth phenotype in the notum. However, all the three forms of HH failed to activate DPP in the peripodial membrane itself. None of the three HH forms affected WG expression in either the pouch or the notum.

to the activation of the signaling cascade by binding to their respective receptors. The receptors too are found at the apical surfaces and thus apical surfaces of epithelial cells are the sites of signaling activities. Here we have shown that PM cells have their polarity reversed with respect to the polarity of DP cells. Thus, the two types of cells have their apical sides facing each other (Fig. 3E). As PM and DP arise from a common pool of embryonic precursor cells (Pallavi and Shashidhara, 2003), it is intriguing how the two cell layers have such an arrangement. It is possible that the imaginal primordium first forms a single layer of disc epithelium, which may fold over itself into a sac-like structure, such that the overlying cells will have their apical side facing the apical side of underlying cells. Specification of the overlying cells as peripodial membrane with squamous epithelial morphology may precede or be concurrent to this folding event. With the help of ectopic activation of the N pathway in PM, we have shown that the apical domain of PM cells does function as a signaling site and thereby could activate WG expression in the underlying DP. Thus, the arrangement of PM and DP cells vis-à-vis cell polarity confirms the potential of PM cells to signal to DP cells.

Patterning of PM along the A/P axis

Both peripodial and disc epithelia are derivatives of a single embryonic imaginal primordium. However, peripodial epithelium does not express WG or VG, which are required for the specification of the wing pouch, nor does it express Iro-C complex genes (Baena-Lopez et al., 2003), which specify notum identity. Nevertheless, PM cells show similarity to DP cells at the levels of patterning events along the AP axis. Only a part of the PM expresses EN. In particular, medial edge cells do not express EN. Between EN[+] and EN^{−} PM cells is a stripe of DPP-expressing cells, which may mark the AP boundary of the peripodial epithelium. Similar to EN[+]-DP cells, EN[+]-PM cells do not express DPP even when we overexpress HH, but they express DPP in response to over expression of CI. However, in the absence of HH and CI expression in PM cells, it is intriguing how DPP is expressed in a narrow row of cells abutting EN[+] and EN^{−} PM cells. Furthermore, overexpression of PTC in PM cells did not affect DPP expression, suggesting a possibility that DPP expression in PM cells is independent of HH function. Thus, regulation of DPP expression observed in a small subset of PM cells needs further investigation. Nevertheless, differences at the morphological level and certain similarities in patterning events between PM and DP further strengthen their utility as a model system.

Interestingly, CI-induced ectopic DPP in the PM did not affect growth properties (Fig. 4F) nor the levels of pMAD (data not shown) in DP. It is possible that activation of DPP in few PM cells may not imbalance the growth of DP cells. However, even overexpression of a DPP::GFP fusion protein (Teleman and Cohen, 2000) directly in PM cells did not affect the growth properties of DP cells, nor did we detect any GFP in those cells (data not shown). These results, thus, suggest that DPP cannot diffuse from PM to DP. Earlier reports suggest that DPP movement is mediated by an endocytic pathway (Gonzalez-Gaitan and Jackle, 1999; Entchev et al., 2000; Arquier et al., 2001; Bharathi et al., 2004). However, a recent report suggests that DPP moves along the cell surface by restricted extracellular diffusion, which is regulated by glypican proteins Dally and

Dally-like (Belenkaya et. al., 2004). In either case (glypican-regulated or endocytosis-mediated movement of DPP), PM cells may not support movement of DPP, although they are capable of receiving DPP signals from DP (Gibson et al., 2002). Interestingly, PM cells do express Dally, a protein required for the diffusion of DPP (K. Makhijani and L.S.S., unpublished observations). It would be interesting to know what mechanisms operate to make DPP diffusion unidirectional. Again, PM and DP may prove to be useful system to study directional movement of such signaling molecules.

Cholesterol modification causes restricted diffusion of Hedgehog

As both PM and DP are made up of epithelial cells with their apical ends facing each other, overexpression of HH in PM would be a good assay system to examine the mechanism of diffusion of HH. The absence of endogenous HH signaling in PM cells would be an added advantage because there would not be any interference to the ectopically expressed HH. Cholesterol modification of HH is believed to be required for its efficient sequestration rather than for signaling per se (Burke et al., 1999). However, recently it has been suggested that cholesterol modification of HH helps in its localization to the apical ends of epithelial cells and is responsible for the activation of only a subset of HH targets (Gallet et al., 2003).

We tested the ability of three different forms of HH, when overexpressed exclusively in PM cells, to activate DPP in DP cells: the wild-type HH, which is cholesterol modified, and two mutant forms, which are not cholesterol modified. Of the latter two, HH-N is mutant only for cholesterol modification (Gallet et al., 2003), whereas HH::CD2 is derived by fusing HH-N protein to the transmembrane domain of the rat CD2 protein (Stringini and Cohen, 1997). HH::CD2 thus lacks cholesterol modification and also does not freely diffuse between cells. It can signal only when the producing and receiving cells make direct cell-cell contacts. We have observed that all the three forms of HH are capable of activating DPP in the pouch, where both the membranes are juxtaposed to each other. In the notum, however, only HH-N could induce DPP expression. Previous reports suggest that cholesterol modification of HH is required for its apical targeting in expressing cells (Gallet et al., 2003). In their experiments, HH-N could partially activate WG (a target of HH in the anterior compartment) expression, whereas HH::CD2 failed to activate WG. The authors have attributed this to the inability of the mutant forms of HH to localize to the apical ends of producing cells (Gallet et al., 2003). In our assay system, we have observed that both HH-N and HH::CD2 could activate DPP expression in the anterior pouch, when expressed in PM cells. However, overexpression of HH-N and not HH::CD2 in PM cells could activate DPP expression in the anterior notum. Thus, our observations suggest that cholesterol modification on HH necessitates cell-cell contact and confirms earlier reports that cholesterol modification makes HH a short-range signaling molecule.

Overexpression of wild-type HH caused activation of DPP in the entire anterior compartment of the pouch as one continuous domain of expression, whereas misexpression of HH::CD2 caused activation of DPP in small patches. HH::CD2 is membrane tethered and therefore it cannot diffuse and is capable of activating DPP only at the places of direct contact

between the two layers. Thus, cells that show DPP expression in the anterior pouch in response to HH::CD2 expression in PM may correspond to those DP cells that form direct contacts with PM cells. Previous reports suggest that PM and DP cells are in 1:80 ratio in the wing disc (Pallavi and Shashidhara, 2003). Although PM cells are large enough to cover the entire DP, it is possible that only a subset of PM cells actually make contacts (of functional significance) with DP cells. Such mapping of cell-cell contact points between PM and DP may provide a useful tool for further studies on possible role/s of PM in patterning wing pouch.

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