EGF receptor attenuates Dpp signaling and helps to distinguish the wing and leg cell fates in Drosophila

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SUMMARY

Wing and leg precursors of Drosophila are recruited from a common pool of ectodermal cells expressing the homeobox gene Dll. Induction by Dpp promotes this cell fate decision toward the wing and proximal leg. We report here that the receptor tyrosine kinase EGFR antagonizes the wing-promoting function of Dpp and allows recruitment of leg precursor cells from uncommitted ectodermal cells. By monitoring the spatial distribution of cells responding to Dpp and EGFR, we show that nuclear transduction of the two signals peaks at different position along the dorsoventral axis when the fates of wing and leg discs are specified and that the balance of the two signals assessed within the nucleus determines the number of cells recruited to the wing. Differential activation of the two signals and the cross talk between them critically affect this cell fate choice.

Key words: Limb, Cell fate, Cross-talk, MAD, MAP kinase, Drosophila

INTRODUCTION

It has been shown that development of insect wings and legs involves intercellular communication mediated by evolutionary conserved signaling pathways such as Hedgehog (Hh), Wingless (Wg) and Decapentaplegic (Dpp) (reviewed in Campbell and Tomlinson, 1995; Brook et al., 1996). The signals are expressed in distinct spatial patterns, defining the unique shape of each appendage and regulating cell growth and differentiation. Studies of such cell signaling in insect appendage development provide a framework to understand a key question of embryology; how the positional information in the two-dimensional field of early embryos is used to specify appendages with three-dimensional organization. In addition, a comparison of the genetic circuitries that specify the leg and wing should help us to understand the ontogenic relationship between the two types of appendages.

Studies on dipteran insects Ducas (Anderson, 1963) and Drosophila (Wieschaus and Gehring, 1976) demonstrated that wings and legs share a common developmental origin. Wing and leg development in Drosophila first becomes evident in early stage 11 embryos when a group of cells (limb primordium) in the lateral ectoderm start to express the homeobox gene Distal-less (Dll) due to induction by Wg (Cohen, 1990; Fig. 1A, staging is according to Campos-Ortega and Hartenstein, 1997). Although Wg is expressed in stripes along the anteroposterior compartment boundary, its inductive effect is limited to the lateral position by the early functions of Dpp and EGF receptor (EGFR) that control global dorsoventral patterning. Dll expression at stage 11 is repressed in the dorsal ectoderm by Dpp and in the ventral ectoderm by EGFR (Fig. 1A, staging is according to Campos-Ortega and Hartenstein, 1997). At this stage, Dpp initiates a second phase of expression in the lateral ectoderm as a spot at the dorsal side of each limb primordium (Goto and Hayashi, 1997b; Figs 1B, 2B). High and low activities of Dpp are required for the specification of wing and proximal leg disc cells, respectively (Goto and Hayashi, 1997b), suggesting that Dpp exerts a graded effect on the wing and leg development. Cell migration separates these cells to form the wing and leg imaginal discs (Cohen et al., 1993; Fig. 1B). The proximodistal axis becomes established in the leg disc by stage 15 when it is separated into the proximodistal subdomains (Goto and Hayashi, 1997b). A wing disc-specific genetic program is turned on when migrating wing disc cells begin to express vestigial (vg; Williams et al., 1991), followed by expression of two related zinc-finger genes escargot (esg) and snail (sna), which have the overlapping function of maintaining commitment to the wing disc cell fate (Fuse et al., 1996). These findings demonstrate that Dpp regulates both wing and leg cell fates.

Several studies suggest that the ectodermal cells expressing Dll at stage 11 are not committed to the wing, leg or epidermal fate. Cell tracing experiments demonstrated that a subset of those cells migrate dorsally within the ectodermal cell layer, and the dorsalmost cells invaginate and form the wing disc. Cells at the intermediate position between the wing and leg discs were not incorporated into either of the
Discs, and most likely differentiate as larval epidermis (Goto and Hayashi, 1997a). In double mutants for esg and sna, cells that began the dorsal migration fail to maintain the expression of wing-specific markers and differentiate as epidermis. In contrast, elevated expression of Dpp caused an increase of wing cell number, apparently due to the recruitment of those intermediate cells. These results indicate that determination of the wing disc fate is a two-step process, allocation to the limb primordium followed by commitment to the wing disc. Failure in the latter step results in epidermal differentiation. It is likely that the leg disc is determined in a similar two-step mechanism. What remains to be determined is how the choice of wing and leg cell fates is made within the limb primordium.

Dpp is a member of the TGFβ family of secreted signaling molecules that are thought to reach distant cells and evoke several distinct responses depending on the level of receptor activation (reviewed in Neumann and Cohen, 1997; Raftery and Sutherland, 1999). Drosophila Mad is a founding member of the SMAD family of signal transducers essential for TGFβ-like signaling (Raftery et al., 1995; Sekelsky et al., 1995; Newfeld et al., 1996; Massague, 1998) and regulates Dpp-dependent transcription of the target gene vestigial (vg) in the wing disc by directly binding to the wing-specific enhancer (Kim et al., 1997). Biochemical studies on vertebrate TGFβ-related signaling demonstrated that the binding of ligands to their receptors activates serine-threonine kinases in the intracellular domains of the receptors and phosphorylation of the C terminus of SMAD proteins. Phosphorylated SMADs translocate from the cytoplasm to the nucleus and bind to specific target genes to control transcription. It was shown previously that the vertebrate RAS-MAP kinase pathway antagonizes the BMP/TGFβ pathway by phosphorylating and inhibiting SMADs (Kretzschmar et al., 1997a, 1999). However, whether such a cross talk occurs in development is not known.

In this work, we identified EGFR as additional signal acting together with Dpp to promote wing and leg disc development. EGFR is a receptor tyrosine kinase and regulates several cell fate decisions through activation of RAS-MAP kinase cascade (reviewed in Schweitzer and Shilo, 1997). Using reagents that detect nuclear transduction of EGFR and Dpp signals, we show that cells responding to EGFR are positioned ventrally to those responding to Dpp. EGFR positively regulates proper differentiation of the leg disc. In addition, EGFR negatively regulates wing disc development, in part by counteracting Dpp signaling after its transduction to the nucleus. The results suggest that EGFR acts in the limb primordium to help determine wing versus leg disc development.

MATERIALS AND METHODS

Fly strains and temperature shift protocols

The fly strains used and their sources were as follows: Dll-lacZ reporters Dll 304-β-gal and Dll 215-β-gal (Vachon et al., 1992) from Dr Stephen Cohen; UAS-sppitz (Schweitzer et al., 1995), UAS-EGFR.DN (O’Keefe et al., 1997) from Dr Ben-Zion Shilo; ato1 (Jarman et al., 1994), rho6, Egfr6, and Egfr7 from Dr Masataka Okabe; DsorF115 (Tsuda et al., 1993) from Dr Yasuyoshi Nishida; UAS-dad (Tsuneizumi et al., 1997) from Dr Tetsuya Tabata; Egfr11 from the Tubingen Stock Center; tkv1 and UAS-dpp from Bloomington stock center. Zygotic mutant chromosomes were balanced over lacZ-marked balancers. See FlyBase (1994) for more information. Temperature-shift experiments were done by collecting embryos at 25°C for 1 hour, and embryos were incubated at 18°C before shifting up to 29°C at appropriate time points.

Preparation of pSSVS antibody

The rabbit anti-pSSVS antibody was raised against a 13 amino acid peptide QMGPHNAISSVS(Pi)VS(Pi) corresponding to the phosphorylated form of the MAD C-terminal domain. The serum was affinity purified with the phosphopeptide-conjugated column, and the antibody that binds to the non-phosphorylated form was removed by absorption to a column conjugated with QMGPHNAISSVS peptide. The whole procedure was carried out at Peptide Institute Inc., Osaka, Japan.

Immunostaining and histochemical analyses

In situ hybridization was performed with a digoxigenin-labeled RNA probe (Tautz and Pfeifle, 1989; Goto and Hayashi, 1997a) using cDNAs of Dll (Panganiban et al., 1994), rho (Bier et al., 1990; from Dr Masataka Okabe), dad (Tsuneizumi et al., 1997), and dpp (Padgett et al., 1987). Anti-Esg (Fuse et al., 1994), anti-Vg (Williams et al.,

Fig. 1. Models of Dpp and EGFR function at two stages of limb development. Limb primordia expressing Dll are labeled red in A and B (left). (A) At early stage 11, Wg is expressed along the AP compartment boundary and induces Dll expression in the limb primordium (Cohen, 1990). Dll expression is inhibited by the early activities of Dpp and EGFR in the dorsal and ventral regions, respectively (Goto and Hayashi, 1997b). (B) Activity of Dpp and EGFR signaling at stage 11 (left) and their effects on the development of wing and leg discs at stage 15. At late stage 11, dpp is expressed at the dorsal side of the limb primordium (left) and controls the later development of the wing and proximal leg disc cells in a dosage-dependent manner (blue arrows; Goto and Hayashi, 1997b). EGFR is activated in the midventral region (left) and inhibits and promotes the development of wing and leg discs, respectively (green lines, this study). Note that the cellular response to Dpp forms a gradient and the response to EGFR forms a sharp boundary, and that each response peaks at different positions along the dorsoventral axis (see text).
1991), anti-dpMAPK (Gabay et al., 1997a) and anti-β-galactosidase (Cappell) were used for immunostaining, which was sometimes amplified with a TSA indirect kit (NEN). Antibody binding was detected with Cy2- or Cy3-conjugated secondary antibodies or streptavidine (Amersham) and observed with a confocal microscope (LSM410, Carl Zeiss).

RESULTS

Activation of EGFR-MAPK pathway in the limb primordia

In a screen for genes expressed in the embryonic limb primordia, we found that rhomboid (rho; Bier et al., 1990) is transiently expressed in the central part of Dll-expressing limb primordia in stage 11 embryos. rho transcription is the rate-limiting step of the activation of an EGFR ligand Spitz (Spi; Schweitzer et al., 1995; Golembo et al., 1996). As expected from the role of rho as a stimulator of EGFR, we detected a transient expression of an activated, phosphorylated form of MAPK (dpMAPK; Gabay et al., 1997a,b) in the nucleus of limb primordial cells surrounding the rho-expressing cells. The dpMAPK expression started after the initiation of Dll transcription (Fig. 2C,D) and diminished before the separation of the wing and leg disc primordium (Fig. 2E). The dpMAPK expression was undetectable in null mutants of rho or Egfr (data not shown). The peak of dpMAPK expression was located ventrally to the cells expressing dpp (Fig. 2B; Goto and Hayashi, 1997b). The results suggest that rho-mediated stimulation of EGFR and MAPK occurs at the time of cell fate specification of wing and leg discs.

Differential distribution of cells responding to EGFR and Dpp signals

We next studied the spatial distribution of cells responding to Dpp and its relationship to EGFR signals. To this end, an antibody specific to phosphorylated C-terminal sequence of MAD was produced (see Materials and Methods). The phosphorylated sequence corresponds to the site at which the type I BMP receptor phosphorylated SMAD1 (Abdollah et al., 1997; Kretzschmar et al., 1997b; Souchelnytskyi et al., 1997). An affinity-purified antibody detected an antigen distributed in a pattern similar to, but broader than, that of dpp mRNA (Fig. 2F; a detailed description of the embryonic expression pattern will be reported elsewhere). This immunoreactivity was dependent on Dpp signaling, as it was absent in stage 11 mutants of thick veins (tkv) encoding type...
I Dpp receptor (Nellen et al., 1994; Fig. 2H) and in dpp mutants (data not shown). This indicates that other extant TGFβ-related signaling molecules present in Drosophila embryos (Khalsa et al., 1998; Brummel et al., 1999) do not substitute for Dpp to induce this immunoreactivity. Conversely, ectopic expression of Dpp resulted in high accumulation of this immunoreactivity (see below; Fig. 4K, O). These results suggest that the antibody detects a Dpp-specific signaling event, most likely the phosphorylation and nuclear transport of MAD. Hereafter, we call the immunoreactivity detected by this antibody pSSVS.

pSSVS was found mainly localized in the nucleus and distributed in regions a few cells wider in diameter than those of dpp-expressing cells (Fig. 2F). These properties are consistent with the previous findings that MAD transduces the Dpp signal to the nucleus. Double labeling of pSSVS and Dll mRNA showed that pSSVS expression was higher in the dorsal region of Dll-expressing cells (Fig. 2G). Combined with the double-labeling results of dpMAPK and Dll or dpp (Fig. 2B,D), we concluded that cells responding to Dpp and EGFR overlap, but the peak of the responses are shifted. Such differential distribution of the two signals results in an arrangement of cells responding to a different strength of Dpp and EGFR along the dorsoventral axis.

**EGFR is required for leg disc development**

To study the role of EGFR at the stage of wing and leg cell fate determination, we examined specific marker gene expression in EGFR signaling mutants. Dll mRNA is expressed in the entire limb primordium at stage 11 (Fig. 3A) and becomes restricted to distal leg cells at stage 15 (Fig. 3B). Esg protein expression was used to detect both wing and proximal leg cells (Fig. 3B; Goto and Hayashi, 1997b). In rho mutants, the size of limb primordia at stage 11 was the same as the control (Fig. 3A,C), but the later development of leg discs was abnormal. The number of leg disc cells expressing Dll and/or Esg at stage 15 was reduced, and these cells no longer showed the circular arrangement typical of leg disc precursors (Fig. 3D, compare with Fig. 3B). Amorphic mutation of Egfr caused a ventral expansion of limb primordia (Fig. 3E) as a result of a loss of the early function of EGFR (Raz and Shilo, 1993), but the expression of leg markers was severely reduced at stage 15 (Fig. 3F). The loss of leg disc cells in rho and Egfr mutants was confirmed using a Dll-215-lacZ marker (Vachon et al., 1992), which is fortuitously expressed in the entire proximal domain and a part of the distal domain of the leg disc (data not shown). A similar phenotype was observed in mutants lacking both maternal and paternal copies of Dsor1, which encodes a MAP kinase kinase (Tsuda et al., 1993; Fig. 3G,H). In all cases described above, Esg-expressing cells at the dorsal part of leg

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**Fig. 3.** EGFR-MAPK cascade is required for the specification of legs, but not for wings. Embryos at stage 11 (left) and at stage 15 (right) were stained for Esg (green), and/or Dll mRNA (brown in A,C,E,I; red in B,D,F,G,H,J,L) to reveal wing and leg disc cells. Large arrowheads, limb primordia (K); arrowheads, leg discs; small arrows, wing discs. Ventral midline, white dotted lines in ventrally oriented embryos. (A,B) Control embryos; (C,D) rho6 embryos, Dll expression was slightly expanded in stage 11 (C), and the number of leg disc cells was reduced in stage 15 (D). (E,F) In Egfrf6 embryos, limb primordial cells were increased in number (E), but the final number of leg disc cells was greatly decreased (F). (G,H) Embryos lacking both maternal and zygotic copies of Dsor1 show the same phenotype essentially as those in Egfr mutants. (I,J) Embryos of Egfrf1 temperature-sensitive allele were grown at the permissive temperature (18°C) up to 6 hours AEL and then shifted up to the restrictive temperature (29°C) and fixed at stage 11 (I) and stage 15 (J). Initial Dll expression was normal (I) but the leg disc cells were greatly lost (J). (K) The expression pattern of Dll-Gal4 was revealed by UAS-lacZ reporter. (L) Expression of a dominant-negative EGFR by Dll-Gal4 resulted in the phenotype similar to rho and Egfrf1. Scale bar: (A,K) 100 μm; (B,G) 50 μm. C,E and I are the same magnification as A. D,F,H,J and L are the same magnification as B.
EGFR interferes with wing disc development

In contrast to the severe defects in leg discs, none of the mutations in EGFR signaling interfered with wing disc formation. In these mutants, wing primordia consistently expressed Esg (Fig. 3D,F,H,J,L) and another wing disc marker Vestigial (Williams et al., 1991; data not shown), and invaginated to form discs. However, we noted an increase in the number of wing disc cells in EGFR signaling mutants. We have chosen to analyze this effect in rho mutants in which, unlike in EGFR mutants, the number of limb primordial cells at stage 11 was the same as the control (Fig. 3C). The number of Esg-expressing wing disc cells in rho mutants was increased (24.7±4.2, n=131) compared to the control (20.2±2.6, n=100), while the number of the proximal leg disc cells was severely reduced (6.7±2.7; compared to the control 15.9±1.8). In each case, the difference was statistically significant (P<0.05). We concluded that EGFR signaling is required to limit wing disc cell differentiation in limb primordial cells that are not yet fully committed. We infer that a subset of prospective leg cells that did not receive a sufficient amount of EGFR signaling failed to differentiate as proximal leg and instead adopted a wing fate.

Antagonistic activities of Dpp and EGFR on wing discs

The increase in the number of wing disc cells in rho mutants resembled the overexpression phenotype of Dpp (Goto and Hayashi, 1997b) and raises a possibility that EGFR might prevent wing disc development by negatively regulating Dpp signaling. Such a cross talk could occur at several levels including the following: (1) regulation of dpp transcription, (2) signal transduction from Dpp receptors to the nucleus, and (3) transcriptional regulation of downstream target genes. Our analyses excluded the first two possibilities. Firstly, the expression pattern of dpp mRNA was unaffected by the mutation of rho (Fig. 2J). The previous report by Raz and Shilo (1993) showing an expansion of dpp expression in Egfr mutants probably reflects the global patterning role of EGFR in the earlier stage. Secondly, pSSVS expression around limb primordium did not change in rho mutants (Fig. 2J). Conversely, the expression pattern of dpMAPK was not changed by a null mutation of tkv (Fig. 2I). These results suggest that the differential distribution of cells responding to Dpp and EGFR is set up independently of each other’s activity.

We found that Dpp and EGFR antagonize each other after signal transduction into the nucleus (Fig. 4). Hyperactivation of EGFR by an ectopic expression of an EGFR ligand Spitz (Schweitzer et al., 1995) caused a great accumulation of dpMAPK (Fig. 4F). As expected from the negative effect of EGFR on the wing development (Fig. 3), this treatment completely eliminated wing disc formation and, in addition, caused a malformation of the leg disc (Fig. 4E). Since we found that cells migrating out of the leg primordium express dpMAPK (Fig. 4F), it is unlikely that the failure in wing disc formation is due to the prevention of cell migration or to cell death. We suggest that hyperactivation of EGFR prevented limb primordial cells from adopting the wing cell fate. It is likely that those cells adopt the epidermal fate instead. Overexpression of Dpp caused an accumulation of pSSVS (Fig. 4K) and an increase in the number of wing disc cells (Goto and Hayashi, 1997b; Fig. 4I). Coexpression of Dpp with Spi partially restored the development of both wing and leg discs (Fig. 4M), suggesting that wing disc development overcomes the negative effect of EGFR if provided with a sufficient amount of Dpp. The restored wing primordium migrated with high level of pSSVS (Fig. 4O) and dpMAPK (Fig. 4N), further supporting the notion that Dpp and EGFR signals are transduced independently of each other.

*dad* is an immediate transcriptional target gene of Dpp (Tsuneizumi et al., 1997), the expression of which closely parallels that of pSSVS expression in embryos (Fig. 4D) and is inducible by Dpp (Fig. 4L). *dad* expression was not affected in *Egfr* or *rho* mutants (data not shown). Furthermore, elevated *dad* expression induced by Dpp was not affected by sSpi (Fig. 4P), suggesting that at least one of the immediate transcriptional responses to Dpp is unaffected by elevated EGFR signaling.

Dpp is a principal inducer of wing disc

The antagonism between Dpp and EGFR during wing disc development raises a question as to what is the default state of the wing and leg primordia in the absence of the two signals. We therefore examined double mutant phenotypes of Dpp and EGFR signaling. *tkv* mutants lack wing discs and their leg discs are malformed (Goto and Hayashi, 1997b; Fig. 5A). This genotype reflects a disc cell autonomous requirement for Dpp
signaling, because the phenotype was reproduced by the disc-specific inhibition of Dpp signaling by *dad*, which inhibits MAD (Tsuneizumi et al., 1997; Fig. 5B). Phenotype of *tkv; rho* and *tkv* *Egfr* double mutants was a simple addition of each mutation, in which wing discs were lost completely and leg discs were severely reduced (Fig. 5C,D). Since *Dll*-expressing limb primordial cells are present in *tkv* *Egfr* double mutants in stage 11 (Fig. 5E), we concluded that these cells failed to differentiate as wing discs and their ability to differentiate as leg discs was also compromised. A few Esg-positive cells remained at the position of the leg, and we speculate that this reflects the presence of a second leg-inducing signal (see Discussion). These results suggest that Dpp is absolutely required for wing disc development irrespective of the activity of EGFR.

Fig. 4. Antagonistic activities of EGFR and Dpp. Lateral views of second thoracic segments of control embryos (A-D) *Dil-Gal4* embryos carrying *UAS-sspi* (E-H), *UAS-dpp* (I-L), and *UAS-sspi* and *UAS-dpp* (M-P). (A,E,I,M) Embryos at stage 15 were stained for Esg (green) and *Dil* mRNA (red). Note that the ectopic *sspi* completely inhibited wing disc formation (E), and that this phenotype was partially restored by coexpression of *dpp* (M). Ectopic expression of *dpp* alone increased the number of wing disc cells (I). Leg discs of *sspi*-expressing embryos also showed an elongated shape (E), and this phenotype was restored by coexpression of *dpp* (M). (B,F,J,N) Early stage 12 embryos stained with anti-dpMAPK. Control embryos no longer express dpMAPK (B), while *sspi* induced a high level of dpMAPK throughout the limb primordium (F; outlined). Cells that are migrating dorsally were marked with brackets (F,N). Coexpression of *dpp* did not affect the pattern of dpMAPK (N). (C,G,K,O) Expression of pSSVS in early stage 12 was inducible by ectopic *dpp* (K) and was not affected by ectopic *sspi* (G,O). (D,H,L,P) Expression of *dad* mRNA was inducible by *dpp* (L). Expression of *sspi* did not affect the pattern (P). Brackets mark prospective wing cells. Scale bar: A, 50 µm.

Fig. 5. Wing disc development absolutely requires Dpp, while leg disc requires both Dpp and EGFR. Embryos were stained for Esg (green) and/or *Dil* mRNA (red in A-D; white in E). Arrowheads indicate leg discs and arrows indicate wing discs. (A) In *tkv* mutants, wing discs were lost completely and the number of proximal leg disc cells was reduced. The leg phenotype was sometimes variable, probably due to the fluctuation of maternal contribution. (B) Embryos overexpressing *dad* by *Dil-Gal4* have a fewer number of wing and leg disc cells, indicating a disc cell autonomous requirement for Dpp signaling. The wing disc phenotype was weaker than that in *tkv* mutants, because of the early loss of Dpp enhancer activity in wing primordia. (C,D) In *tkv*; *rho* (C) and *tkv* *Egfr* (D) double mutants, only a few Esg positive cells remained, suggesting that the phenotype of double mutants is the sum of each mutation. (E) Ventral view of a stage 11 embryo bearing both *tkv* and *Egfr* mutations. The ventral limit of *Dil* mRNA at stage 11 was expanded to the ventral midline, suggesting that the induction of the limb primordium itself took place. The later loss of wing and leg discs must be due to a failure in specifying wing and leg disc cells. Scale bar: A,E, 50 µm. B,C,D are the same magnification as A.
Dpp is not required for the dorsal migration of wing disc

The dorsal migration of the limb primordial cells is intimately coupled to the determination of wing cell fate. Since Dpp controls another cell migration event, dorsal closure, we were interested to know whether Dpp also regulates the migration of the wing primordial cells. We observed the behavior of cells expressing Dil-304β-gal, transcription of which in the limb primordium occurs transiently in stage 11 (Cohen et al., 1993). Highly stable β-galactosidase allowed us to trace the position of cells after the decay of the transcript (Cohen et al., 1993; Goto and Hayashi, 1997b). In tkvδ embryos where the wing primordium is completely missing (Fig. 5A), dorsal migration of Dil-304β-gal-expressing cells was indistinguishable from the control (data not shown). We concluded that Dpp is not required for the migration of limb primordial cells.

DISCUSSION

An understanding of the genetic mechanisms controlling the specification of the insect wing and leg helps define their ontogenetic relationship. Limb primordial cells at stage 11 have two developmental options, to become the wing or the leg. EGFR affects the choice of these options differently, it promotes leg development whilst inhibits wing development. These two activities of EGFR are the earliest of known events of leg specification, and occur prior to the establishment of proximal-distal axis in the leg.

Specification of wings and legs by the dorsoventral positional cue

In the absence of late functions of Dpp and EGFR, limb primordia are specified (Fig. 5E) but fail to differentiate into wing disc and most of leg disc (Fig. 5D). We thus propose that early limb primordium at stage 11 consists of cells not yet fully committed to either wing or leg disc fate, and the cells are being exposed to different amounts of Dpp and EGFR signaling according to their dorsoventral location (Fig. 1B). Dpp recruits the cells to the wing disc fate. EGFR antagonizes the cellular response to the wing-inducing function of Dpp and allows the development of wing discs only in the dorsal region. Thus the dorsoventral difference in Dpp and EGFR signaling in the limb primordium provides key information to the separation and differentiation of the wing and leg discs.

In contrast to the opposing roles of Dpp and EGFR on wing disc development, leg discs require both signals. The effect of the loss of EGFR activity on leg disc development (Fig. 3D,F) was not compensated for by a simultaneous loss of Dpp signaling (Fig. 5C,D), indicating that EGFR has an additional activity to promote leg development separately from its role to antagonize Dpp. Because dorsal and ventral limb primordial cells respond to EGFR differently, we speculate that at least one additional dorsoventral factor influences leg disc formation at stage 11. This idea is consistent with the fact that residual proximal leg cells can still be induced in the almost complete absence of EGFR and Dpp activity (Fig. 5). One candidate for the factor is Wg, which is expressed in the limb primordium (Cohen et al., 1993; Goto and Hayashi, 1997a).

We have shown that the nuclear transduction of the Dpp signal, as visualized by the distribution of pSSVS and expression of dad, was unaffected by EGFR (Figs 2J, 4). The results suggest that the antagonistic effect of EGFR on Dpp signaling occurs after transduction into the nucleus. Therefore, the mechanism of SMAD inhibition by direct phosphorylation by MAP kinase (Kretzschmar et al., 1997a, 1999) does not play a major role in this case.

Separation of wing and leg primordium is essential for wing development

Our finding that EGFR is activated in the limb primordium and prevents wing disc formation suggests that EGFR is a key factor in the diversification of the wing and leg fate. We propose that the differential activation of Dpp and EGFR, and the dorsal cell migration brings a subset of limb primordial cells out of the range of EGFR signaling, and thereby allow Dpp to induce wing development. It follows that dorsally migrating cells acquire the wing cell identity only after the separation from leg-promoting signals. Consistent with this idea, expression of wing-specific markers Vg and Sna, start only after the separation of the two primordia. Mechanisms that promote the dorsal cell migration remain to be identified.

Given that the basic genetic components for the induction of the wing and leg have been identified in the model organism Drosophila, we can now ask the question as to how the genetic mechanism of wing and leg specification has evolved by comparing the expression and function of these genes in limb primordial cells of primitive insects.

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