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The proteoglycan Trol controls the architecture of the extracellular matrix and balances proliferation and differentiation of blood progenitors in the *Drosophila* lymph gland

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ABSTRACT

The heparin sulfate proteoglycan Terribly Reduced Optic Lobes (Trol) is the *Drosophila melanogaster* homolog of the vertebrate protein Perlecan. Trol is expressed as part of the extracellular matrix (ECM) found in the hematopoietic organ, called the lymph gland. In the normal lymph gland, the ECM forms thin basement membranes around individual or small groups of blood progenitors. The pattern of basement membranes, reported by Trol expression, is spatio-temporally correlated to hematopoiesis. The central, medullary zone which contain undifferentiated hematopoietic progenitors has many, closely spaced membranes. Fewer basement membranes are present in the outer, cortical zone, where differentiation of blood cells takes place. Loss of *trol* causes a dramatic change of the ECM into a three-dimensional, spongy mass that fills wide spaces scattered throughout the lymph gland. At the same time proliferation is reduced, leading to a significantly smaller lymph gland. Interestingly, differentiation of blood progenitors in *trol* mutants is precocious, resulting in the break-down of the usual zonation of the lymph gland. which normally consists of an immature center (medullary zone) where cells remain undifferentiated, and an outer cortical zone, where differentiation sets in. We present evidence that the effect of Trol on blood cell differentiation is mediated by Hedgehog (Hh) signaling, which is known to be required to maintain an immature medullary zone. Overexpression of *hh* in the background of a *trol* mutation is able to rescue the premature differentiation phenotype. Our data provide novel insight into the role of the ECM component Perlecan during *Drosophila* hematopoiesis.

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Introduction

Animal cells secrete proteins that assemble into an extracellular matrix (ECM). The ECM plays important roles in determining the mechanical properties of cells and tissues, as well as in intercellular communication. ECM can also play a role in facilitating integrin mediated cell interactions (Subramanian et al., 2007). In many cells, the ECM forms a layered structure called the basal lamina or the basement membrane. In epithelia, the basement membrane assembles over the basal cell surface; in other, mesenchymal tissues, such as muscle or cartilage, basement membranes surround cells on all sides (Durbeej, 2010). Many basement membranes are composed primarily of Laminin, type IV Collagen, Nidogen and heparin sulfate proteoglycans (HSPGs), which include Perlecan and Agrin (reviewed in Yurchenco et al., 2004; Laurila and Leivo, 1993). The heparin sulfate chains of these proteins are mainly responsible for the involvement of basement membranes in cell–cell signaling. They bind (and thereby sequester or release in a controlled manner) ligands like Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Wnt, Hedgehog (Hh) and Transforming Growth Factor (TGFβ) (Lin, 2004; Lindner et al., 2007; Yurchenco et al., 2004).

Perlecan is a large macromolecule which consists of a core protein associated with three glycosaminoglycan side chains and that occurs in basement membranes around most cells. The core protein has five distinct domains. Domains I and V, located at the N and C terminal sides, respectively, both contain binding sites for heparin sulfate side chains (reviewed in Whitelock et al., 2008). These two domains also interact with other ECM proteins, including Laminin and Collagen IV, as well cell adhesion molecules, such as integrins. Domain II is thought to interact with lipids (Fuki et al., 2000; reviewed in Whitelock et al., 2008). Domains III and IV interact with other secreted signals and ECM proteins (Friedrich et al., 1999; Iozzo, 2005; reviewed in Whitelock et al., 2008).

Given its widespread expression in basement membranes and its ability to bind to a multitude of known adhesion and signaling molecules, it is to be expected that Perlecan (along with other HSPGs) plays many different roles in tissue development and

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maintenance. Both in vitro and in vivo studies confirm this expectation. In vertebrates, Perlecan plays particularly important roles in the growth and morphogenesis of the skeleton and vascular system by modulating cell adhesion and/or the effect of the signaling molecules FGF, PDGF, and VEGF on chondrocytes, smooth muscle cells, and endothelial cells (Melrose et al., 2008; Segev et al., 2004; Whitelock et al., 2008). In *Caenorhabditis elegans*, Perlecan mediates the binding of muscle cells to epidermal cells via integrins (Rogalski et al., 2001). In *Drosophila*, the Perlecan homolog was originally identified as a mutation affecting postembryonic growth of the central nervous system. In larvae carrying this mutation, called *trol*, neuroblasts show a strongly decreased rate of proliferation (Datta, 1995). This phenotype was shown to be mediated by an effect of *trol* on FGF and Hh signaling (Caldwell and Datta, 1998; Lindner et al., 2007; Park et al., 2003). The effect of *trol* on proliferation is not confined to the CNS; experiments looking at hemocyte number in *trol* mutants have also shown a significant drop in circulating plasmatocyte numbers (Lindner et al., 2007). Further studies have also shown the expression of *trol* in the cardioblasts, visceral mesoderm and hindgut of stage 15 *Drosophila* embryos (Friedrich et al., 2000).

Comparisons of the human Perlecan gene to *trol* have found 34% sequence identity in domain III, 24% identity in domain IV and 30% identity in domain V. No significant similarity was seen in domains I or II (Murdoch et al., 1992; Park et al., 2003). In *Drosophila*, Trol is expressed strongly in basement membranes of embryonic tissues, where it interacts with Laminin and Collagen (Urbano et al., 2009). It also appears around hemocytes, vascular cells, and the hematopoietic organ ("lymph gland") of the late embryo. Given the reported effect of *trol* on blood cell number, we embarked on a more detailed analysis of the role of *trol* in *Drosophila* hematopoiesis.

The blood, or hemolymph, of *Drosophila* contains three major types of blood cells (hemocytes), called plasmatocytes, crystal cells and lamellocytes. Plasmatocytes act as macrophages during development, and together with crystal cells, play a role in immunity and response to injury (Crozatier and Meister, 2007; Martinez-Agosto et al., 2007). These two cell types comprise the hemocytes most commonly seen under non-immune challenged conditions. Lamellocytes are very rare under normal conditions. In cases of immune challenge, their numbers increase and they act to neutralize objects too large to be phagocytosed.

Hemocytes are produced during two phases of development. The first phase of hematopoiesis takes place in the head mesoderm of the early embryo; hemocytes produced during this phase populate the embryo and the circulating hemolymph of the larva. The second phase of hematopoiesis takes place in the lymph gland of the larva, a solid hematopoietic organ located alongside the dorsal vessel ("heart"). The lymph gland derives from a small population of hematopoietic blood progenitors that first appear in the trunk mesoderm of the embryo, consolidate into the lymph gland, and then proliferate during the larval stage. By late larval stages, the lymph gland has grown into a series of several paired lobes which flank the dorsal vessel. Differentiation of hematopoietic progenitors into mature blood cells takes place in the periphery (cortex) of the large, anteriorly located primary lobe. A specialized subpopulation of hemocytes, called the posterior signaling center (PSC), signal to the medullary zone via the Hh pathway to maintain cells in an undifferentiated state (Mandal et al., 2007). Aside from Hh, the Wg signal (expressed in the medullary zone) and Adenosine deaminase growth factor A (Adgf-A), produced by differentiating cells in the cortical zone, antagonize prohemocyte differentiation (and prolong proliferation) in the medullary zone (Sinenko et al., 2009; Mondal et al., 2011). Differentiated hemocytes are released from the lymph gland into circulation during early metamorphosis (Lanot et al., 2001; Grigorian et al., 2011). During this phase, the entire lymph gland dissociates; adult flies lack a solid hematopoietic organ. A lymph gland similar to that described for *Drosophila* has been documented for many insects (reviewed in Grigorian and Hartenstein, 2013). Similarities to the hematopoietic tissue of vertebrates are present; even though a prominent "stroma" (represented in the vertebrate bone marrow by the network of capillaries and reticular cells) is missing in invertebrates, cells described as "reticular cells", surrounding prohemocytes and possibly acting as stem cells, have been described in several insect species (Hoffmann et al., 1979). In all insects investigated, profuse lamellae of ECM, formed by proteins that are found ubiquitously in basement membranes and other ECM assemblies of Drosophila and vertebrates (reviewed in Grigorian and Hartenstein, 2013), were observed.

In this paper, we show that Perlecan/Trol is expressed in basement membranes that both surround the surface of the lymph gland and that form discrete chambers within the lymph gland interior. Loss of *trol* is associated with a dramatic change in the texture of the ECM: instead of forming thin membranes around blood cells, the matrix appears as a spongy mass filling large areas of the lymph gland. Concomitantly, proliferation of blood progenitors is reduced, and the lymph gland is significantly reduced in size and cell number. Finally, the altered composition of the ECM resulting from a loss of *trol* is associated with premature hemocyte differentiation. This phenotype can be rescued by the ectopic expression of Hh. We propose that *trol* is required for the organization of the lymph gland ECM, and the proper release and/or distribution of the Hh ligand throughout the lymph gland.

Material and methods

Fly lines

y *trol*^{8} w/Bnsn (Park et al., 2001), y *trol*^{4} w/Bnsn (Park et al., 2001), y *trol*^{SD} w/Bnsn (Datta and Kankel, 1992), ZCL1973X (Kelso et al., 2004), *trol*-GFP (Lindner et al., 2007; Medioni and Noselli, 2005; Kelso et al., 2004), *domeless*-Gal4 (Jung et al., 2005; Mandal et al., 2007; Ghiglione et al., 2002), *Antennapedia*-Gal4 (Mandal et al., 2007; Emerald and Cohen, 2004), *elav*-Gal4 (Bloomington Stock Center), *Tubulin*-Gal4 (Bloomington Stock Center), *Collagen*-Gal4 (comprised of a regulatory region, located between the adjacent collagen type IV genes *Cg25C* and *Viking*, fused to a Gal4; Asha et al., 2003), UAS-*trol* Rnai (Vienna *Drosophila* RNAi Center; ID# 24549), heatshock-*hh* (*hh* fused downstream of a Hsp70 promoter; Ingham, 1993; Park et al., 2003), *hh*^{AC} (Bloomington Stock Center), *egfr*^{f2} (Bloomington Stock Center), Oregon R (Bloomington Stock Center), *Hemolectin*Δ-DsRed (Makhijani et al., 2011).

Immunohistochemistry

α-Antennapedia (Mouse; 1:4; Developmental Hybridoma Bank), α- BrdU (Rat; 1:100; Abcam), α-Cleaved Caspase 3 (Rabbit; 1:100; Cell Signaling Technology, Inc.), α-Collagen IV (Mouse; 6G7; 1:25–1:50; Murray et al., 1995), α-GFP (Mouse; 1:400; Sigma), α-GFP (Rabbit; 1:2000; Molecular Probes), α-Laminin (Rabbit; 1:500; Fessler et al., 1987), α-Odd (Rabbit; 1:500; Ward and Skeath, 2000), α–P1 (Mouse; 1:10; Kurucz et al., 2007), α-Perlecan domain V (Rabbit; 1:1000; Friedrich et al., 2000), α-Peroxidasin (Mouse; 1:500; Nelson et al., 1994), α-ProPo (Rabbit; 1:1000; Muller et al., 1999). All secondary antibodies were acquired from Jackson Immunoresearch. Both Cy3 and FITC were used at a dilution of 1:200. Cy5 was used at a dilution of 1:100. Immunohistochemistry was carried out following standard protocols (Ashburner, 1989). Some preparations were counterstained with

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