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 \textit{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, \textit{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 \textit{Ducas} (Anderson, 1963) and \textit{Drosophila} (Wieschaus and Gehring, 1976) demonstrated that wings and legs share a common developmental origin. Wing and leg development in \textit{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 \textit{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. \textit{Dll} expression at stage 11 is repressed in the dorsal ectoderm by Dpp and in the ventral ectoderm by EGFR (Fig. 1A). 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 \textit{vestigial (vg); Williams et al., 1991}, followed by expression of two related zinc-finger genes \textit{escargot (esg)} and \textit{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 \textit{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
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).

disks, and most likely differentiate as larval epidermises (Goto and Hayashi, 1997a). In double mutants for esg and snai, cells that began the dorsal migration fail to maintain the expression of wing-specific markers and differentiate as epidermises. 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 disc 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 

**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-sspitiz (Schweitzer et al., 1995), UAS-EGFR.DN (O’Keefe et al., 1997) from Dr Ben-Zion Shilo; ato1 (Jarman et al., 1994), rho6, Egfrb6 and Egfrb7 from Dr Masataka Okabe; DorsalG18S (Tsuda et al., 1993) from Dr Yasuyoshi Nishida; UAS-dad (Tsuneizumi et al., 1997) from Dr Tetsuya Tabata; Egfrb1 from the Tübingen 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 QMGSPHNAISSVS 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 QMGSPHNAISSVS phosphate. 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.,
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

![Fig. 2. The activation of EGFR-MAPK and Dpp pathways in the limb primordium. Lateral views of three thoracic segments are shown at stage 11, except for C, which is at stage 10. Arrowheads, the limb primordium in second thoracic segment. (A) rho mRNA expression was detected within Dll-304-β-gal-expressing cells. (B) dpMAPK expression was detected at the ventral side of cells expressing dpp. (C-E) The temporal change of dpMAPK expression at stage 10 and 11. dpMAPK was not expressed when Dll transcription starts at stage 10 (C). It became highly expressed at early stage 11 (D) and turned off at late stage 11 (E). (F-G) Distribution of cells expressing pSSVS and the relationship to cells expressing dpp (F) and Dll (G). (H) Expression of pSSVS was eliminated in tkv mutants (H). (I) Expression patterns of dpMAPK and Dll were not altered in tkv mutants. (J) Expression patterns of pSSVS and dpp were not altered in rho mutants. ato mutants were used for staining (in B–E) to remove rho and dpMAPK expression derived from chordotonal organ precursors near the limb primordium. ato mutants lack chordotonal organ precursors-derived signals, but develop with normal wings and legs (Jarman et al., 1994), and were used here as controls. Scale bar in A, 50 μm.
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 andDll 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 Egr 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 Egr 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
discs were most frequently lost, suggesting that the development of dorsoproximal leg cells is most sensitive to the loss of EGFR activity. In contrast, wing and leg disc development was normal in vein mutants (Schnepf et al., 1996; data not shown), suggesting the putative ligand of EGFR encoded by this gene is dispensable. These results suggest that MAPK activation induced by rho and Egfr is essential for normal leg development.

The temporal requirement for EGFR was studied by the temperature-sensitive allele EgfrΔ1 (Raz and Shilo, 1993). When the temperature was increased to the restrictive temperature at 5 hours after egg laying (AEL) prior to the induction of the limb primordium, the expression of Dll was expanded to the ventral midline (data not shown) as was also observed with the strong Egfr mutants (Fig. 3E). When the temperature was increased at 6 hour AEL, the initial Dll expression was not altered (Fig. 3I), but the leg disc development was severely affected (Fig. 3J). Only mild defect was found in leg discs when the temperature was increased at 7 hour AEL, suggesting that EGFR must function between 6 and 7 hour AEL to correctly specify the leg cell fate. This is the time when the transient activation of MAPK was observed (Fig. 2).

Furthermore, we tested whether EGFR is required autonomously in limb primordial cells by expressing a dominant-negative form of EGFR (O’Keefe et al., 1997) by the Dll-Gal4. Expression of this driver starts in the limb primordium at stage 11 (Fig. 3K; Calleja et al., 1996) and persists in a subset of wing discs and in entire leg discs at stage 15 because of the persistence of Gal4 activity. Imaginal disc-specific inhibition of EGFR interfered with leg disc development, while leaving the wing disc intact (Fig. 3L). These results demonstrated that a transient activation of EGFR in stage 11 limb primordia is essential for the leg disc development.

**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. 2I). 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 primordia 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 phenotype 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\(^8\); rho\(^6\) and tkv\(^8\) Egfr\(^f6\) 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\(^f6\) 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) Dll-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 Dll 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 Dll mRNA (red in A-D; white in E). Arrowheads indicate leg discs and arrows indicate wing discs. (A) In tkv\(^8\) 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 Dll-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 Dll enhancer activity in wing primordia. (C,D) In tkv\(^8\); rho\(^6\) and tkv\(^8\) Egfr\(^f6\) 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\(^8\) and Egfr\(^f6\) mutations. The ventral limit of Dll 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 disc 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-304-β-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 tkv− embryos where the wing primordium is completely missing (Fig. 5A), dorsal migration of Dil-304-β-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 proximodistal 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 dud, 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 Sn, 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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