Regulation of Drosophila matrix metalloprotease Mmp2 is essential for wing imaginal disc:trachea association and air sac tubulogenesis

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ABSTRACT

The Drosophila Dorsal Air Sac Primordium (ASP) is a tracheal tube that grows toward Branchless FGF-expressing cells in the wing imaginal disc. We show that the ASP arises from a tracheal branch that invades the basal lamina of the disc to juxtapose directly with disc cells. We examined the role of matrix metalloproteases (Mmps), and found that reducing Mmp2 activity perturbed disc-trachea association, altered peritracheal distributions of collagen IV and Perlecan, misregulated ASP growth, and abrogated development of the dorsal air sacs. Whereas the function of the membrane-tethered Mmp2 in the ASP is non-cell autonomous we find that it may have distinct tissue-specific roles in the ASP and disc. These findings demonstrate a critical role for Mmp2 in tubulogenesis post-induction, and implicate Mmp2 in regulating dynamic and essential changes to the extracellular matrix.

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Introduction

Organogenesis integrates signaling, proliferation, patterning and structural processes to generate the variety of forms and functions essential to viability. Many organs develop from tubular outgrowths that assemble, grow and mature by a variety of mechanisms (reviewed in Hogan and Kolodziej, 2002). The only identified example of tubulogenesis in Drosophila that involves both cell proliferation and directed extension is the Dorsal Air Sac Primordium (ASP; Sato and Kornberg, 2002). This tubular outgrowth of the larval tracheal system is induced during the third instar (L3) period by Bnl/FGF expressed in the wing imaginal disc. Within 16 h of its induction, the ASP grows from a simple epithelial bud to a sac with many cells and with a distinct stalk and tip. During the subsequent pupal period, further growth and branching morphogenesis lead to its forming the Dorsal Air Sacs, the major tracheal organs of the adult fly that deliver oxygen to the thoracic flight muscles (Cabernard and Affolter, 2005; Guha and Kornberg, 2005; Sato and Kornberg, 2002). Although ASP development, structure and physiology are largely unexplored, the ASP promises to be an excellent system for investigating basic mechanisms of organogenesis. Here we report that regulation of the extracellular matrix (ECM) is essential for both cell proliferation and morphogenesis of the ASP and that the matrix metalloprotease Mmp2 plays a critical role.

Epithelial tissues are typically associated with an ECM layered over their basal surface. The ECM is a meshwork of proteins and carbohydrate polymers that when viewed with an electron microscope appears as a basal lamina (BL) that has a tissue-proximal lamina lucida and an outer dense sheet, the lamina densa. BL thickness and composition vary in different tissues and during development. An example is the mammary ducts, whose BL appearance and composition change during branching morphogenesis (Fata et al., 2004). The importance of BL assembly, disassembly and architecture during development and for tissue homeostasis is underscored by changes in the BL that accompany many disorders. Metastasis of tumors is thought to require BL-disassembly. While there are many candidate genes that potentially could regulate the ECM, the essential functions for maintenance and remodeling have not been definitively identified.

Mmps constitute a large family of Zn-containing endopeptidases that proteolyze ECM proteins. Although in vivo evidence for Mmp roles is limited and most Mmp mutant mice develop without apparent consequence (Andrews et al., 2000; Brinckerhoff and Matrisian, 2002), there are several notable exceptions. Ectopic expression of Mmp3/Stromelysin perturbs both mammary gland development and influences breast cancer (Sternlicht et al., 1999). The phenotype of mice deficient for Gelatinase B/Mmp9 establishes a role for this proteinase in angiogenesis (Vu et al., 1998). Evidence from mice deficient for the membrane-tethered MT1-Mmp implicates this enzyme in connective tissue metabolism and white adipose tissue development, and suggests a role in degrading and remodeling pericellular collagen I (Chun et al., 2006; Holmbeck et al., 1999).

Since the principal constituents of ECM (collagen IV, Laminin, Perlecan, Nidogen/Entactin; LeBleu et al., 2007) are conserved in metazoa (Hopf et al., 2001), it seems likely that the mechanisms for ECM-remodeling are also shared. Indeed, Mmps are conserved (Page-McCaw, 2008), and Drosophila has two—Mmp1 and Mmp2 (Llano et al., 2002; Llano et al., 2000; Page-McCaw et al., 2003). The Drosophila
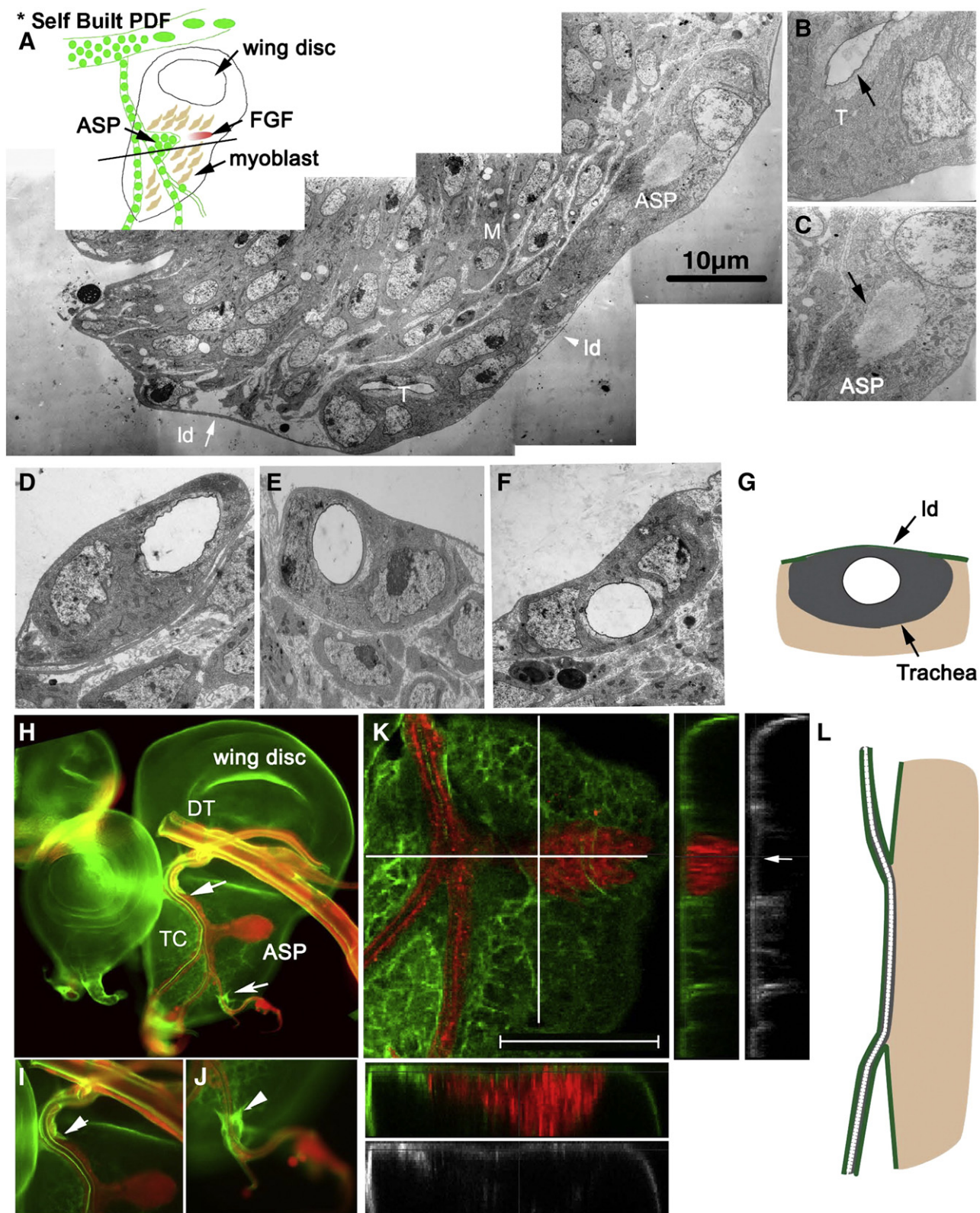
Fig. 1. Invasive coupling of trachea and wing disc. (A) Drawing of a L3 wing disc and associated tracheal branches showing the adepithelial myoblasts (brown), Bnl/FGF-expressing columnar epithelial cells (red), and tracheal cell nuclei (green). Black line shows approximate position of thin sections assembled into the montage. The section transects the transverse connective, ASP and myoblasts (M), revealing that these tracheal branches are underneath the disc BL. The thickness of the lamina densa (ld) overlying the ASP (arrowhead) is less than other regions (arrow). (B, C) Higher magnification views of the disc-associated trachea and ASP in (A) showing that the disc-associated trachea (B) has an apical taenidium (T, arrow) that is absent in the ASP (C). (D–F) Sections 5 μm apart showing the trachea outside (D) and underneath (E, F) the disc BL. The space between the tracheae and myoblasts is not uniformly electron-dense. (G) Drawing showing the disc-associated trachea within the lamina densa of disc. (H) Collagen IV:GFP (green) illuminates the BL in the wing, haltere and leg discs and in associated tracheae (arrows in all panels indicate points of entry and exit). Tracheal cells (*btl*-RFP, red). (I, J) High magnification views showing the points at which the transverse connective enters (I) and exits (J) the disc BL. (J) A unicellular tracheole whose nucleus is outside the disc, is encapsulated by BL only where is outside the disc. (K) Confocal-derived XZ (bottom) and YZ (right) reconstructions of collagen IV:GFP fluorescence (green, and black and white) and tracheal cells (red, *btl*-Cherry) in a late L3 show disc-associated tracheae and ASP located underneath a collagen IV:GFP layer. Levels of collagen IV:GFP above the ASP (arrow in YZ reconstruction shown in the right panel) are lower than in other regions of the notum. Scale bar: 90 um (L) Drawing illustrating invasive coupling of the disc with thinner BL over the trachea contacting the disc.

Mmps (Dm-Mmp1 and Dm-Mmp2) are required for the morphogenesis of various tissues (Miller et al., 2008; Page-McCaw et al., 2003) and appear to play roles in tumor invasion (Beaucher et al., 2007; Page-McCaw et al., 2003; Pastor-Pareja et al., 2004; Srivastava et al., 2007; Uhlirova and Bohmann, 2006). The sequences of the two Mmps suggest that they are structurally distinct: Dm-Mmp1 is predicted to be secreted and Dm-Mmp2 to be membrane-tethered by a glycosyl-phosphatidyl inositol anchor (Llano et al., 2000, 2002; Page-McCaw et al., 2003).

We were alerted to a possible role for ECM-remodeling in the morphogenesis of the ASP when we examined the disc:tracheal interface. We found that the ASP arises from a region of a tracheal branch that is directly juxtaposed to the wing disc. This tracheal segment lies underneath the disc BL and lacks a conspicuous, tracheal-specific BL. We investigated the role of Mmps in the regulation of this architecture to find that Dm-Mmp2 regulates levels of collagen IV and Perlecan in the ECM around disc-associated trachea and the ASP, and this function is essential to properly juxtapose tracheal and disc cells, for growth of the ASP and for development of the Dorsal Air Sacs.

Materials and methods

Fly strains

btl-Gal4 (Shiga et al., 1996), *ap*-Gal4 (Flybase) are promoter- or enhancer trap Gal4 transgenes that express Gal4 in trachea (*btl*), the dorsal compartment of the wing disc but not in trachea (*ap*) and were used to drive the expression of UAS transgenes. Tracheal cells were illuminated by the expression of GAP-GFP (UAS GAP-GFP, Sato and Kornberg, 2002), UAS-RFP and UAS-Cherry (gift from S. Roy). BL

distribution was observed using Perlecan protein traps (Flytrap; GFP Protein Trap Database: http://flytrap.med.yale.edu; G00022, ZCL1700, ZCL1973) and *Viking* (collagen IV; G00454). $Mmp2^{W307}$, UAS TIMP, and UAS $Mmp2$ (Page-McCaw et al., 2003), FRT $Mmp2^{K07511}$ FRT $Mmp2^{W307}Mmp1^{Q112}$ (gift from A. Page-McCaw), UAS $Mmp1^{RNAi}$, UAS $Mmp2^{RNAi}$ (Uhlirova and Bohmann, 2006), and Mmp2 Gal4 (Srivastava et al., 2007) were used for the analysis of Mmp function. Bnl/FGF clones and ectopic expression were induced as described in Sato and Kornberg (2002).

Fluorochromes and histochemistry

Nuclei were stained with DRAQ5 (Biostatus, Ltd.). Immunostainings of discs with mouse α-Discs large (1:50, DSHB) and mouse α-Dp-ERK (1:100, Sigma) were performed with a Cy3-conjugated donkey anti-mouse secondary antibodies (1:750, Jackson Labs). RNA in situ hybridization was performed according to O'Neill and Bier (1994) and Klebes et al. (2002). DIG labeled antisense probe was generated by *in vitro* transcription (Megascript) from *Hin*dIII digested Bnl cDNA (pBSKII, Sutherland et al., 1996)) and fragmentation at high pH. Alkaline phosphatase conjugated α-DIG antibody (Roche) was used to detect the DIG labeled probe *in situ*.

Mounting and imaging fixed and unfixed imaginal discs

All discs were mounted using the "hanging drop" method (Sato and Kornberg, 2002) or in flat preparations (Vectashield) and were imaged at room temperature. All epifluorescence images of collagen IV:GFP and Perlecan-GFP were acquired using the "hanging drop" method on a Zeiss Axioplan 2 microscope with a 10X Fluar, 0.5 NA objective, a PCO Sensicam CCD camera (Cooke Corporation, USA)
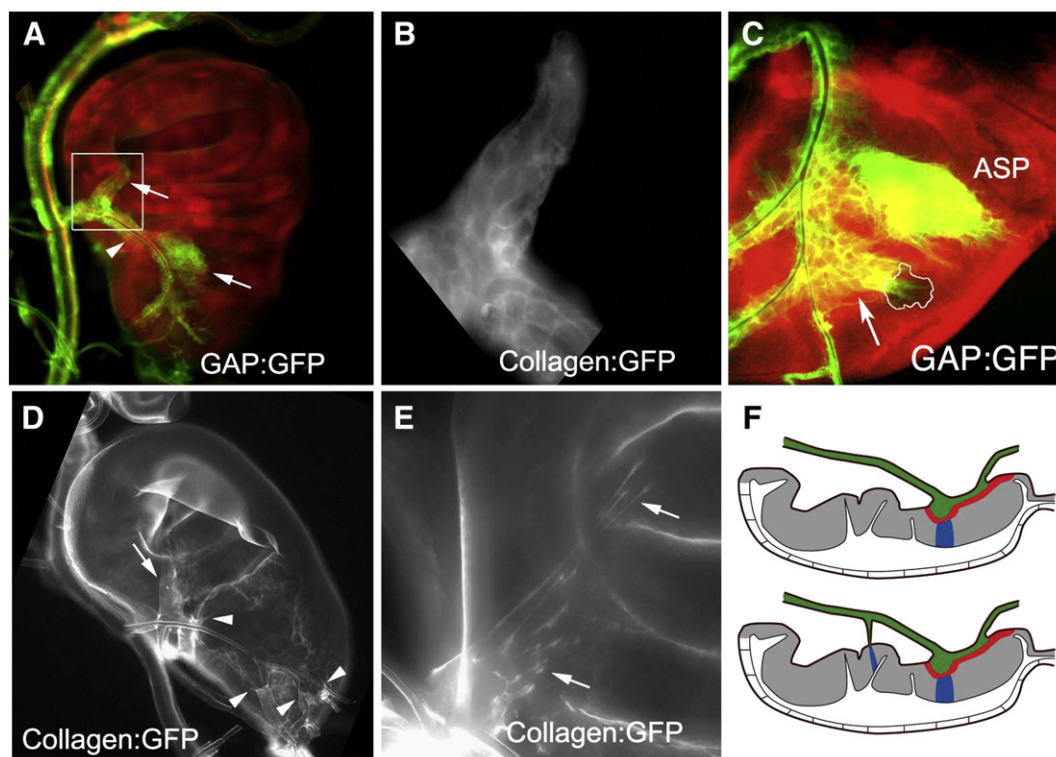
Fig. 2. Tracheal tunneling does not restrict responsiveness to Bnl/FGF. Clones expressing ectopic Bnl/FGF (dark areas) attract outgrowths of the trachea from a region outside the BL (green; GFP). Region of disc in (A) delineated by white box is shown at high magnification in (B) to reveal the multicellular composition of the outgrowth. (C) Gap:GFP expressed in tracheal cells illuminates the ASP and, from an adjacent tunneled region, an ectopic outgrowth (arrow) that extends toward Bnl-expressing cells (encircled by white line). (D) Collagen IV:GFP marks enlarged ASP and ectopic outgrowth. Arrowheads indicate "points of entry/exit" through the disc BL. (E) Collagen IV:GFP (arrows) labels extensions that bridge the transverse connective and disc. (F, upper) Drawing showing normal association of transverse connective (green) with the wing disc (gray), and identifying Bnl/FGF-expressing cells (blue) and adepithelial myoblasts (red). BL (brown) encapsulates both trachea and disc except regions of invasive coupling. (F, lower) Drawing showing ectopic outgrowths from transverse connective, penetrating the disc BL to contact disc clones expressing Bnl/FGF.

using Slidebook acquisition software (Intelligent Imaging Innovations). The 20× Plan-NeoFluar, 0.5NA and 40× Plan-NeoFluar objectives were used for other epifluorescence images. Optical sections were acquired on Leica TCS SP2 confocal microscope system using a Leica DMRXE microscope with an HC PL APO 20×, 0.7 NA objective. Identical acquisition settings were used for wild type and mutant tissue; all confocal images were acquired using a pinhole size of 1.0 Airy Unit. Brightfield images were acquired on a Leica DMR microscope equipped with SPOT CCD Camera (Diagnostics Instruments) and SPOT acquisition software. Adult flies were mounted in halocarbon oil for imaging dorsal air sacs.

Electron microscopy

Larvae were dissected in 0.12 M sodium cacodylate buffer (CaCo; pH 7.0) and fixed either overnight at 4°C or at room temperature for 1 h in 0.12 M CaCo, 2% glutaraldehyde. Following primary fixation, wing discs were dissected and stained with tannic acid (1%) and post-fixed in osmium tetroxide (2%). Samples were dehydrated in an ascending series of ethyl alcohol and embedded in a medium mixture of Embed 821 resin. Ultra-thin sections were stained for contrast using 1% aqueous uranyl acetate and Reynold's lead citrate and examined and photographed using either a JEOL 100CX transmission electron microscope or a FEI Technai Spirit transmission electron microscope.

Results

### The transverse connective, ASP and wing imaginal disc are invasively coupled

The ASP originates near a point of bifurcation of the transverse connective and extends over the notum primordium of the wing disc in an oblique, dorsoventral orientation where myoblasts overly the columnar epithelial cells (Fig. 1A). Contact between the transverse connective and disc is sufficiently strong that the transverse connective frequently accompanies wing discs when wing discs are dissected from larvae. Although association of the disc and transverse connective can also be observed in intact larva, the nature of the contact between the disc and tracheal tube has not been characterized previously. Our finding that Bnl/FGF signals from the disc to the transverse connective to induce the outgrowth of the ASP (Sato and Kornberg, 2002) suggested that contact between the transverse connective and the disc has a functional role. We therefore undertook a fine structure analysis to gain insight into the nature of their association.

Tracheae are tripartite tubes with an ECM on the outer basal surface, a tube consisting of epithelial cells, and apically, a cuticle (taenidium) that lines the lumen. Serial ultra-thin sections of discs and associated trachea revealed that the ASP differs from other tracheal tubes in two respects (number of specimens examined = 2). The ASP lacks a taenidium (compare Figs. 1B, C). In addition, although the BL that lines segments of the tracheal branch that do not contact the disc has a thick lamina densa that was similar in appearance to the one that encapsulates the disc (Fig. 1D), the portions of the transverse connective that are directly apposed to the disc does not (Figs. 1A, E, F). Instead, they are situated underneath the BL of the disc and were directly juxtaposed to the disc (shown schematically in Fig. 1G). The surfaces of tracheal cells situated underneath the disc BL are associated with lamina densa that is significantly thinner and less uniform than the lamina densa elsewhere in the disc. In some sections (not shown), tracheal cells of the transverse connective are directly juxtaposed to the columnar epithelium, the source of Bnl/FGF. We refer to the placement of the trachea underneath and inside the disc BL as "invasive coupling".
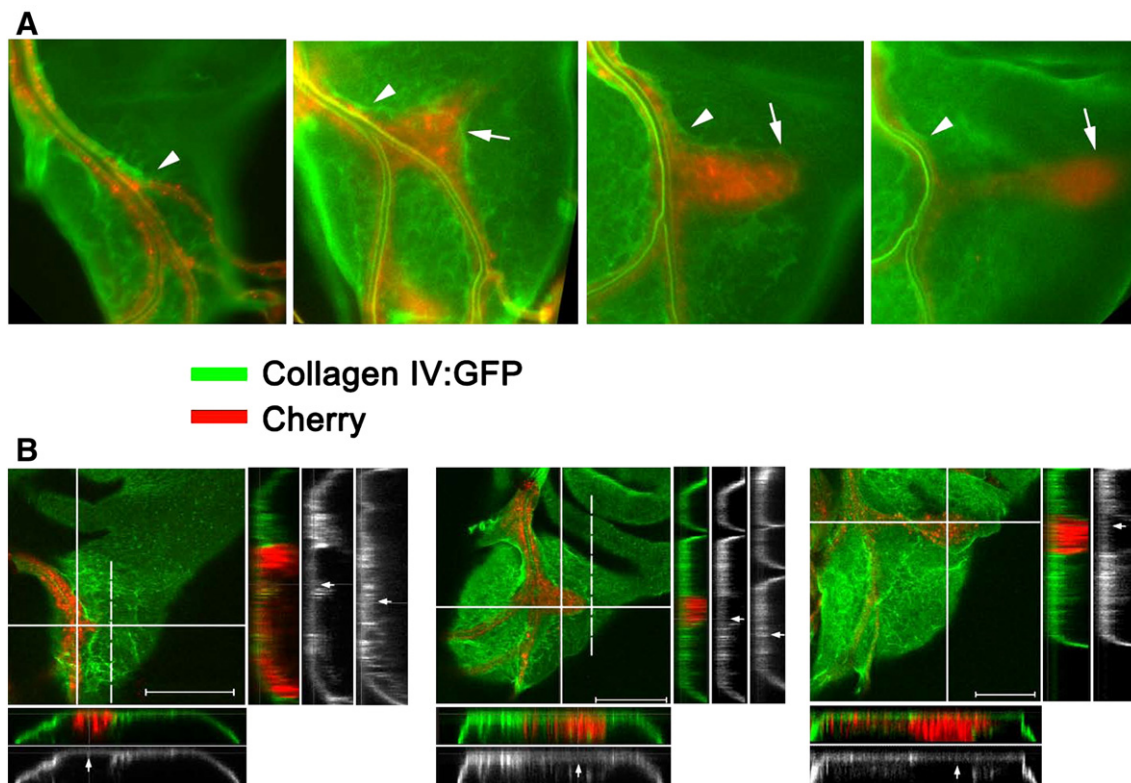
Fig. 3. ECM dynamics during ASP morphogenesis. Collagen IV:GFP fluorescence marks ECM in wing disc and trachea in preparations isolated from early L3 larvae prior to ASP induction (left panels, A, B) and at successively later stages (middle and right panels). Red: cherry expressed in *btl* domain in trachea. (A) Arrowheads indicate "points of entry/exit" through the disc BL; arrows indicate regions of dynamic changes in collagen IV levels. (B) Confocal images with adjacent XZ (bottom) and YZ (right) reconstructions showing that the abundance of collagen IV:GFP around the trachea and ASP (solid lines) is lower (arrows) than in other regions (dashed lines). Number of preparations examined: pre-ASP: 5; early: 6; late 5.
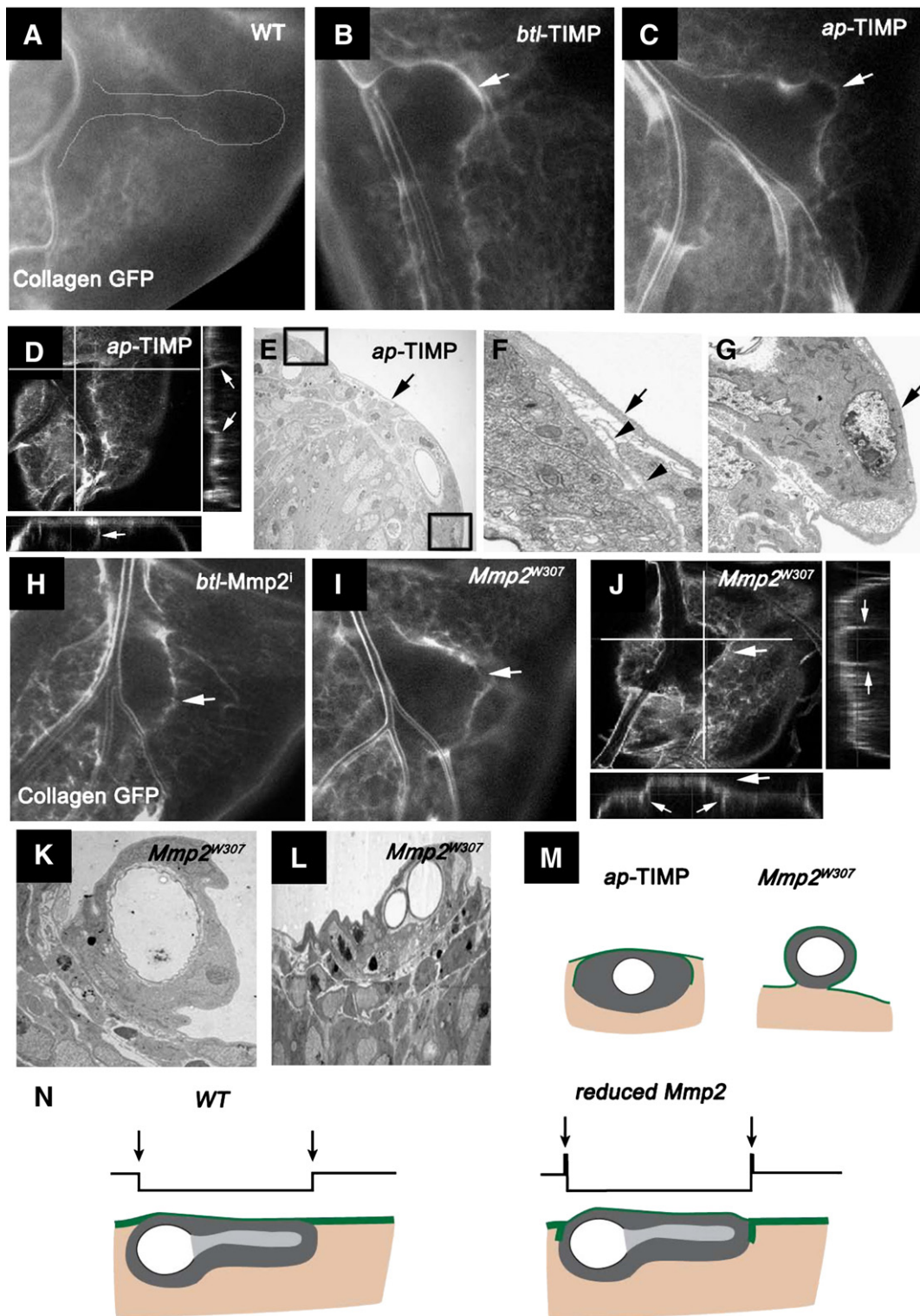
Fig. 4. ECM abnormalities in animals expressing TIMP and in *Mmp2* mutants. (A–C) In contrast to normal animals (A, ASP outlined in white), *btl*-TIMP (B) and *ap*-TIMP (C) specimens have elevated collagen IV:GFP levels at edges of disc-associated transverse connective and ASP (arrows). (D) XZ (bottom) and YZ (right) reconstructions of collagen IV:GFP distribution in *ap*-TIMP disc showing that collagen IV:GFP accumulates around the distal tip of the ASP (XZ, arrow) and that disc-associated trachea and ASP extrude from the disc (YZ, arrows). (E–G) Ultrastructure of *ap*-TIMP discs reveals defects in the ECM. Trachea is under the lamina densa (arrow), but ectopic lamina densa is detected around tunneled trachea. (F) Higher resolution views of left boxed region in (E) showing disc lamina densa (arrow) and ectopic lamina densa (arrowheads), and extrusion from the disc (G, right boxed region in (E)). (H) RNAi knockdown of *mmp2* in the trachea recapitulates the phenotypes in (B) and (C). In *mmp2*W307 (I, J), ectopic collagen IV:GFP is detected around disc-associated tracheae. Note both extrusion of the trachea (J, XZ arrows) and accumulation of collagen-GFP along the lateral margins (right arrow in XZ, arrows in YZ) of the ASP (K, L) Ultrastructure of *mmp2*W307 discs at two positions along the disc–trachea interface showing the trachea underneath the disc lamina densa but extruded from the disc proper. (M) Diagram depicting defects in disc–tracheal association and lamina densa in *ap*-TIMP and *Mmp2*W307 animals. (N) Diagram showing the distribution and levels of collagen IV and Perlecan in specimens with wild type and reduced Mmp2 activity.
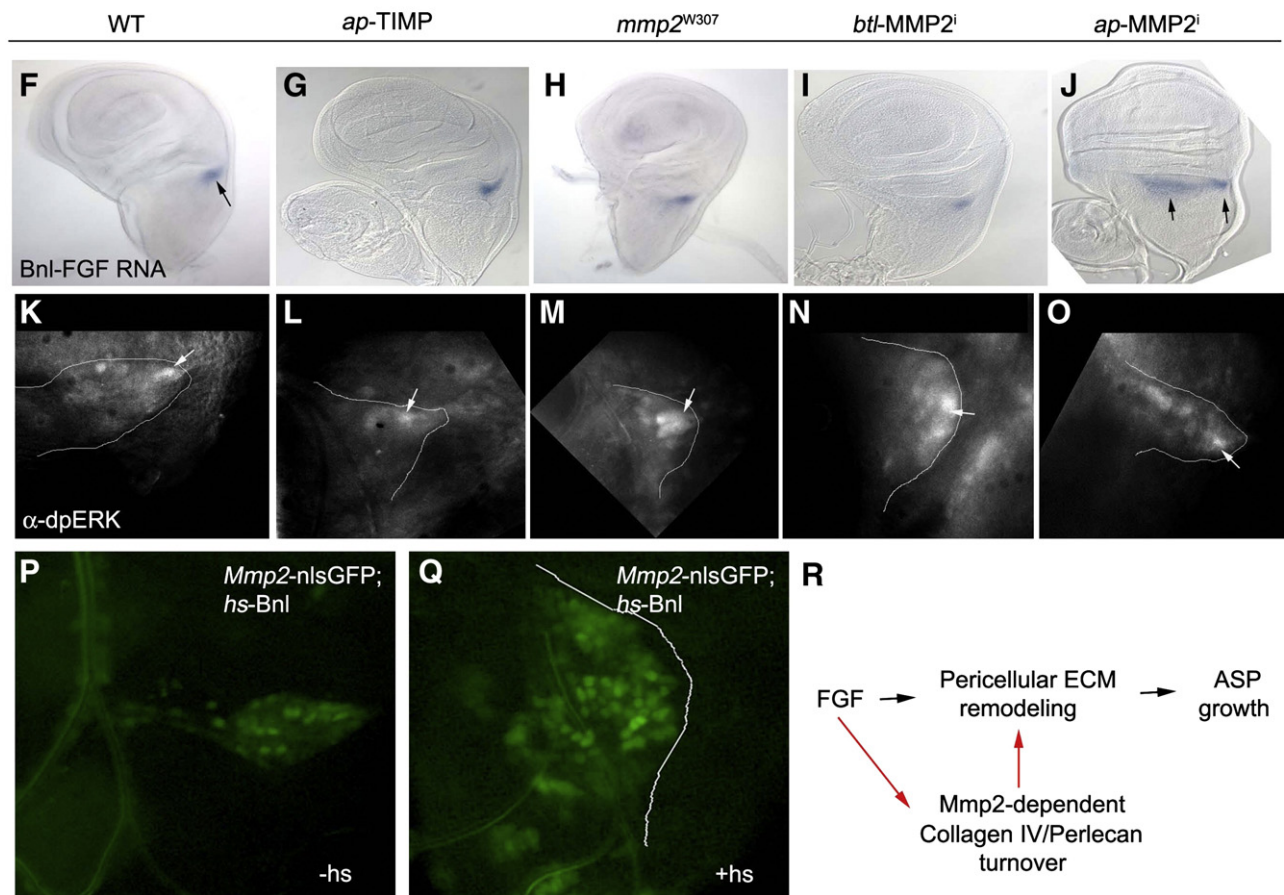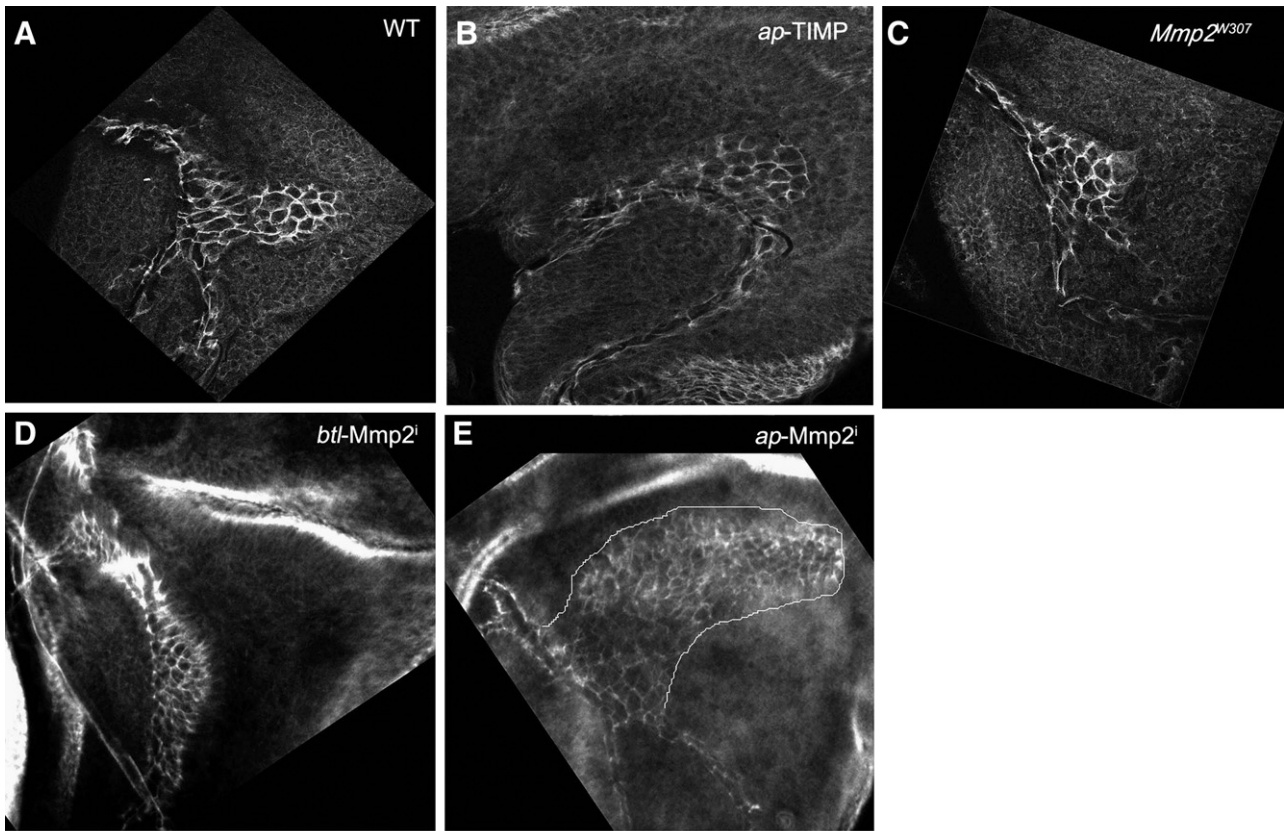
To further characterize the tracheal-wing disc association, we examined protein trap strains that express GFP in the ECM. We examined a line that tags collagen IV (Figs. 1H-K) and three lines that tag Perlecan (not shown). In specimens dissected from L3 larvae of all four lines, we detected similar patterns of strong fluorescence around the disc and around most of the tracheal branches (Fig. 1H). However, we did not detect strong fluorescence from the regions of the trachea that contact the disc (Figs. 1H–J). In addition to the absence of strong fluorescence around the trachea in this region of contact, the intensity of fluorescence in this region was lower than in other regions of the wing imaginal disc. The pattern of fluorescence correlated well with the appearance of the lamina densa in ultrastructural studies—we detected a prominent, thick layer over the disc and around tracheal branches, except the portion of the transverse connective that contacts the disc. In these regions, the lamina densa overlying the trachea was significantly thinner. Confocal microscopy of collagen IV:GFP expressing discs also showed that both disc-associated trachea and the ASP were underneath the disc BL, directly juxtaposed to the disc (Fig. 1K). In addition, both XZ and YZ reconstructions showed that the levels of Collagen IV:GFP overlying the ASP were significantly lower than other regions of the notum (Figs. 1K, YZ). Epi-fluorescence images clearly revealed the points of tracheal "entry" and "exit" (Figs. 1H–J) that define the extent of "invasion"; these points were similar in all specimens we examined. Fig. 1L summarizes the disposition of the transverse connective with respect to the disc and BL.

Based on these observations we conclude that the wing disc and trachea are invasively coupled and that the ASP grows out from this invasively coupled tracheal segment. Since we were unable to detect a continuous BL around the tracheal segments that are coupled to the disc (no lamina densa was present at the transverse connective:disc interface, it may be that disc-derived cues suppress BL assembly locally. We note that the unicellular tracheole that emanates from the transverse connective at a point distal to the ASP (Figs. 1H, J) has portions both inside and outside the disc BL. Since only the portion of the tube that is outside the disc BL had collagen IV, BL assembly by this tracheole is not cell autonomous.

The BL is functionally transparent to FGF signaling

Growth of the ASP from a region of the trachea that invasively couples with the disc raises the question: is direct contact necessary for FGF signaling to induce the ASP? Would interposition of BL between the disc and tracheal cells abrogate signaling? In order to determine if direct contact is essential for FGF signaling, we induced clones of Bnl/FGF-expressing cells and monitored responses of trachea by examining fluorescence of either *btl*-GAP:GFP or *btl*-collagen IV:GFP. Since we observed association of disc and trachea and detected collagen IV:GFP in the ECM around these tissues in preparations from first instar larvae (data not shown), induction of Bnl/FGF expression in first and second instar larvae would allow us to address the responses of the trachea after initial BL assembly. Ectopic Bnl/FGF expression induced outgrowths from the transverse connective, both from the portions that had invasively coupled with the disc as well as outlying regions that were outside the disc BL (Figs. 2A, C, D). The latter type of outgrowths were multi-cellular (Fig. 2B) and oriented toward Bnl/FGF-expressing disc cells, and some had thin collagenous structures that connected trachea and disc that could be

discerned (Fig. 2E). Since these ectopic structures suggested that signaling is not blocked by the disc BL (schematized in Fig. 2F), we suggest that direct juxtaposition of disc and trachea is not essential for FGF signaling and tubulogenesis, and that the BL is functionally transparent to FGF signaling.

Matrix metalloprotease is required during ASP outgrowth for ECM remodeling at the disc:trachea junction

The disc-associated trachea and ASP are situated beneath a layer of collagen IV:GFP-containing ECM from the earliest stages of ASP induction (Figs. 3A, B). However, during the early stages of growth, collagen IV-GFP distribution accumulated along the lateral margins of the disc-associated trachea and along the lateral and distal margins of ASP. These bands of fluorescence were variable, but at late stages, most specimens had no apparent pericellular collagen IV:GFP (Fig. 3A). The levels of collagen IV:GFP in the BL that overlies both the invasively coupled TC and ASP were lower at all stages of growth (Fig. 3B). We interpret both the lower levels of collagen IV:GFP in the BL overlying the ASP and the transient elevation of pericellular collagen IV:GFP fluorescence along the lateral and distal edges of the ASP at early stages of ASP growth as evidence of active ECM remodeling during growth and extension of the ASP.

The invasive nature of the disc–tracheal association, together with the dynamic changes in the ECM around disc-associated tracheae, suggested that enzymes involved in ECM remodeling might play important roles. The Drosophila genome encodes two MMPs, Dm-Mmp1 and Dm-Mmp2 (Llano et al., 2000, 2002; Page-McCaw et al., 2003), as well as a tissue inhibitor of MMPs (TIMP; Brew et al., 2000). Previous studies reported that Dm-Mmp1 is expressed in the trachea and the ASP, and that Dm-Mmp2 is expressed in the trachea, ASP and wing disc (Llano et al., 2002; Page-McCaw et al., 2003; Srivastava et al., 2007; Uhlirova and Bohmann, 2006). Drosophila TIMP is thought to inhibit both Dm-Mmp1 and Dm-Mmp2 in the extracellular milieu, although its endogenous distribution and roles are not well understood.

To assess the roles of Mmps, we examined animals in which TIMP was ectopically expressed, mutant animals defective for MMP activity, as well as animals in which RNAi was expressed to reduce Mmp1 and Mmp2 levels. As detailed in the subsequent paragraphs, these approaches led us to focus on the role of Mmp2. While ectopic TIMP expression, which reduces both Mmp1 and Mmp2 activities perturbed disc:tracheal association and ASP growth, the presence of *Mmp1^{RNAi}* in the *btl* expression domain had no apparent effect. And although most *Mmp1* mutants exhibited early larval lethality, some *Mmp1^{Q273}* mutant animals grew slowly and reached the pupal stage, but the imaginal discs, trachea and ASP in these mutants appeared normal. Thus, we found no role for Mmp1 in disc:tracheal association, ASP growth, or ECM remodeling (data not shown).

When TIMP was expressed in the *apterous* (*ap*-GAL4 UAS-TIMP, (*ap*-TIMP); number of specimens examined = 12) or *btl* domains (*btl*-GAL4 UAS-TIMP, (*btl*-TIMP); number of specimens examined = 8), the general level of collagen IV:GFP fluorescence was comparable to wild type, as was the fluorescence of tracheal branches not in contact with the disc (Figs. 4A-D). However, collagen IV:GFP fluorescence increased at specific locations at which the invasively coupled

Fig. 5. Role of Mmp2 in FGF-dependent ASP growth. (A) Disc from WT animal (48–50 h, L3) stained with α-Dlg. Similar preparations from *ap*-TIMP (B) and *Mmp2^{W307}* (C) animals (48–50 h, L3) revealed ASP induction, but no directional growth. A requirement for Mmp2 expression in the trachea was revealed in preparations from *btl*- Mmp2^{RNAi}-expressing animals (D). Mmp2 knockdown in the disc (*ap*-Mmp2^{RNAi}) resulted in hyperplastic ASP growth (E). Knockdown of Mmp2 did not diminish Bnl/FGF expression (arrow in (F)) in the disc (F–I) or FGF signaling in the ASP (K–O). Knockdown of Mmp2 in the disc (*ap*-Mmp2^{RNAi}) resulted in expansion of the domain of Bnl/FGF expression (arrows, J). (P) Expression of *mmp2GAL4* (*Mmp2*-nls GFP) was detected at higher levels at the distal edge of the ASP. (Q) Animals in which FGF expression was induced by heat shock showed hypertrophy of the ASP (outlined with a white line) and increased levels of Mmp2-nls GFP expression. (R) Model for the role of Mmp2 in ASP morphogenesis. Proliferation and growth of the ASP is accompanied by ECM remodeling (black arrows), mediated, in part, by FGF-dependent expression of Mmp2 in the ASP and consequent modulation of collagen IV/Perlecan levels (red arrows).

transverse connective and the ASP make contact (Fig. 4D). *ap*-TIMP-expressing discs had a similar pattern of Perlecan distribution, as indicated by Perlecan:GFP fluorescence (see Supplementary Figure) or immunolabeling with anti-Perlecan antibody (not shown). Ectopic basal lamina around the tunneled tracheae was also apparent in serial thin sections of five *ap*-TIMP-expressing discs (Figs. 4E–G). In addition to increasing BL where the invasively coupled tracheal tubes and disc make contact, TIMP expression changed the depth at which the disc-associated transverse connective and ASP position within the disc. In wild type, both the transverse connective and ASP tunnel so that the plane of disc BL is relatively flat (Figs. 1, 4B). In *ap*-TIMP animals, however, slight extrusion was apparent both in fluorescence images (Fig. 4D, YZ plane) and in thin sections (Fig. 4G). These results suggest that MMP is required to limit synthesis of BL that would otherwise form around the tunneled tracheal tubes.

$Mmp2^{W307}$ mutant larvae had similar phenotypes. Although their tracheal systems were without detectable structural or cell proliferation defects, mutant wing discs and associated transverse connective and ASP had phenotypes comparable to *btl*-TIMP and *ap*-TIMP. High levels of both collagen IV:GFP (Figs. 4I, J; number of specimens examined = 8) and Perlecan-GFP (see supplementary figure) accumulated around the ASP. Confocal images (number of specimens examined = 3) and serial ultra-thin sections (number of specimens examined = 2) of $mmp2^{W307}$ also revealed invasive coupling of the transverse connective and ASP, and showed that the mutant trachea extruded from the plane of the disc BL throughout the length of their contact (Figs. 4K, L and summarized in Figs. 4M, N). Knockdown of Mmp2 in the trachea (*btl*-GAL4 UAS-$Mmp2^{RNAi}$ (*btl*-$Mmp2^{RNAi}$)) increased levels of collagen IV:GFP in patterns that were comparable to ectopic expression of TIMP and $Mmp2^{W307}$ (Fig. 4H; number of specimens examined = 5).

In addition to the increases in BL, reduced levels of Mmp2 in *ap*-TIMP, *btl*-TIMP, *btl*-$Mmp2^{RNAi}$ and $Mmp2^{W307}$ animals stunted ASP growth (Figs. 4B, C, H, I; Figs. 5A-D). These stunted ASP tubes were populated by polarized epithelial cells (Figs. 5A-E), but extension and growth was arrested beyond the stages shown in these figures. Viable adults that expressed TIMP in the trachea (*btl*-TIMP) lacked normal Dorsal Air Sacs (Fig. 6), showing that Mmp2 function is required to make these adult organs.

Study of *Mmp2* mutant clones revealed the non-autonomy of its function within the ASP. We generated clones of $mmp2^{K07511}$ and $mmp2^{W307}mmp1^{Q112}$ cells (number of clones = 10 and 8, respectively) in the ASP using the MARCM system, and found no change in the distribution, or reduction in the number or size of the clones when compared with control (number of specimens examined = 20). Thus, our data show that in the ASP, Mmp2 has a tissue-specific but non-autonomous role.

In sum, reduction of Mmp2 function resulted in three distinctive and consistent mutant phenotypes: (1) ECM proteins collagen IV and Perlecan accumulated to abnormally high levels at the junctional interfaces of tunneled trachea and disc; (2) tunneled trachea and ASP extruded from the plane of the disc BL; and (3) development of the ASP and dorsal airs sacs were abnormal. As described below, ASP development depends upon Mmp2 function in both the ASP and wing disc.

Autonomy of Mmp2 function for ASP development

The experiments described in the previous section show that reduction of Mmp2 function in trachea alone (*btl*-$Mmp2^{RNAi}$) or in both trachea and disc (*ap*-TIMP and $mmp2^{W307}$) have similar effects on the the BL distribution, the topology of the trachea within the disc BL and on ASP growth. These results establish that the $Mmp2^{RNAi}$–induced phenotypes are specific to knockdown of Mmp2 and are not due to off-target effects. Expression of $Mmp2^{RNAi}$ to reduce Mmp2 function in the disc alone (*ap*-$Mmp2^{RNAi}$) had an unexpected and strikingly different phenotype. RNAi knockdown of Mmp2 in the *ap* domain did not affect disc development (not shown), but caused hyperplastic growth of the ASP (Fig. 5E). This effect shows that expression of Mmp2 in both disc and ASP is essential for ASP development and that Mmp2 has tissue-specific roles. During normal development presumably, proteolysis of BL components by ASP-produced Mmp2 enables extension and growth of the ASP, whereas proteolysis of BL components by disc-produced Mmp2 suppresses ASP growth.

Bnl/FGF signaling drives Mmp2 expression

Since ASP induction and growth are dependent on Bnl/FGF produced in the disc, we monitored discs with altered levels of Mmp2 for Bnl/FGF expression. In late L3, Bnl/FGF RNA was expressed in a discrete group of cells in the posterior compartment of the disc (Fig. 5F). Neither its abundance nor its distribution changed in *ap*-TIMP, $Mmp2^{W307}$, and *btl*-$Mmp2^{RNAi}$ discs (Figs. 5G–I). Discs were also probed for evidence of FGF signal transduction with α-DpERK antibody. We detected nuclear Dp-ERK at the leading edge of the ASP in specimens from all backgrounds (Figs. 5K–O). Although these
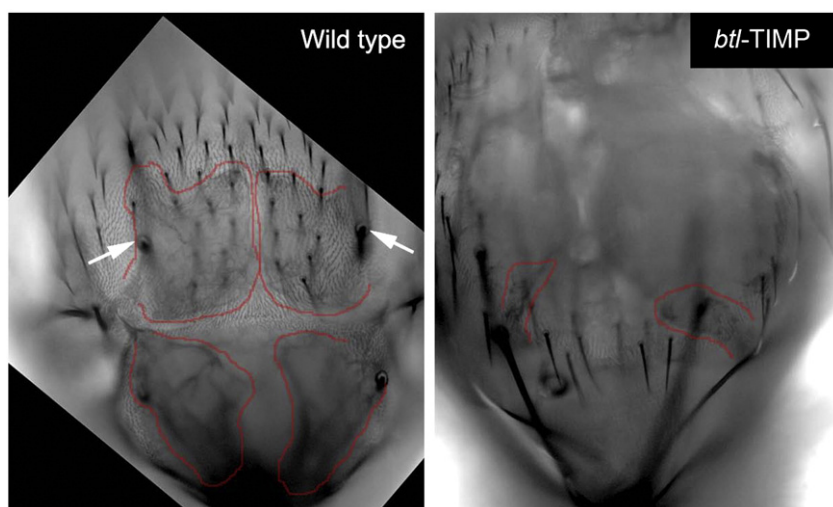
Fig. 6. Dorsal Air Sacs development is inhibited by TIMP. Red lines outline the medioscuteal (upper) and scutellar (lower) air sacs in the thorax of adults imaged with bright field optics by mounting flies in halocarbon oil. Arrows indicate macrochaete. Medioscutal and scutellar lobes are stunted or missing in animals expressing TIMP in tracheal (*btl* domain) cells.

assays for FGF expression and pathway activation are not quantitative, we conclude that the growth defects were not a consequence of a qualitative change in FGF signaling. In contrast, both the levels and area of Bnl/FGF expression increased in *ap-Mmp2*^RNAi discs (Fig. 5J). This increase is consistent with the ASP hyperplasia (Fig. 5E).

As shown above, Mmp2 must be expressed in the trachea to promote ASP growth. Previous studies of its expression pattern revealed that it is expressed at elevated levels in the ASP (Llano et al., 2002; Srivastava et al., 2007). Since growth of the ASP is dependent upon Bnl/FGF signal transduction (Sato and Kornberg, 2002), we tested if Bnl/FGF drives Mmp2 expression. We over-expressed Bnl/ FGF with a heat shock-Bnl/FGF construct in an *Mmp2* GAL4 UAS-nls-GFP background, and observed both increased proliferation and elevated levels of GFP in the ASP (Figs. 5P, Q). Elevated Dm-Mmp2 expression is consistent with the hypothesis that Dm-Mmp2 expression in the ASP is an outcome of Bnl/FGF signaling.

Discussion

The invasive coupling of the wing disc with the Tr2 transverse connective and the ECM remodeling that accompanies ASP growth led us to investigate how the presence of a BL impacts FGF signaling and the roles of MMPs. Based on the capacity of Bnl/FGF to signal through the disc and tracheal BL, we conclude that these BL are functionally transparent to FGF. Based on the effects of *btl*-TIMP, *ap*-TIMP, *btl*-MMP2 RNAi and *ap*-MMP2RNAi, and on the pheno-types of *Mmp2* mutants, we conclude that Dm-MMP2 has essential roles sculpting the disc-trachea association and remodeling the ECM during ASP induction and growth. We now consider the mechanisms underlying disc–trachea association, disc to trachea FGF signaling and the role of Dm-Mmp2 in tissue contact, ECM remodeling and organ morphogenesis.

Invasive coupling of wing disc and trachea and the role of Dm-MMP2

Invasive coupling of the wing disc and trachea must involve several distinct processes. First, progenitor disc and trachea cells, which originate independently in the embryo, must establish contact. Studies by Inoue et al. (2007) revealed that disc cells migrate towards the trachea during embryogenesis, leading to direct juxtaposition. Collagen IV was not detected at this stage (Inoue and Hayashi, 2007), so it seems unlikely that the BL had fully formed. Our experiments did not address whether Mmps are required for the early association of disc and trachea. However, we found that L3 discs remain associated with trachea in *Mmp2*^w307 (Fig. 4) and *Mmp1*^Q273 (data not shown) mutants. Since the *Mmp2*^w307 allele harbors a nonsense mutation and is a genetic null, our findings suggest that Mmp2 is not required to join these tissues.

Second, the arrangement of BL over the invasively coupled tracheal segment requires precise position-specific synthesis as well as continuous remodeling as the disc and trachea grow. Since core proteins of the ECM (e.g. collagen IV) are expressed by only a few disc cells (Butler et al., 2003), most BL components are presumably recruited from circulating stores in the hemolymph; little is known of the processes that bring these components to appropriate locations or regulate their assembly. We do not know, for instance, whether the absence of a distinct tracheal BL where the transverse connective contacts the disc is a consequence of insufficient levels of components. Alternatively, if availability of BL components is not limiting, whether the enzymes that synthesize BL are absent from these locations, or whether proteases that degrade the core components might be activated there. The changes in levels of Mmp2 that we engineered had significant effects on the ECM and its components. The presence of ectopic BL around disc-associated trachea in *ap*-TIMP animals suggests that neither components nor synthetic enzymes are limiting. Moreover, the accumulation of collagen IV and Perlecan in *Mmp2*

mutants revealed that proteolysis of ECM components is dependent upon Mmp2 and regulates BL assembly around disc-associated tracheae. collagen IV and Perlecan are either substrates of Mmp2 or their levels are dependent on another component that is. The distinct effects of reducing Mmp2 activity in the trachea (increased collagen IV and stunted ASP growth) or in the disc (hypertrophic ASP growth) show that location and level of Mmp2 activity is critically important for normal association and growth of the ASP. They also imply that the location and level of Mmp2 expression must be precisely regulated and that Mmp2, a membrane-tethered enzyme, might have different substrates in the disc and trachea extracellular milieu. A possible explanation is that despite the absence of a lamina densa separating the ASP and disc, distinct collagen-containing layers overly each tissue (see Fig. 7).

Third, the invasively coupled transverse connective and ASP nestle within the plane of the disc such that the overlying ECM forms a relatively flat sheet. However, reducing Mmp2 activity led to the partial extrusion of the disc-associated trachea (Fig. 4). This phenotype revealed that the character of the disc:trachea association is sensitive to the composition of the ECM, and is impaired if the system's capacity to remodel the ECM is reduced.

The BL is not a barrier to FGF signaling

The BL is rich with proteins that bind and sequester growth factors (LeBleu et al., 2007), and it therefore has the potential to block movement of proteins such as FGF. However, ectopic expression of Bnl/FGF in the disc induces invasive outgrowths from regions of the trachea that do not contact the disc and are separated from disc cells by two layers of BL (Fig. 2). Invasive coupling and direct contact are not therefore prerequisites for signaling and growth. Our ectopic expression assay is a qualitative measure of FGF signaling and does not ascertain whether direct apposition facilitates signaling; however, our results suggest that the BL is functionally transparent to FGF signaling. Tunneling may be needed for other purposes, for example, for the ASP to interact with the disc and to develop together with other thoracic structures during pupal development. We speculate that the functional transparency of the BL is likely to be general property, that the BL may be generally transparent to signaling proteins and growth factors. Such transparency would be relevant to the mechanisms that distribute signaling proteins, since constraining
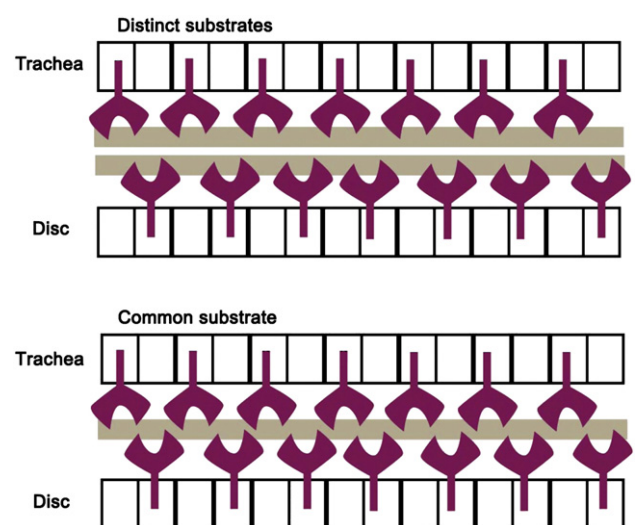
Fig. 7. Fine substructure may underlie the tissue specificity of Mmp2. Two generic possibilities are that both the invasively coupled trachea and the disc have distinct collagen IV-containing layers that are regulated independently by tracheal- or disc-generated Mmp2 (above), or that there is a single layer that both enzymes proteolyze (below).

signaling proteins to restrict their influence to only their intended targets would seem to be an essential feature (Kornberg and Guha, 2007).

ASP morphogenesis and the role of Mmp2

Although tube formation is essential to generate many vertebrate organs, Drosophila offers few relevant models. Strategies for making tubes have been classified according to the apical-basal polarity of the founding cells (Hogan and Kolodziej, 2002). Some, such as the vertebrate mammary gland, hair follicle and early pancreas, form from clusters of cells that initially lack polarity but acquire apical-basal polarity as they coalesce around a central lumen. Others, such as the vertebrate liver, lung and neural tube and the Drosophila salivary glands, form directly from morphogenetic movements of polarized epithelial sheets. The progenitors of these tubes retain their apical-basal polarity as they generate tubular extrusions.

The ASP is an example of the latter type of tubulogenesis. The cells of the ASP retain the apical-basal polarity of the tracheal epithelium from which they emerge (Cabernard and Affolter, 2005). Many of the cells in the ASP are mitotically active, distinguishing the ASP from the Drosophila salivary gland, whose cells invaginate from an epithelial sheet but do not divide. The process of ASP tubulogenesis is therefore more like that of the vertebrate liver, lung and neural tube, which also grow by coupling cell division to invagination and morphogenesis.

Mmps have been implicated in organ morphogenesis in a variety of contexts (Page-McCaw et al., 2007). A relevant example is HGF-induced tubulogenesis by MDCK cells cultured in 3D-matricies. Initial stages of tube morphogenesis required ERK activation, after which tube growth was dependent on Mmps but independent of ERK (O'Brien et al., 2004). Since the Drosophila ASP was induced but its growth was stunted in genetic backgrounds that reduced Mmp function, Mmps also appear to have a stage-specific role in ASP morphogenesis.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.005.

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