PERICARDIAL CELLS AND EXTRACELLULAR MATRIX REMODELLING

THE ROLE OF PERICARDIAL CELLS IN *DROSOPHILA MELANOGASTER* EXTRACELLULAR MATRIX REMODELLING AT THE DORSAL VESSEL

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Abstract

The cardiovascular system of *Drosophila melanogaster* consists of a cardiac tube composed of myogenic cardiomyocytes and associating non-contractile pericardial cells, pumping hemolymph into the open circulatory system. The cardiac tube, known as the dorsal vessel, is embedded in a highly regulated extracellular matrix environment, required to maintain cellular integrity and cardiac function. After embryogenesis, the dorsal vessel undergoes extensive physiological changes, relying on the extracellular matrix to adapt and remodel accordingly. Three extracellular matrix proteins are investigated throughout this thesis: Type IV Collagen, Laminin and Pericardin. Due to their localization, morphology, and role in early development, the pericardial cells are candidate cells responsible for dorsal vessel extracellular matrix deposition and regulation throughout post-embryonic growth. Using immunofluorescence techniques in combination with confocal microscopy, I characterize the association between pericardial cells and extracellular matrix proteins, and quantify extracellular matrix protein deposition at the dorsal vessel throughout post-embryonic development. Gene knock-down experiments assess pericardial cell contribution to extracellular matrix synthesis and deposition at the dorsal vessel in third instar larva. Moreover, I quantify extracellular matrix protein deposition at the dorsal vessel in the absence of pericardial cells. These data suggests that pericardial cells regulate extracellular matrix protein deposition, localization and contribute to proper cardiac morphology in post-embryonic development.

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ABBREVIATIONS AND SYMBOLS:

+	Wild type chromosome
α	alpha- or anti-
β	beta
μm	micrometer
μl	microliter
A5	Abdominal segment A5
BMP	Bone morphogenic protein
CB	Carrdioblast
Cg25C	Collagen at 25C
CyO	"Curly O" balancer chromosome
da	"daughterless" ubiquitous gene
DV	Dorsal vessel
ECM	Extracellular matrix
GFP	Green fluorescent protein
Hl	Heart lumen
klf15	*"*Kruppel Like Factor 15" gene
LanA	LamininA
LanB	LamininB
PBS	Phosphate Buffer Saline
PBT	PSB + 0 .1% Triton
Pc	Pericardial cell
ROI	Region of interest
Prc	Pericardin
RFP	Red fluorescent protein
SEM	Standard error of the mean
tin	Tinman
UAS	Upstream activation sequence
Vkg	"Viking" Collagen IV gene
WT	Wild Type

1.0: Chapter One: Introduction

A play begins as a script, a blueprint, outlining the role of each actor and the story the play wants to tell. When the actors first come together on stage, a script begins telling a coherent story, with a specific rhythm and flow. A script pumps life into its actors, differentiating them into the unique characters they are meant to embody and portray.

After a script is written and its ensemble comes together, a play takes on a new life: actors are not only required to know their characters and memorize their lines, but also interact with the production crew behind the curtain. Interactions between actors and crew members are critical: they determine whether a play runs smoothly. Communication between a play's actors and crew allows them to react and adapt as the play develops act by act, scene by scene.

A play's costumes, set design, props, and lighting are put in place during the dress rehearsal so that a play may come together and take off on opening night.

Like a play, the *Drosophila melanogaster* heart (known as the dorsal vessel) transforms from a "blueprint", during embryogenesis, into a fully functioning contractile organ. The cells of the dorsal vessel represent the actors in a play, coming together to perform their specific roles. The extracellular matrix represents members of the production crew, changing the set after each act, adapting to shifts in the script, and providing structure and organization along the way.

This thesis examines the interplay between cardiac specific cells of the heart and their surrounding extracellular matrix environment. As the dorsal vessel undergoes enormous physiological changes after embryogenesis, the extracellular matrix (ECM) is required to respond and adapt accordingly. This thesis explores the role of pericardial cells of the dorsal vessel in regulating ECM deposition and organisation.

Pericardial cells are non-contractile endocytic cells that maintain close association with the dorsal vessel throughout development. I chose to explore the role of pericardial cells in regulating post- embryonic ECM deposition because they: 1) are in close physical proximity to the heart, 2) have an extensive extracellular matrix around their cell surface, and 3) are implicated in producing extracellular matrix proteins in embryonic development (Chartier et al 2002; Drechsler et al 2013). Before assessing the pericardial cells' instructive role in ECM deposition, it is necessary to characterize how the ECM is localized to the dorsal vessel in normal development.

Objectives

My thesis' initial objective sought to characterize extracellular matrix protein levels and localization at the dorsal vessel throughout post- embryonic development; to do so required exploring the structural morphology of the dorsal vessel, along with the associated changes in ECM deposition. I looked at three extracellular matrix proteins to achieve this objective: Type IV Collagen, LamininA, and Pericardin. The first objective "sets the stage" to explore the question at the heart of my thesis: to implicate pericardial cells in ECM deposition.

The second objective asked whether pericardial cells produce ECM proteins at the dorsal vessel in post- embryonic development. Pericardial cells are involved in ECM deposition during embryogenesis, secreting collagen-like protein Pericardin to the extracellular matrix environment (Chartier et al 2002; Drechsler et al 2013). This objective sought to investigate the role of post- embryonic pericardial cells in ECM deposition because it is scarcely discussed in scientific literature.

My thesis' third objective was to assess whether pericardial cells are required for proper ECM localization and deposition at the dorsal vessel. As in the play analogy, if an actor falls sick in the middle of the performance, the cast and crew will likely adapt and try to compensate for the loss. Although audience members may not notice any irregularities in the overall quality of the performance, the cast and crew's cues behind the scenes may become disorganized and suboptimal. Just as the play's ensemble would likely suffer due to the absence of an actor, I expected the ECM deposition and organization would likely be impaired in the absence of pericardial cells. The interplay between pericardial cells and the associating dorsal vessel ECM will give insight into a novel role for post-embryonic pericardial cells, and a deeper understanding of the intrinsic and dynamic extracellular environment.

The introductory sections of this thesis discuss: the significance of *Drosophila melanogaster* as a model organism for heart development, the structure of the dorsal vessel, the cells associated with the dorsal vessel, and three extracellular matrix proteins that this thesis will focus on. The final section of this introductory chapter provides

evidence that explains why post- embryonic pericardial cells are ideal candidate cells to facilitate the organization and deposition of the dorsal vessel ECM.

1.1 Significance of Drosophila melanogaster as a model organism for heart development.

As a model for cardiac development and ECM remodelling, the *Drosophila* dorsal vessel combines the advantages of invertebrate genetics, advanced molecular tools, and developmental homology to provide meaningful insight into the vertebrate cardiac system. The *Drosophila* dorsal vessel shares similarities in physiology and molecular signalling events with vertebrate heart development. In early cardiac development of both *Drosophila* and vertebrates, bilateral rows of cardiac progenitor cells migrate towards the midline and enclose a linear tube with a central luminal domain (Bier and Bodmer, 2004; Venkatesh et al., 2000; Cripps). Progenitor heart cells are determined through conserved signalling pathways involving homologous transcription factors common to *Drosophila* and vertebrate systems (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Venkatesh et al., 2000). Forward genetic techniques using the *Drosophila* dorsal vessel as a model system have provided genetic insight into signalling pathways and specific regulatory transcription factors reflected in early vertebrate heart development. For example, Zaffran and colleagues (2006) found that stage specific *tinman* mutant embryos share a similar phenotype with their vertebrate homolog Nkx2.5, displaying inability to differentiate into adult dorsal vessel, and inability to undergo cardiac looping, respectively. Nkx2.5 and the *Drosophila* homolog, tinman (tin), act downstream of BMP (decapentaplegic) signalling to specify cardiac progenitor cells during early heart development (Shi Y et al 2000;

Zaffran et al. 2006). There is an abundance of literature that discusses the relevance of dorsal vessel specification during embryogenesis; while, exploring the application of dorsal vessel function and physiology in later developmental stages has only recently attracted similar attention. More recently, the adult dorsal vessel has been used as a model to explore aspects of cardiac ageing relevant to vertebrates. Like the vertebrate heart, performance and function of the dorsal vessel declines with age. Owing to the complexity of cardiac ageing in humans, the short lifespan of *Drosophila*, and the reduced genetic redundancy, makes the dorsal vessel an ideal model to investigate cardiac aging.

This thesis focuses primarily on cardiac growth and ECM remodelling throughout larval and early adult development. During larval development, *Drosophila* grows approximately five times in size, shifting from a languid 1st instar larva to a wandering 3rd instar larva. Along with a dramatic increase in body size, an associated increase in ECM turnover and remodelling accounts for dramatic physiological changes. The larval dorsal vessel can therefore act as a model to explore the effects of increased cardiac load and consequent ECM remodelling.

1.2 Structure of the *Drosophila melanogaster* dorsal vessel.

As discussed in the previous section, the dorsal vessel shares several structural similarities to the primitive vertebrate heart tube, making it a meaningful model for understanding cardiac development and disease. Outlining the structure and morphology of the dorsal vessel will familiarize the reader with key components and structures

addressed throughout this thesis. The section introduces the reader to the structural changes of the dorsal vessel at the following developmental stages: embryonic, larval, and adult.

Structure at embryonic development

Upon the completion of embryogenesis (stage 17), the dorsal vessel is a functioning contractile organ, pumping hemolymph in a posterior-anterior direction into the open circulatory system of the fly (Bodmer and Frasch 1999; Rizki 1978). The dorsal vessel extends across the anterior-posterior axis under the dorsal ectoderm (Rizki 1978; Bodmer and Frasch 1999; Lehmacher et al. 2012). Segments of the dorsal vessel coincide with thoracic and abdominal segmentation of the body cavity (Figure 1.1). Towards the end of embryogenesis, the dorsal vessel extends from segment T3-A8 across the anterior-posterior axis. An inner row of myogenic cardioblasts is flanked by an outer row of non-contractile pericardial cells along the dorsal vessel (Figure 1.1). At the end of embryogenesis, differentiated cardioblasts are comprised of:

- cardiomyocytes, the contractile cells of the dorsal vessel
- the ostial cells, which form inflow tracts where hemolymph passively enters the posterior heart proper, and
- the valve cells, responsible for maintaining a unidirectional flow of hemolymph and subdividing the heart into chambers (Lehmacher et al. 2012; Molina and Cripps 2001).

Structure at larval development

Throughout larval development, the heart is divided into two distinct chambers separated by a cardiovascular valve (Lehmacher et al. 2012). One of the chambers, the aorta, is characterized by a narrow luminal domain, extending from abdominal segment T3-A4, terminating at the anterior lymph and ring glands (Lehmacher et al. 2012). Three pairs of ostial cells are located in the other chamber, the posterior heart chamber. The ostial cells act as passive inflow tracts for hemolymph to enter the dorsal vessel (Lehmacher et al. 2012; Molina and Cripps 2001). The posterior heart chamber is characterized by a larger luminal domain, extending from abdominal segment A5-A8, responsible for actively pumping hemolymph to the aorta (Lehmacher et al. 2012).

Larval development is characterised by enormous growth. Understandably, the dorsal vessel must increase in proportion to the growing body cavity. The cells of the dorsal vessel are non-proliferative, so instead of growing in number, they grow in cell volume (Lehmacher et al 2012). Although the volume of the dorsal vessel increases throughout larval development, its structure remains similar to the mature embryonic dorsal vessel (Figure 1.2).

Structure in Adult

The adult dorsal vessel is structurally different from the dorsal vessel in embryonic and larval development due to the remodelling events of metamorphosis (Curtis et al 1999; Lehmacher et al. 2012). During metamorphosis, programmed cell death (PCD) eliminates

segments A6-A8 of the abdomen, and consequently the majority of the larval posterior heart chamber (Figure 1.3) (Zeitouni et al 2007). Abdominal segment A5 persists through metamorphosis but loses contractility and transdifferentiates into the terminal chamber of the adult heart (Curtis et al 1999; Zeitouni et al 2007). The cells of the larval aorta differentiate into functioning contractile cardiomyocytes, forming the functioning adult heart (Curtis et al 1999; Zeitouni et al 2007). The adult dorsal vessel extends from segment A1-A5, with three pairs of valve cells, and five pairs of ostial cells throughout the heart chamber (Figure 1.3) (Curtis et al 1999; Lehmacher et al. 2012). Ventral longitudinal muscles extend across the ventral surface of the adult dorsal vessel (Figure 1.3) (Curtis et al 1999; Schaub et al 2015; Lehmacher et al. 2012).

Associated structure: Alary muscles of the dorsal vessel

Alary muscles anchor the dorsal vessel to the epidermis, maintaining dorsal vessel orientation and providing stability (Bataillé et al 2015, Lehmacher et al. 2012). Six pairs of alary muscles are present in larval development, reducing to four pairs after metamorphosis (Figure 1.2;1.3) (Curtis et al 1999). The alary muscles attach to the dorsal vessel indirectly through pericardial cells and the intermediate ECM (Figure 1.2) (Ivy et al 2015; Bataillé et al 2015, Lehmacher et al. 2012). The alary muscles' interaction with the ECM is required to maintain dorsal vessel orientation.

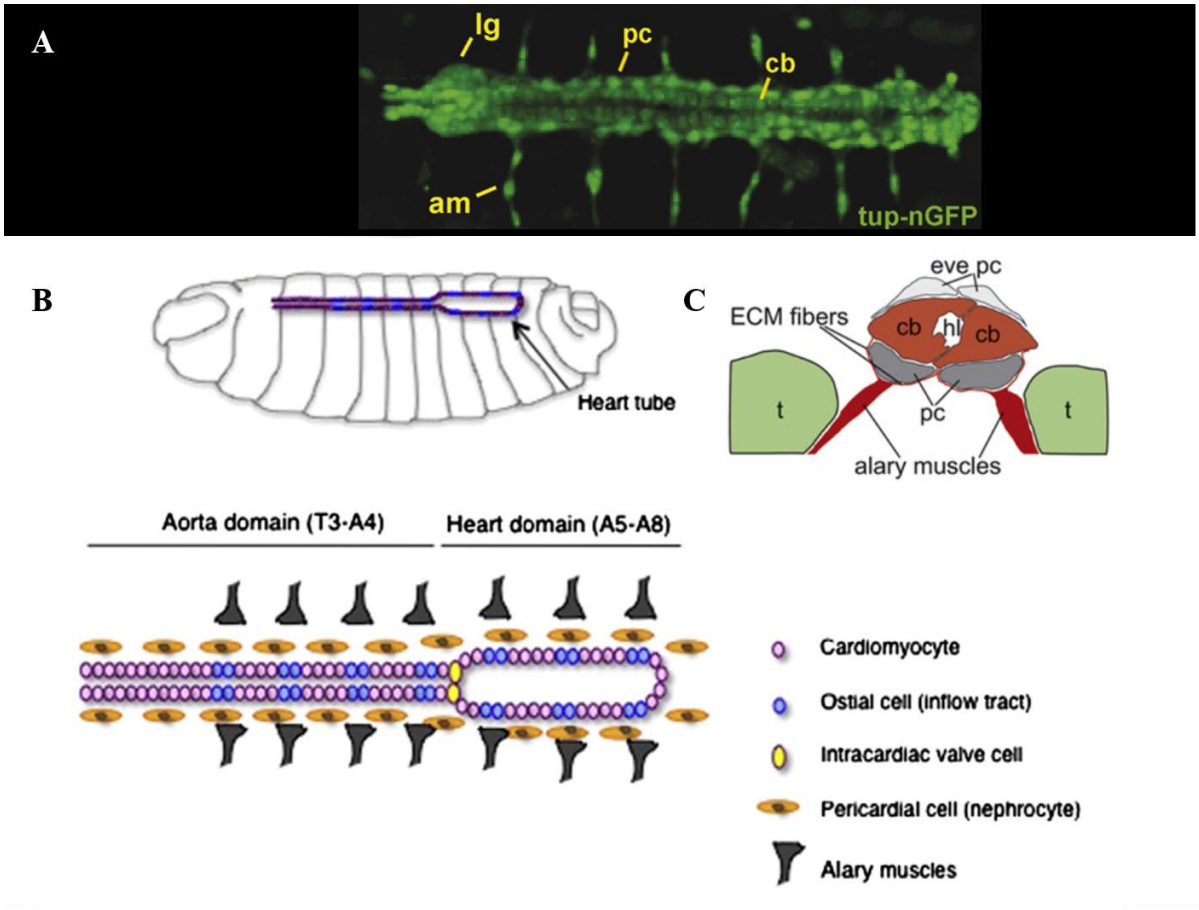


Figure 1.1. Structure of embryonic dorsal vessel at the end of embryogenesis. (A) Confocal image of embryonic heart. lg: Lymph gland, pc: pericardial cell, cb: cardioblast, am: alary muscle . (B) Animated representation of dorsal vessel. (C) Cross sectional representation of dorsal vessel. cb: cardioblast, hl: heart lumen, pc: pericardial cell, ECM fibers: Extracellular matrix fibers, t: trachea. Adapted and modified from: (A) Tao and Schulz 2007, (B) Volk et al 2014, (C) Lehmacher et al 2012.

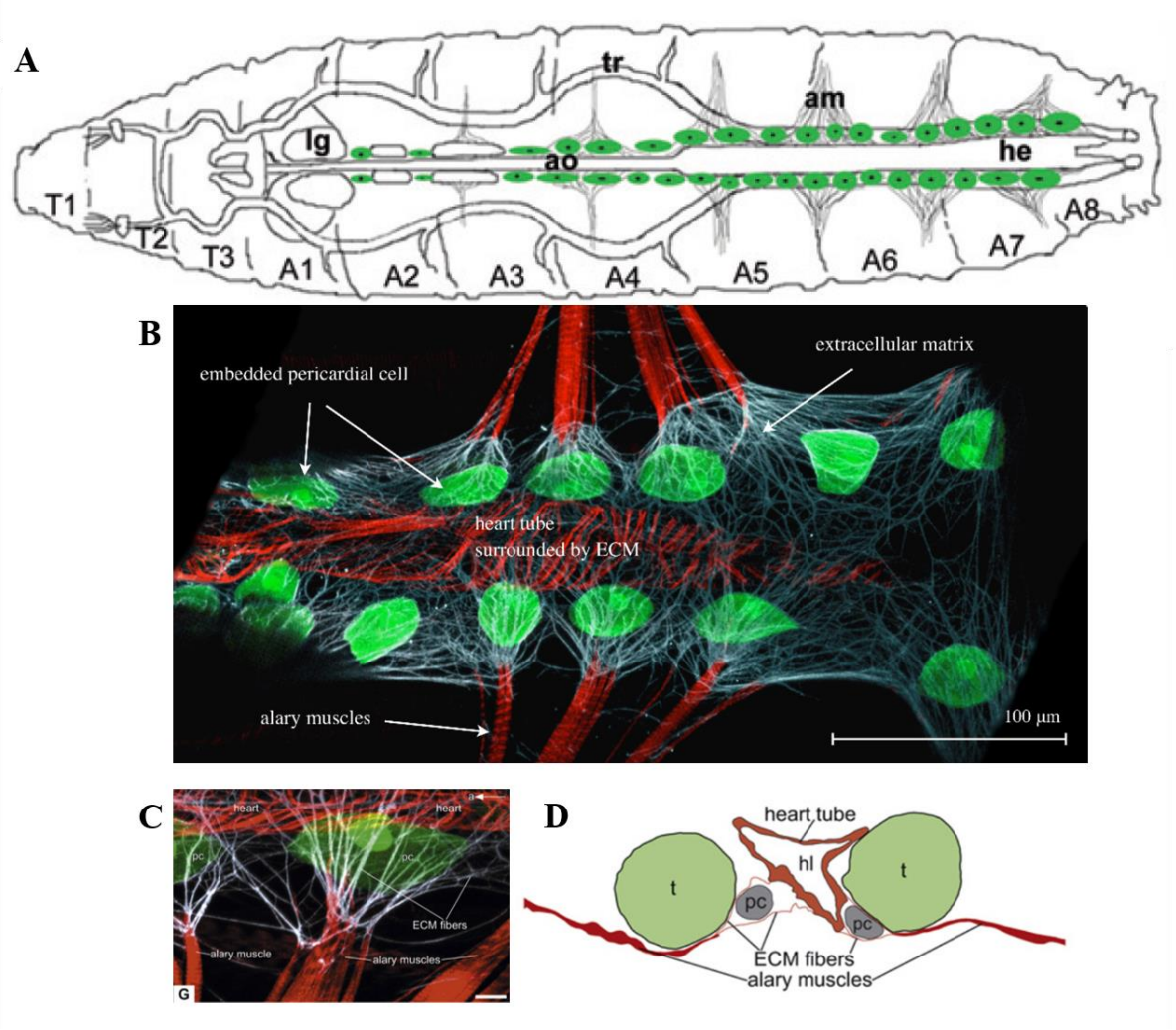


Figure 1.2. Structure of the dorsal vessel at 3rd instar larval development. (A) Animated representation of larval dorsal vessel. lg: lymph gland, tr: trachea, am: alary muscle, he: posterior heart chamber, ao: aorta, green: pericardial cells. (B) Confocal image of larval dorsal vessel. Green: pericardial cells, White: extracellular matrix protein Pericardin, red: heart musculature and alary muscles (labeled for F-actin). (C) Higher magnification of (B), visualizing pericardial cells embedded in extracellular matrix network. (D) Animated cross sectional representation of the dorsal vessel. t: trachea, pc: pericardial cell, hl: heart lumen. Adapted and modified from: (A) Das et al 2007 (B,C,D) Lehmacher et al 2012.

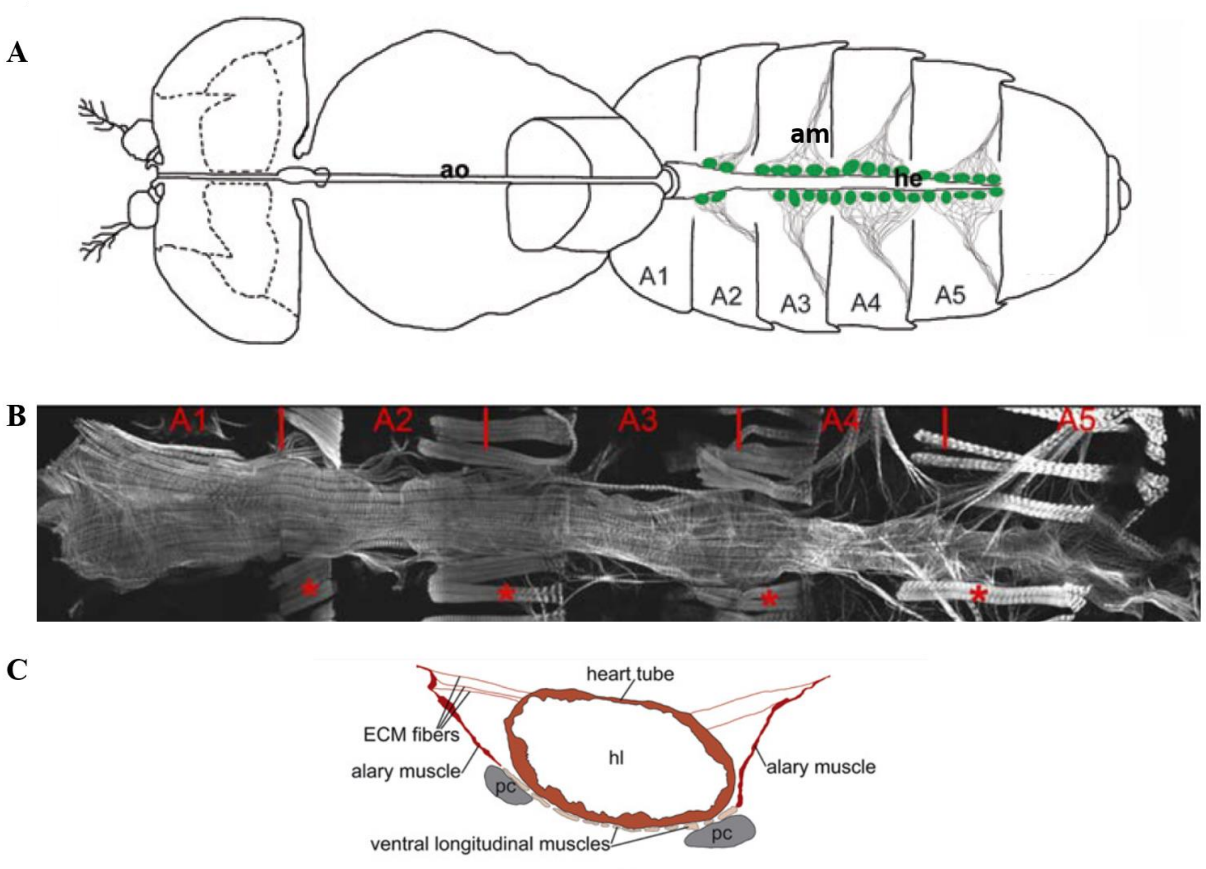


Figure 1.3: Structure of adult dorsal vessel. (A) Animated representation of adult dorsal vessel. am: alary muscle, he: heart chamber, ao: aorta, green: pericardial cells. (B) Confocal image defining abdominal segments of the adult dorsal vessel. (C) Cross sectional animation of adult dorsal vessel. hl: heart lumen, pc: pericardial cell. Adapted from: (A) Das et al 2007, (B) Zeitouni et al 2007.

1.3: Structural proteins of the extracellular matrix and matrix- related proteins of the dorsal vessel.

The extracellular matrix is a highly regulated environment that constantly adapts in response to extra and intra-cellular cues. It is a non-cellular microenvironment that surrounds all tissues and cells in multicellular organisms (Frantz 2010). The ECM regulates cellular processes by providing a means of communication between the cell and the external environment. It is able to alter its mechanical properties in response to external stimuli, triggering changes in cell signalling and behaviour (Harburger and Calderwood 2009; Urbano et al 2009). The ECM also responds to larger scale physiological changes throughout *Drosophila* development.

As discussed earlier, the dorsal vessel undergoes extensive physiological changes throughout development resulting in an increase in cardiac load and metabolic stress. The dorsal vessel relies on the ECM to adapt and remodel in accordance to the growing heart, especially during larval development. Since the dorsal vessel lacks stem cells, no new cardiac cells arise during development. Therefore, the interaction and communication between dorsal vessel specific cells and the extracellular matrix environment is critical to maintaining cellular integrity and cardiac function. For this reason, the *Drosophila* dorsal vessel is a promising model to explore the regulation and mechanism of ECM remodelling.

This thesis focuses on three particular proteins that are components of the extracellular matrix: Type IV Collagen, Laminin, and a novel dorsal vessel collagen-like protein, Pericardin. These three proteins differ in regard to their abundance, localization, and assembly at the dorsal vessel ECM, and will be discussed in the sections below.

Basement membrane of the dorsal vessel.

In order to understand the context within which the ECM proteins localize, assemble, and accumulate, it is first necessary to describe a specialized component of the ECM known as the basement membrane. The basement membrane adheres to the basal surface of the dorsal vessel, connecting to pericardial cells and associated alary muscle fibers. It is visible at both the luminal and abluminal surface of the dorsal vessel (Bogatan et al 2015) providing structural support and environmental cues to fine tune cellular function. The major components of the basement membrane include: Type IV Collagen, Laminin, Nidogen and Perlecan. Laminin expression is strictly localized to the basement membrane of the ECM (Urbano et al 2009). Type IV Collagen and Laminin self- assembly is thought to drive basement membrane assembly and influence basement membrane stability (Hollfelder et al 2014).

Integrin

Integrins are heterotrimeric transmembrane receptors composed of alpha and beta-subunits, mediating ECM interaction with the cytoskeleton of the cell (Gotwals et al 1994). It is important to note that integrin beta subunit (βPS) powerfully labels the cell surface of pericardial cells throughout all stages of development; suggesting a persistent association between pericardial cells and the ECM environment throughout development.

Type IV Collagen

Type IV Collagen is the most abundant component of the ECM, localized at the basement membrane and the interstitial ECM environment (Kalluri, 2003). *Drosophila* has two genes encoding for Type IV Collagen: *viking (vkg)* and *Collagen at 25C (Cg25C)* (Yasothornsrikul et al 1997; Parco et al 1986). To explore the regulation of Type IV Collagen in the dorsal vessel ECM, I used a GFP protein trap inserted into the *Drosophila vkg* gene, rendering a functional Vkg-GFP fusion protein tracing endogenous Vkg throughout development. During embryogenesis, hemocytes are responsible for contributing Type IV Collagen to the dorsal vessel ECM (Bunt et al 2010; Martinek et al 2008). It has recently been shown that hemocytes and the fat body secrete Type IV Collagen to the dorsal vessel ECM during larval development (Cevic 2016; Pastor-Pareja and Xu, 2011). Although hemocytes and the fat body have been implicated as regulators of post-embryonic Type IV Collagen deposition, the potential role of pericardial cells has not yet been explored.

Laminin

Laminin is the most abundant non-collagenous protein, strictly localized to the basement membrane of the ECM. The *Drosophila* genome encodes two Laminin alpha-chains, one beta chain, and one gamma chain, forming two distinct Laminin heterotrimeric molecules (LamininA and LamininW) (Urbano et al 2009). Pericardial cells are embedded in Laminin ECM network throughout development. In *lamininA (lanA)* and *lamininB (lanB)* mutant embryos, pericardial cells appear disorganized and detached from their associated cardioblasts (Yarnitzky and Volk 1995). Laminin appears to regulate pericardial cell localization and dorsal vessel integrity during embryogenesis. This thesis explores whether pericardial cells have a regulatory role in Laminin throughout post- embryonic development.

Pericardin and Lonely Heart

Pericardin (Prc) is a collagen- like ECM protein unique to the *Drosophila* dorsal vessel (Chartier et al 2002). Prc does not localize to the basement membrane; rather, it concentrates around the basal surface of cardiomyocytes, around pericardial cells, and near the dorsal ectoderm (Chartier et al 2002; Fogerty et al 1994). Lonely Heart (Loh) recruits Pericardin and regulates its accumulation in the dorsal vessel ECM (Drechsler et al 2013). During embryonic development, both Lonely Heart and Pericardin are secreted by embryonic pericardial cells (Drechsler et al 2013). After embryogenesis, the larval fat body secretes both Lonely Heart and Pericardin (Drechsler et al 2013). Throughout all

developmental stages, Pericardin is required to mediate pericardial cell adhesion to cardiomyocytes (Chartier et al 2002). In *prc* mutants, pericardial cells detach from the dorsal vessel (Drechsler et al 2013). Pericardin likely plays a fundamental structural role in maintaining pericardial cell localization and association with the dorsal vessel. Although pericardial cells are no longer responsible for secreting Pericardin to the dorsal vessel ECM, this functional role for pericardial cells has not necessarily been lost. This thesis investigates the regulatory role pericardial cells play in ECM deposition and localization at the dorsal vessel.

The ECM is essential to maintaining the structural integrity of the dorsal vessel and related cell types. Exploring which cells contribute and regulate ECM deposition at the dorsal vessel will shed light on novel cellular functions and ECM organization.

1.4 Dorsal vessel associating hemocytes

Hemocytes serve as *Drosophila*'s white blood cells. During embryogenesis, the first hemocyte population is derived from the anterior mesoderm (Tepass et al 1994). That population later migrates from the anterior mesoderm, dispersing throughout the body cavity of the embryo and acting primarily as phagocytic cells (Rugendorff et al 1994; Rizki 1978).

During larval development, the lymph gland is the major organ responsible for hematopoiesis. A subset of sessile larval hemocytes cluster in islets under the dorsal ectoderm, often observed along the posterior region closely associating with the posterior heart chamber (Leitao and Sucena 2015). Throughout larval development, three types of

hemocyte classes are categorized based on their localization and morphology (Rizki and Rizki, 1980): plasmatocytes, lamellocytes, and crystal cells.

Plasmatocytes

Plasmatocytes (representing 90-95% of mature larval hemocytes) are the most abundant class of hemocytes and are localized to the lymph gland and sessile islets clustered under the dorsal ectoderm. Plasmatocytes are also found circulating throughout the body cavity (Tepass et al 1994; Holz et al 2003). Plasmatocytes are small and rounded phagocytic cells targeting apoptotic cells and microbial pathogens (Lavine and Strand 2002).

Lamellocytes

Lamellocytes, the second class of hemocytes, are large flat cells that seldom appear in the circulating hemolymph of healthy larva (Rizki and Rizki 1992). Lamellocytes are not observed during embryogenesis or adult development (Honti et al 2014). They are triggered as an immune response to encapsulate or neutralize foreign molecules (Kurucz et al 2003; Lavine and Strand 2002). Lamellocytes are thought to be differentiated from undifferentiated hemocytes (prohemocytes) in the lymph gland and only release into the body cavity upon immune system induction (Stofanko et al 2010).

Crystal cells

The last class of hemocytes, crystal cells, represent 5% of larval and embryonic hemocytes. Crystal cells are nonphagocytic cells. They are implicated in melanisation as a means of inducing cytotoxicity (Nappi and Ottaviani 2000; Bidla et al 2007).

Plasmatocytes and crystal cells make up the population of circulating hemocytes as well as the sessile hemocyte population.

At the onset of metamorphosis, the lymph gland releases large numbers of plasmatocytes; the plasmatocytes play a critical role in tissue remodelling by engulfing hystolizing larval tissue (Jung et al 2005; Holz et al 2003). In adults, a uniform population of plasmatocytes is observed (Holz et al 2003). Since the lymph gland undergoes degradation during metamorphosis, the adult population of hemocytes is thought to have persisted from lymph gland derived larval hemocytes and hemocytes differentiated during embryogenesis (Lanot et al 2001). In adults, no proliferation or differentiation of hemocytes is observed (Lanot et al 2001).

The role of hemocytes in immune response and phagocytosis has been extensively studied, however the role of hemocytes is not limited to phagocytosis. Hemocytes have been found to cluster around pericardial cells at the dorsal vessel in larva exposed to bacteria; as well they have been implicated in secreting dorsal vessel ECM proteins during larval development (Cevic 2016). The potential recruitment of hemocytes to the dorsal vessel may be mediated through pericardial cell- hemocyte interaction.

1.5: Pericardial cells

Pericardial cells are non-contractile cells which flank the myogenic cardiomyocytes of the dorsal vessel. Pericardial cells maintain a close association with the dorsal vessel and the associated extracellular matrix environment throughout development.

Embryonic development

At the end of embryogenesis, approximately 100-120 pericardial cells flank the myogenic cardiomyocytes of the dorsal vessel (Das et al 2007). Embryonic pericardial cells are characterized and can be divided by their distinct genetic expression profiles: 1) Odd-skipped, 2) Even- skipped, and 3) Tinman expressing pericardial cells (Ward and Skeath, 2000; Lehmacher et al. 2012; Denholm and Skaer 2009).

Although differing with respect to their gene expression profiles, the ultrastructure of embryonic pericardial cells remains consistent (Mills and King 1965; Lehmacher et al 2012). Embryonic pericardial cells have several mitochondria, rough endoplasmic reticulum, and a single nucleus which takes up the majority of the cell volume (Mills and King 1965; Lehmacher et al 2012). This distinct ultrastructure might allow embryonic pericardial cells to synthesize and secrete extracellular matrix proteins.

As discussed earlier in this introductory chapter, embryonic pericardial cells play a critical role in ECM assembly at the dorsal vessel. Embryonic pericardial cells secrete Pericardin (Prc), and its precursor Lonely Heart (loh), at the dorsal vessel ECM (Chartier et al 2002; Drechsler et al 2013). In *prc* and *loh* mutant embryos, pericardial cells detach from cardioblasts, subsequently compromising dorsal vessel structural integrity

(Drechsler et al 2013). In addition, ECM protein Laminin appears to play a regulatory role in pericardial cell localization and dorsal vessel integrity during embryogenesis (Yarnitzky and Volk 1995). Throughout embryonic development, pericardial cells play a critical role in ECM matrix assembly and deposition.

Post- embryonic development

Post- embryonic pericardial cells share a similar ultrastructure and subcellular localization that persists through metamorphosis. The basement membrane appearing at the cell surface of pericardial cells acts as a barrier separating the highly invaginated labyrinthine channels at the outer cortex, and the external circulating hemolymph (Figure 1.4) (Mills and King 1965; Lehmacher et al 2012). Pericardial cell slit diaphragms are localized at the entrance of labyrinthine channels, thought to regulate the inflow of molecules from the external environment (Lehmacher et al 2012). Three distinct types of vacuoles, with specific subcellular localizations, exist among the highly concentrated labyrinthine channels (Lehmacher et al 2012). Towards the center of the cell, an abundance of rough-endoplasmic reticulum are stacked around the nucleus (Lehmacher et al 2012) (Figure 1.4).

Post- embryonic pericardial cells are often described as nephrocytes due to their indicative endocytic ultrastructure. Das and colleagues (2007) investigated the role of post- embryonic pericardial cells in regulating cardiac function. Upon removal of post-embryonic pericardial cells through induced cell death, cardiac function appeared normal, but a decreased lifespan and increased sensitivity to toxic stress was observed. Das and

colleagues (2007) suggested that pericardial cells sequester toxins from the circulating hemolymph, defining them as nephrocyte-like cells. A similar study conducted by Zhang and colleagues (2013), found that *Drosophila* pericardial cells can take up secreted fluorescent-GFP proteins from the hemolymph. A functional role of post- embryonic pericardial cells is their ability to sequester toxic and non-toxic molecules from the hemolymph. Although Das and colleagues claim the absence of post- embryonic pericardial cells does not disrupt cardiac function, they did not explore changes associated with ECM deposition and organization at the dorsal vessel.

In contrast to Das and colleagues (2007), Ivy and colleagues (2015) conducted preliminary studies that suggest cardiac abnormalities are observed in the absence of post-embryonic pericardial cells in *dKlf15* null mutant flies. Recently, the insect ortholog to the mammalian Krüppel-like factor (KLF15), *dKlf15*, was proven essential to pericardial cell differentiation and function in larval development (Ivy et al 2015). In *dKlf15* mutants, pericardial cells are unable to differentiate, leading to subsequent pericardial cell attrition during larval development (Ivy et al 2015). During L3 development, pericardial cells appear infrequently, often having what Ivy and colleagues describe as a distinctive "poly-cellular" phenotype (2015).

An additional study by Hartley and colleagues (2016) confirmed that loss of pericardial cells, or pericardial cell related function, results in abnormal cardiac function. Hartley and colleagues (2016) also identified high levels of circulating SPARC protein in the hemolymph of *dKlf15* mutant flies. SPARC, Secreted Protein Acidic and Rich in Cysteine, is a highly conserved collagen-binding ECM protein, expressed in *C. elegans*,

zebrafish, chicken, mammals and *Drosophila melanogaster* (Bradshaw 2009). SPARC is required for proper Type IV Collagen assembly and localization in the basement membrane of *Drosophila* embryos; however, the role of SPARC in later developmental stages remains largely unexplored (Martinek et al 2008). This data suggests that post-embryonic pericardial cells likely mediate non- cellular processes regulating cardiac function. Taken together, embryonic pericardial cells appear to be distinct from their post-embryonic counterparts with regards to gene expression profile and subcellular organization. Because of this, pericardial cells have been implicated as strictly nephrocyte-like cells, no longer influencing dorsal vessel function or structural integrity after embryogenesis. Recently, studies have emerged showing evidence that post-embryonic pericardial cells regulate dorsal vessel function and influence ECM deposition at the dorsal vessel beyond embryogenesis. This thesis adds to this growing body of literature.

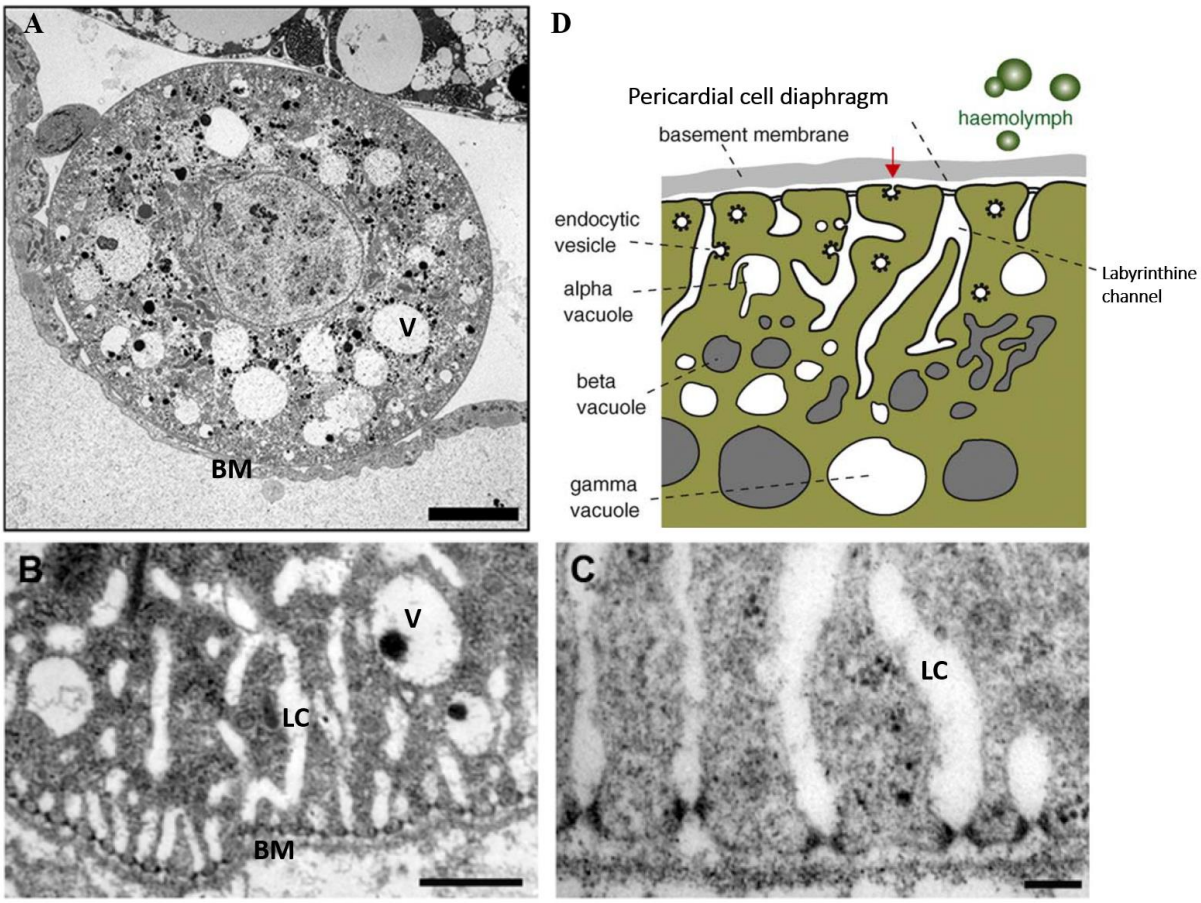


Figure 1.4 Ultrastructure of post-embryonic pericardial cells. (A,B,C) Electron micrographs show ultrastructure of post-embryonic pericardial cell. V: Vacuole, BM: basement membrane, LC: labyrinthine channel (D) Animated representation of post-embryonic pericardial cell. Basement membrane (outer grey layer), pericardial cell slit diaphragm present at labyrinthine channel opening. Vacuoles with specific subcellular localization (alpha, beta and gamma). Adapted and modified from: (A) Ivy et al 2015 (B,C) Zhang et al 2013 (D) Denholm and Skaer (2009).

1.6: Project Outline and Objectives

Objective 1: Characterizing the association between pericardial cells and extracellular matrix proteins at early 3rd instar larva, late 3rd instar larva and early adult stages of development.

1.1 Use pericardial cell specific promoter (DotGAL4 driver) to drive red fluorescent protein expression (under UAS control) to visualize pericardial cells and determine early and late stages of larval development under consistent conditions.

1.2 Using transgenic line and developmental stages from objective 1.1, early 3rd instar larva, late 3rd instar larva and adults 5 days post- eclosion were dissected and labeled with antibodies specific to extracellular matrix proteins of interest in order to examine wildtype localization and expression.

1.3 Using dissections from 1.2, confocal images at identical settings were taken for each experimental group, in order to examine and quantify extracellular matrix protein levels and dorsal vessel growth under wild type conditions.

Hypothesis 1: Throughout larval and early adult development, pericardial cells contribute to extracellular matrix protein levels at the dorsal vessel

2.1 Impair pericardial cells using pericardial cell- specific promoter in order to knockdown extracellular matrix proteins of interest using a UAS-line for LanA RNAi and Vkg-GFP;UAS-GFP RNAi.

2.2 Late 3rd instar larva dissected alongside two control groups, in order assess if pericardial cells are responsible for production of LamininA and/or Type IV collagen associated with the dorsal vessel ECM.

2.3 Using dissections from 2.2, confocal images at identical settings were taken for experimental and control groups in order to quantify and compare the total fluorescence signal between groups.

Hypothesis 2: Extracellular matrix protein levels and localization is impaired in the absence of pericardial cells at late 3rd instar larva and adults 5 days post- eclosion.

3.1 In *klf15* (Kidney Kruppel-like factor) mutant background, pericardial cells degenerate at 3rd instar larval stage of development and are completely absent in adults. These mutants were dissected at late 3rd instar and adults 5 days post- eclosion and immunolabeled with αβPS to ensure pericardial cell degradation and validity of the mutant line.

3.2 In a *klf15* mutant background, late 3rd instar larva and adults 5 days post eclosion were dissected and labeled for extracellular matrix proteins of interest in order to visualize extracellular matrix protein levels and localization in the absence of pericardial cells.

3.3 Using dissections from 3.1, confocal images were taken at identical setting for each experimental group and the total fluorescence signal was quantified to examine the change in extracellular matrix protein levels and localization in the absence of pericardial cells.

3.4 In the absence of pericardial cells I wanted to examine if there was response from other cell types implicated in ECM remodelling at the dorsal vessel. Transgenic line: KLF15; DotGAL4,hmldsRED; UAS-live-actinGFP created through genetic crosses, to examine the recruitment of hemocytes to areas of pericardial cell degradation and potential phagocytosis of degrading cells.

2.0 Chapter Two: Methods

Drosophila stocks: *w;daGal4* (Bloomington 55851), UAS-*lanA* RNAi (lamininA, VDRC 18573), *Hml-dsRed* (Makhijani et al 2011), UAS *GFP* RNAi (Bloomington 9330), UAS CD8 RFP (Bloomington 27399), vkg^{cc0079}, *yw* (Bloomington 6598), UAS LifeAct- GFP (Bloomington 35544), *dotGal4* (Bloomington 26982) is used to drive expression to pericardial cells of the dorsal vessel, *klf15* (Bloomington 18979), line to knock down GFP generated by Duygu Cevic using vkg^{cc0079} *(vkg; UAS GFP RNAi). dotGal4,* vkg^{cc0079}*/Cy.O;UAS-CD8RFP/UAS-CD8RFP* I generated by recombination to visualize Type IV Collagen and fluorescing pericardial cells within the same stable stock, I generated *Klf15*/y;*dot*GAL4/*HmldsRed*;UAS-LivActinGFP/+ was created by me to visualize pericardial cell degradation in a *klf15* mutant background.

Larval Dissections: Larval dissections were done one at a time according to the protocol adapted from Vogler and Ocorr 2009. Larva were immobilized on dissection plate by pinning the anterior and posterior body dorsal side down on the plate using magnetic pins. Each dissection was done alongside its respective control to maintain consistency. Larva were dissected for no longer than 20 minutes.

Dissecting and imaging adults: Adult females were dissected 5 days post-eclosion, according to the protocol adapted from Vogler and Ocorr 2009 in order to visualize the dorsal vessel. Vaseline was used to adhere the fly wings to the surface of the dissection plate. Approximately four dissections were done on the same plate at a time for no longer than 20 minutes. Dissections were then fixed on the dissection plate in 4%

paraformaldehyde/PBS solution for 5 minutes before being transferred to 12 well plate for immunolabelling. Following the immunolabelling protocol described for larvae, dissections were placed at 4 degrees Celsius overnight in 50% glycerol/PBS solution before being mounted on a slide in 70% glycerol/PBS solution. It should be noted that all experimental dissections were done alongside at least two controls for each genotype each session.

Immunolabeling: Labeling protocol was done at room temperature following the established procedure used by Bogatan et al 2015 and Alayari et al 2009. Following dissection, tissues were placed on a low speed belly-dancer shaker, fixing in 1mL of 4% paraformaldehyde/PBS for 20 minutes in a 12 well culture plate. After fixation, samples were washed in 150 ul of PBT for 10 minutes, changing the solution 3 times; with a maximum of 10 dissections were in each well at a time. Dissections were then transferred to a 96 well plate containing, 150ul of PBT and 5 ul of primary monoclonal antibody overnight at 4 degrees Celsius with gentle shaking. The following morning, dissections were washed for 10 minutes in PBT, changing the solution 3 times. Following the 10 minute PBT wash, dissections were transferred to a new well within the 96 welled plate, containing 150ul of PBT solution and 1ul of secondary antibody on shaker for one hour. If phalloidin was used, 1ul of phalloidin was also placed in the 150 ul PBT solution along with the 1ul of secondary antibody for 1 hour. Dissections were then transferred to a 12 well plate and then washed for 10 minutes in PBT solution, changing the solution 3 times. Following the final PBT wash, dissections were placed in a new well with 1xPBS for 10 minutes, then placed in 50% glycerol/PBS solution at 4 degrees Celsius overnight (not on

shaker). When staging dissections for imaging, dissections were mounted in 70% glycerol/PBS solution.

Antibodies used: αβPS (Integrin, DF.6g11- DSHB, monoclonal primary antibody) primarily to label pericardial cells in Figures: 3.5, 3.6 ,3.7. αLanA (LamininA, monoclonal primary antibody, Vanderploeg 2014) and αPrc (Pericardin EC11 anti-Pericardin DSHB). Secondary Alexa 647 anti-mouse (1:200) and Alexa 543 conjugated phalloidin (1:200) (LifeTechnologies, Burlington ON, Canada)

Confocal imaging: All images were taken with Leica SP5 confocal microscope. Ventral Z- stacks were taken 1.3um apart and projected using the 3D-Projection function on the Leica software. All dissections were imaged at identical settings within each experiment to maintain consistency and allow statistical comparisons of fluorescence data.

Larval collections and determining early and late L3 developmental stage:
Approximately 150 flies were put in a fly house (inverted beaker with airholes over a 60 mm apple juice agar plate with a streak of yeast paste down the midline). The flies were able to mate and lay eggs for approximately 2 days flipping the house to a new apple juice agar plate each day. On the third day, the house was flipped every 2 hours, and each plate was collected and stored at 25 degrees Celsius. Each plate was examined 24 later and between 20-30 first instar larva were collected upon hatching within a 30 minute time period and then subsequently transferred to a yeast plate. 48 hours after first instar collections, larva was assessed for molting by visualizing spiracle branching. Larvae were inspected every 2 hours until molting was visualized in order to determine early L3 stage. 48 hours after L3 molt, plates were inspected for early pupae to determine late L3 larval

stage. Late L3 dissections began when the first larva appeared to enter prepupal development until all larva became pupa. For each genotype dissected, early L3 and late L3 developmental stages were determined and recorded. Mahan Ghodrati helped with virgin collections, fly house maintenance and first instar collections.

Fluorescence Quantification:

Following confocal imaging, 1.3 µm z-sections were blurred, converted into TIFF files, and imported into ImageJ for analysis. Z-sections were cropped and displayed as 2D maximum projection using ImageJ software so that only the ventral portion of the heart was measured for fluorescence quantification. A consistent region of interest at abdominal segment A5 was outlined for each dissection. More specifically, a rectangle was made between two adjacent pericardial cells and their contralateral counterparts so that the fluorescence quantified was restricted to the dorsal vessel and did not overlap with adjacent cells. The "integrated density" value from the ImageJ software, is interpreted as the total amount of fluorescence signal emanating from a selected region of interest (ROI). The total amount of fluorescence signal is proportional to the amount of protein in that segment. Therefore, I could assess the total amount of protein within the ventral portion of abdominal segment A5 of the dorsal vessel at different stages in development. Measurements made at each ROI were repeated three times to reduce sampling error. The total amount of fluorescence signal was corrected for background fluorescence. The following formula was used to account for the background fluorescence signal: Corrected total fluorescence (CTCF) = integrated density – (area of ROI× mean fluorescence of background readings). An F-test followed by a two-tailed T-test was used

to compare the total fluorescence signal among experimental groups. This is an established method for assessing total amount of protein localized to a specific region of interest (McCloy et al 2014; Sonam et al 2016).

Abdominal segment A5 Surface area measurements:
The surface area of abdominal segment A5 was measured using ImageJ Software in combination with Microsoft excel. Following confocal imaging, 1.3 µm z-sections were converted into TIFF files, and imported into ImageJ. Each dissection was calibrated for micrometer measurements and cropped at the A5 segment boundaries determined by its location between bordering alary muscles. A line was used to measure the length of the A5 segment in micrometers. The cropped z-stack was then re-sliced to visualize virtual cross-sectional image of the z-stack. The diameter was measured using the line tool in ImageJ and the circumference of the tube was then calculated in excel. In combination with the length of the segment and the circumference of the dorsal vessel at segment A5, the surface area was quantified. Measurements made corresponding to segment A5 length and cross-sectional diameter were repeated three times to reduce sampling error.

Calculated Fluorescence density:
To assess how protein deposition adapts in accordance with changes in size, I set out to calculate the total fluorescence signal per unit area at abdominal segment A5 of the dorsal vessel. This calculation represents the density of protein per μm^2. First, I extrapolated the total fluorescence signal calculated for the ventral portion of abdominal segment A5 to account for the whole circumference of the heart. Second, I divided the total calculated fluorescence signal by the corresponding surface area of abdominal segment A5 per individual.

Stock maintenance: Flies were kept at room temperature on yeast based fly food and flipped approximately every 10 days. Ana Stosic maintained the stocks and prepared fly food and apple juice agar plates.

3.0: Chapter Three: Results

3.1: Characterizing Type-IV Collagen protein levels, localization, and dorsal vessel growth in larval and adult development.

To analyze post- embryonic ECM remodelling at the dorsal vessel, I examined three different ECM proteins: Type IV Collagen, LamininA and Pericardin. I analyzed their protein levels and localization at the dorsal vessel ECM through quantitative and qualitative techniques. Type IV Collagen is the most abundant ECM protein localizing to the basement membrane and interstitial ECM environment (Volk et al 2014).

Pericardial cells flanking the dorsal vessel are embedded in a Type IV Collagen ECM network in early L3, late L3, and adults 5 days post eclosion (Figure 3.1A (A,B,C)). Pericardial cells were visualized by a pericardial cell specific promoter (DotGAL4), driving RFP reporter expression to the pericardial cell membrane. Type IV Collagen was visualized by inserting a GFP exon to the endogenous Type IV Collagen gene, Viking (Vkg-GFP). Cross sectional views of the dorsal vessel (Figure 3.1A (D,E,F)) show Type IV Collagen expression around the circumference of the dorsal vessel, pericardial cells, as well as associating with connecting alary muscles. There was no Type IV Collagen detected within the pericardial cell cytoplasm in L3 and adults 5 days post eclosion.

The total fluorescence signal is proportional to the amount of protein within the ventral portion of abdominal segment A5 of the dorsal vessel. For the remainder of my thesis, the total fluorescence signal will be interpreted as the relative amount of protein deposited within the ventral portion of the dorsal vessel at abdominal segment A5 (see Methods). Type IV Collagen protein levels at the dorsal vessel appeared to increase between the

commencement and the end of L3 development (Figure 3.1A (A,B)).This observation was then tested through quantification of total Vkg-GFP fluorescence signal (see methods; Figure 3.1B (A)). Similar observations were validated between early L3 and adult developmental stages (Figure 3.1A (A,B), Figure 3.1B (A)).

The surface area of the heart chamber at abdominal segment A5 was quantified in early L3, late L3, and adults 5 days post eclosion to observe changes in dorsal vessel growth/shrinkage throughout development (Figure 3.1B (B)). Late L3 had a significantly larger surface area in comparison to early L3 and adults (Figure 3.1B (B)).

A consistent region of interest (ROI) in the dorsal vessel was analyzed in order examine structural and physiological changes occurring at heart chamber throughout development. Abdominal segment A5 represents this region of interest, and its boundaries remained consistently identifiable because of its border delimiting alary muscles (see Methods). I calculated the density of Type IV Collagen at abdominal segment A5 of the dorsal vessel as the calculated total Vkg-GFP fluorescence signal per μm^2 (see methods; Figure 3.1B (C)). This calculation was used for all the florescence quantification experiments conducted throughout this thesis. The density of Type IV Collagen per μm^2 accounts for changes in abdominal segment A5 of the dorsal vessel's surface area throughout development. These results suggest that the density of Type IV Collagen increases from larval to adult development (Figure 3.1C).

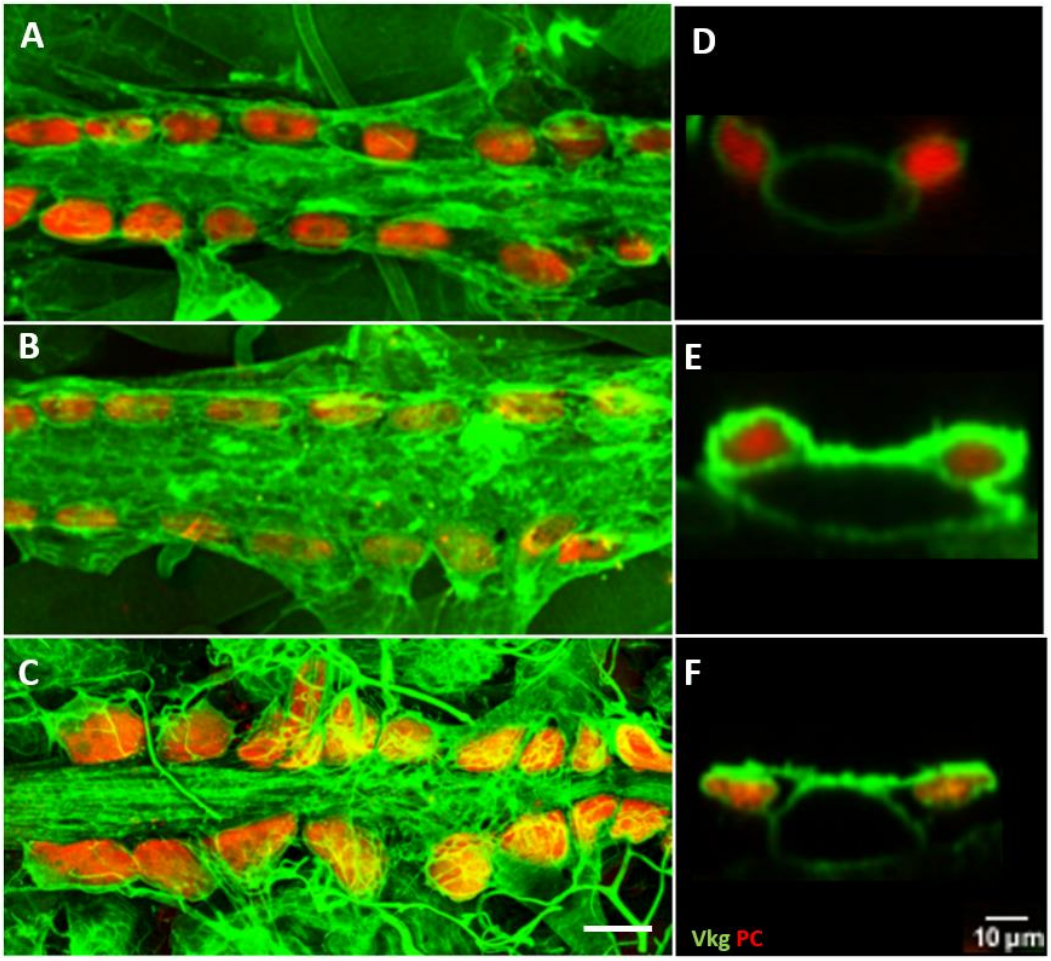


Figure 3.1A: Pericardial cells embedded in Type IV Collagen extracellular matrix network throughout late larval and adult developmental stages. (A,B,C) Ventral and cross sectional (D,E,F) view of *Drosophila* dorsal vessel at early L3, late L3 and adult stages of development. Pericardial cells (PC) flank the dorsal vessel and are embedded in the Type IV Collagen (VKG) ECM network. Type IV Collagen total fluorescence signal appeared to increase from early L3 to adult stages. Cross sectional views of A5 abdominal segment (D,E,F) show changes in dorsal vessel luminal diameter at different developmental stages. Genotype: *dot*Gal4,*vkg*-GFP/+;10XUAS-CD8RFP/+. *dot*Gal4 (*dorothy*-Gal4) is a pericardial cell specific driver used to target pericardial cell- specific reporter expression 10XUAS-CD8RFP (Red). *vkg*-GFP gene trap inserting a GFP exon to endogenous Type IV Collagen gene. PC: Pericardial cell, VKG: Type IV Collagen.

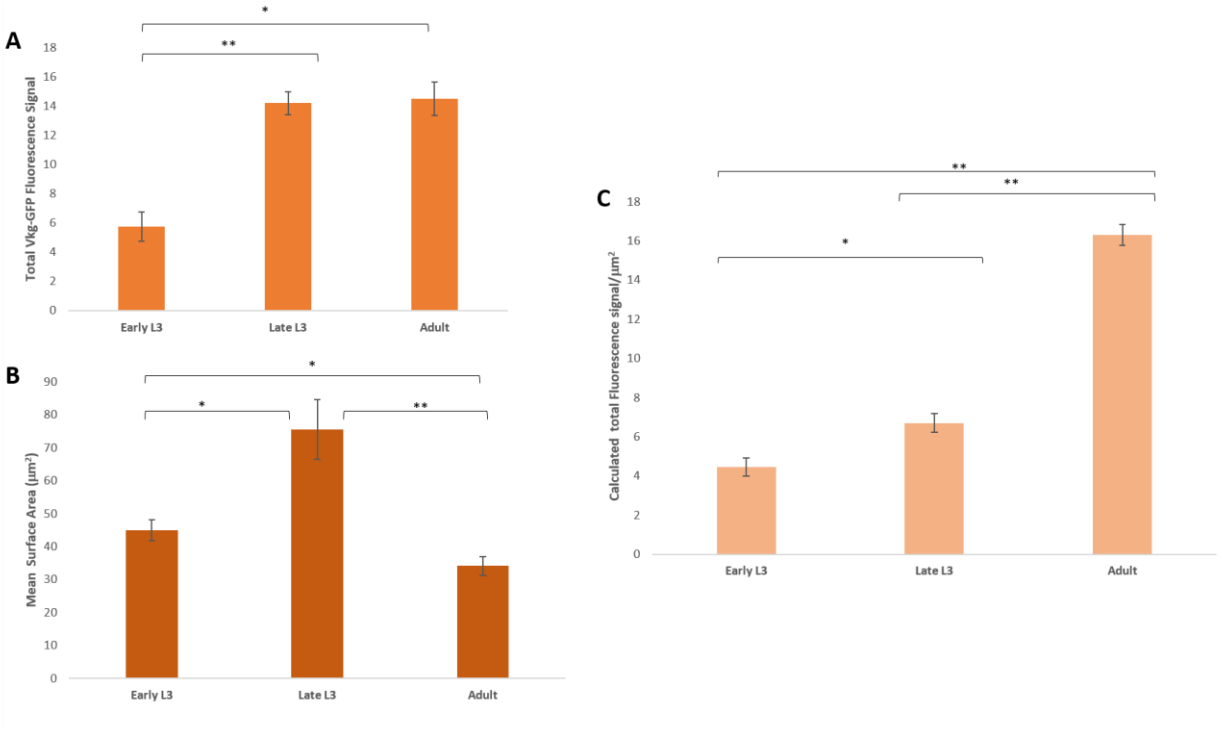


Figure 3.1B: Quantification of total Vkg-GFP fluorescence signal, mean surface area, and calculated density of Type IV Collagen/ µm^2 at abdominal segment A5 of *Drosophila* dorsal vessel. (A) Total Vkg-GFP fluorescence signal was quantified at larval and adult developmental stages. The total Vkg-GFP fluorescence significantly increased from early to late L3 stage and from early L3 to adulthood. (B) Quantification of surface area from early L3 to adulthood. The surface area significantly increased in size from early L3 to late L3, and significantly decreased in adults. (C) Calculated density of Type IV Collagen protein per µm^2 significantly increased from early to late L3 and from early L3 to adulthood. Error bars: SEM. Sample size: Early L3 (n=7), Late L3 (n=8), Adult (n=9). F-test, followed by two-tailed T-test, $P< 0.05$ (*), $P<0.001$(**).

3.2: Characterizing LamininA protein levels, localization, and dorsal vessel growth in larval and adult stages of development.

LamininA is strictly localized to the basement membrane of the ECM, and is critical to basement membrane assembly and composition in both human and *Drosophila* model systems (Yarnitzky and Volk 1995). Characterizing LamininA protein levels and localization provides insight into basement membrane remodelling throughout post-embryonic cardiac development.

Pericardial cells are embedded in LamininA ECM network throughout larval and adult development (Figure 3.2A (A,B,C). Cross sectional qualitative analysis did not detect LamininA protein within the cytoplasm of pericardial cells (Figure 3.2A (D,E,F)). Total LamininA fluorescence signal appeared to increase from larval to adult development (Figure 3.1A (A,B)). This observation was tested, noting a significant increase in total fluorescence signal between early L3 and late L3 developmental stages (Figure 3.1A (A,B), Figure 3.1B (A)) (Figure 3.1B (B)). These findings suggest that the total LamininA protein levels increased at abdominal segment A5 of the dorsal vessel from early to late L3 development, but did not significantly increase from late L3 to adult development.

Changes in surface area were observed and verified through quantitative analysis from larval to adult development (Figure 3.2B (B)). These findings were consistent with Figure3.1B(B) measurements. A significant increase LamininA protein per μm^2 was detected from L3 to adult development Figure 3.1B (C).

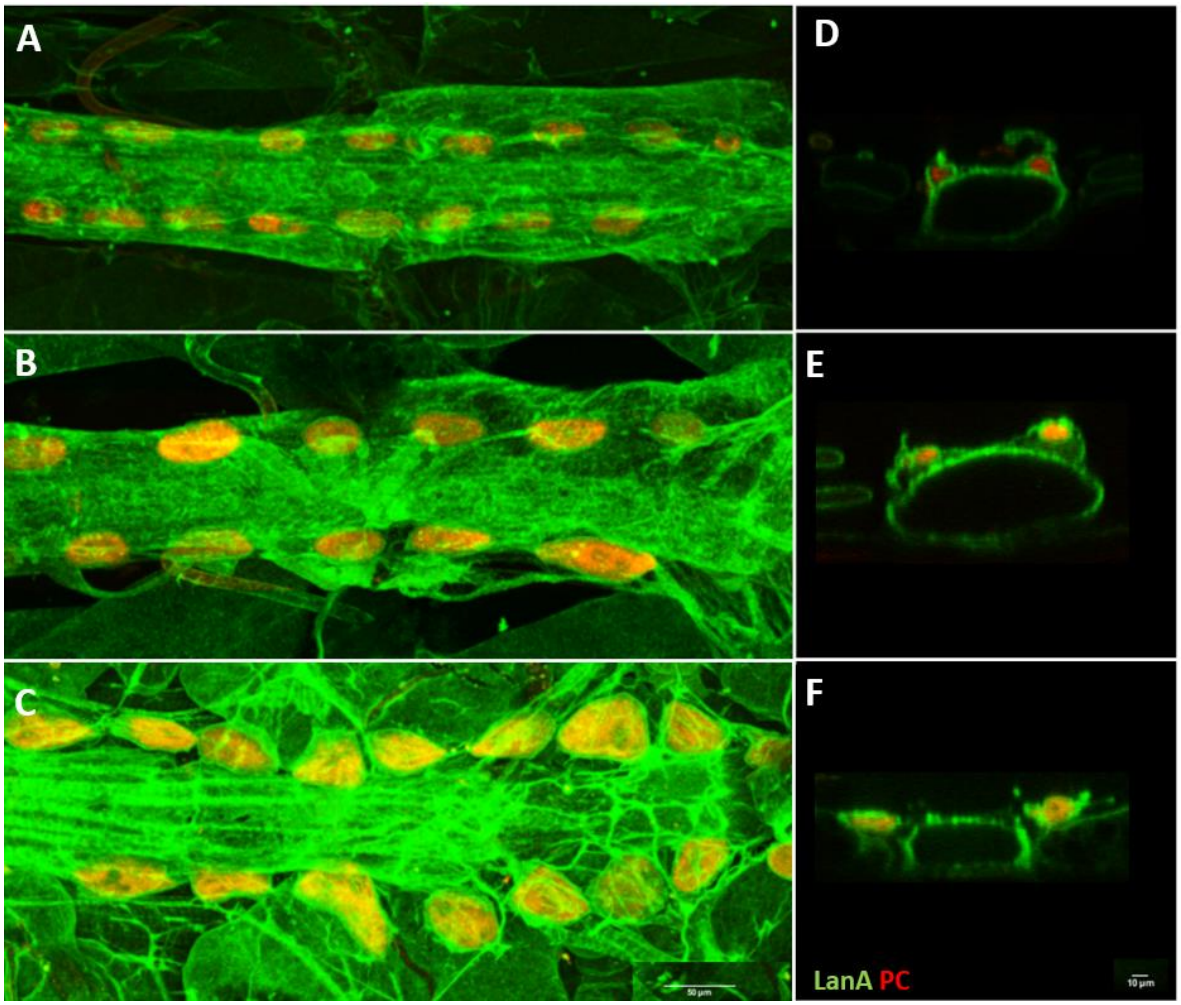


Figure 3.2A: Pericardial cells embedded in LamininA extracellular matrix network throughout late larval and adult developmental stages. (A,B,C) Ventral and cross sectional (D,E,F) view of *Drosophila* dorsal vessel at early L3, late L3 and adult stages of development. Pericardial cells (red) flank the dorsal vessel and are embedded in the LamininA (green) ECM network. αLanA fluorescence appeared consistent through larval development and elevated in adults. Cross sectional views (D,E,F) detected a change in dorsal vessel luminal diameter throughout development. Genotype: DotGal4/+;10XUAS-CD8RFP/+. DotGal4 (Dorothy-Gal4) is a pericardial cell specific driver used to target pericardial cell- specific reporter expression 10XUAS-CD8RFP (Red). αLanA antibody used to visualize LamininA protein. PC: Pericardial cell, LanA: LamininA.

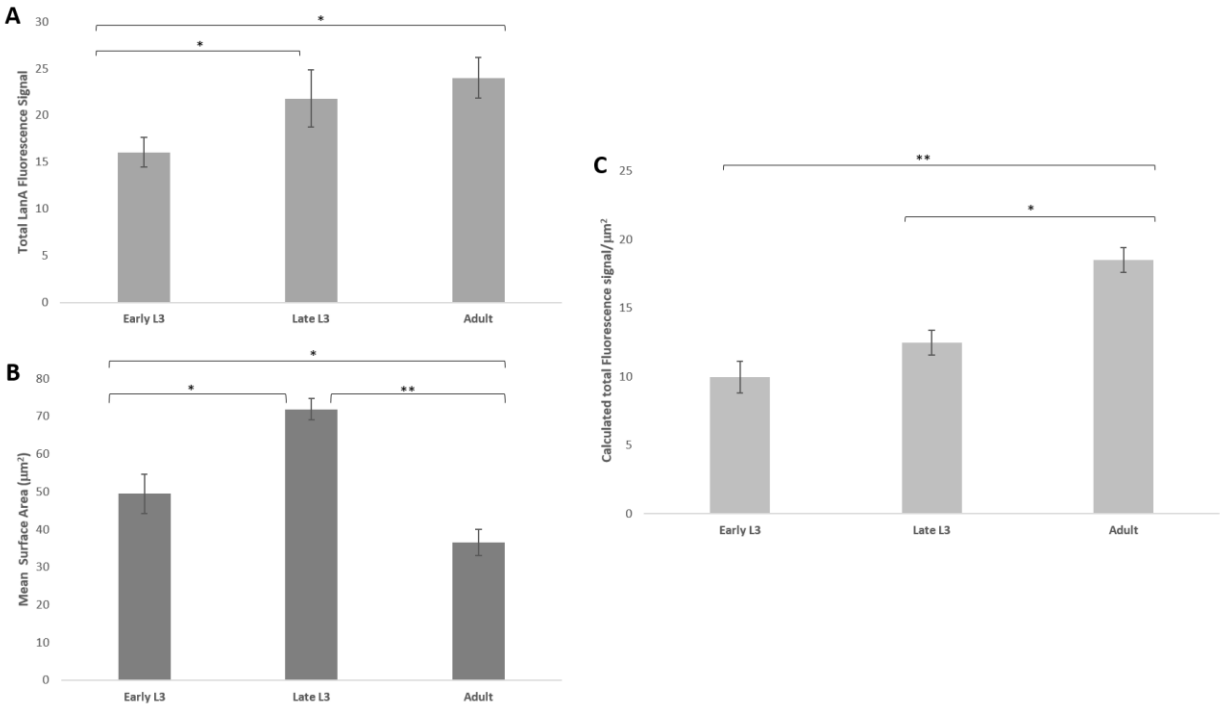


Figure 3.2B: Quantification of total LamininA fluorescence signal, mean surface area, and calculated density of LamininA per µm^2 at *Drosophila* dorsal vessel throughout development. (A) Quantification of total LamininA fluorescence signal in larval and adult development. The total fluorescence signal significantly increased throughout L3 development and did not significantly increase from late L3 to adult development. (B) Quantification of surface area increased significantly from early L3 to late L3, and significantly decreased in adulthood. These results were consistent with previous surface area measurements calculated in Figure 3.1B(B). (C) Calculated density of LamininA protein per µm^2 was quantified and did not change significantly throughout L3 development, but increased from L3 to adult developmental stages. Error bars: SEM. Sample size: Early L3 (n=8), Late L3 (n=7), Adult (n=8). F-test, followed by two-tailed T-test, $P< 0.05$ (*), $P<0.001$(**).

3.3: Characterizing Pericardin protein levels, localization, and dorsal vessel growth in larval and adult stages of development.

Pericardin, which is a dorsal vessel- specific ECM protein in *Drosophila*, localizes to the abluminal surface of the dorsal vessel, unique in its orientation and deposition (Chartier et al 2002). Pericardial cells flanking the dorsal vessel are embedded in Pericardin ECM network in early L3, late L3 and adult developmental stages (Figure 3.3A (A,B,C)). Cross sectional views of dorsal vessel (Figure 3.3A (D,E,F)), show αPrc fluorescence along circumference of the dorsal vessel, around pericardial cells and associating with connecting alary muscles. No Pericardin expression was detected within pericardial cell cytoplasm.

Total Pericardin fluorescence signal did not increase between larval to adult stages of development (Figure 3.3A (A,B)).This observation was then verified through quantification of total αPrc antibody fluorescence signal (Figure 3.3B (A)). These findings demonstrate that the total amount of Pericardin protein localized at abdominal segment A5 of the dorsal vessel remains consistent throughout post-embryonic development.

The surface area of the dorsal vessel at abdominal segment A5 was quantified in early L3, late L3 and adults to observe changes in structure throughout development (Figure 3.3B (B)). The surface area of the dorsal vessel at abdominal segment A5 was significantly larger in late L3 compared to early L3 and adults (Figure 3.3B (B)). A significant increase Pericardin protein per μm^2 was quantified in adult development compared to L3 development Figure 3.1B (C).

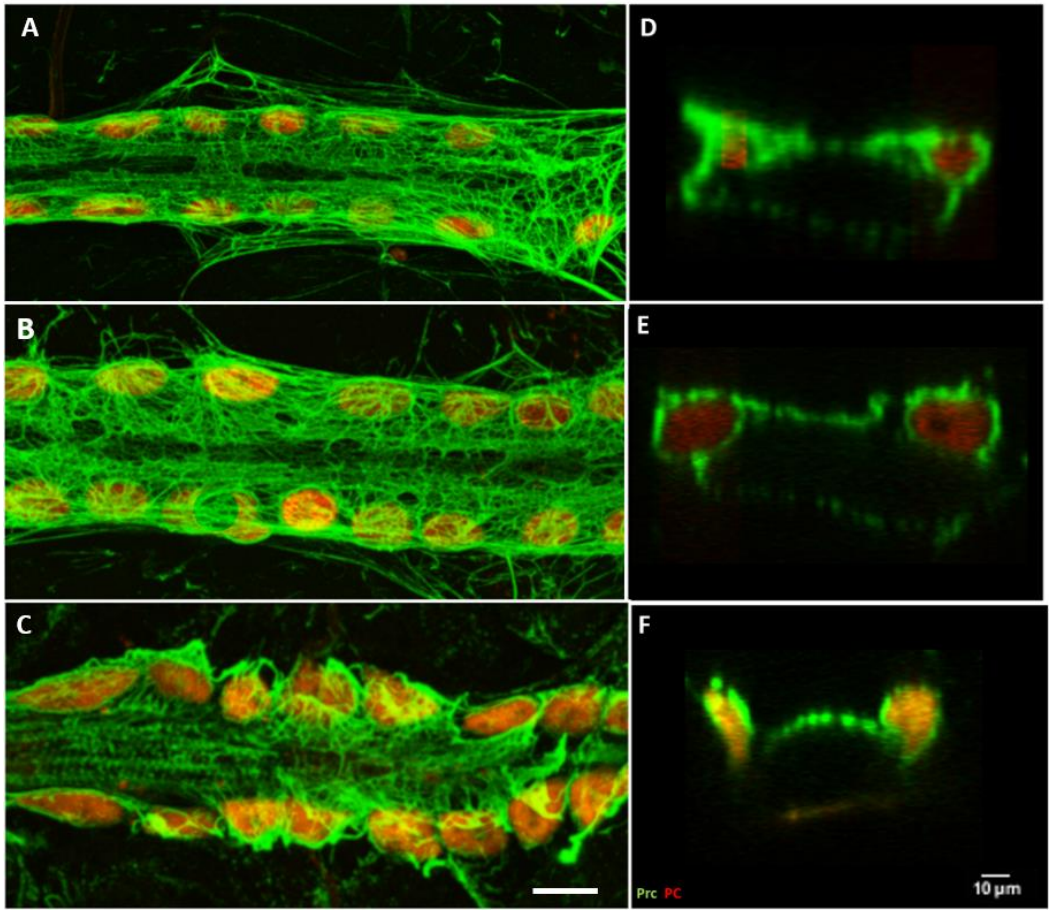


Figure 3.3A: Pericardial cells embedded in Pericardin extracellular matrix network throughout late larval and adult developmental stages. (A,B,C) Ventral and cross sectional (D,E,F) view of *Drosophila* dorsal vessel at early L3, late L3 and adult stages of development. Pericardial cells (red) flank the dorsal vessel and are embedded in the Pericardin (green) ECM network. Pericardin fluorescence signal appears consistent throughout developmental stages. Cross sectional views (D,E,F) detect changes in dorsal vessel luminal diameter at different developmental stages. Genotype: DotGal4/+;10XUAS-CD8RFP/+. DotGal4 (Dorothy-Gal4) is a pericardial cell specific driver used to target pericardial cell- specific reporter expression 10XUAS-CD8RFP (Red). αPrc antibody used to visualize Pericardin protein.

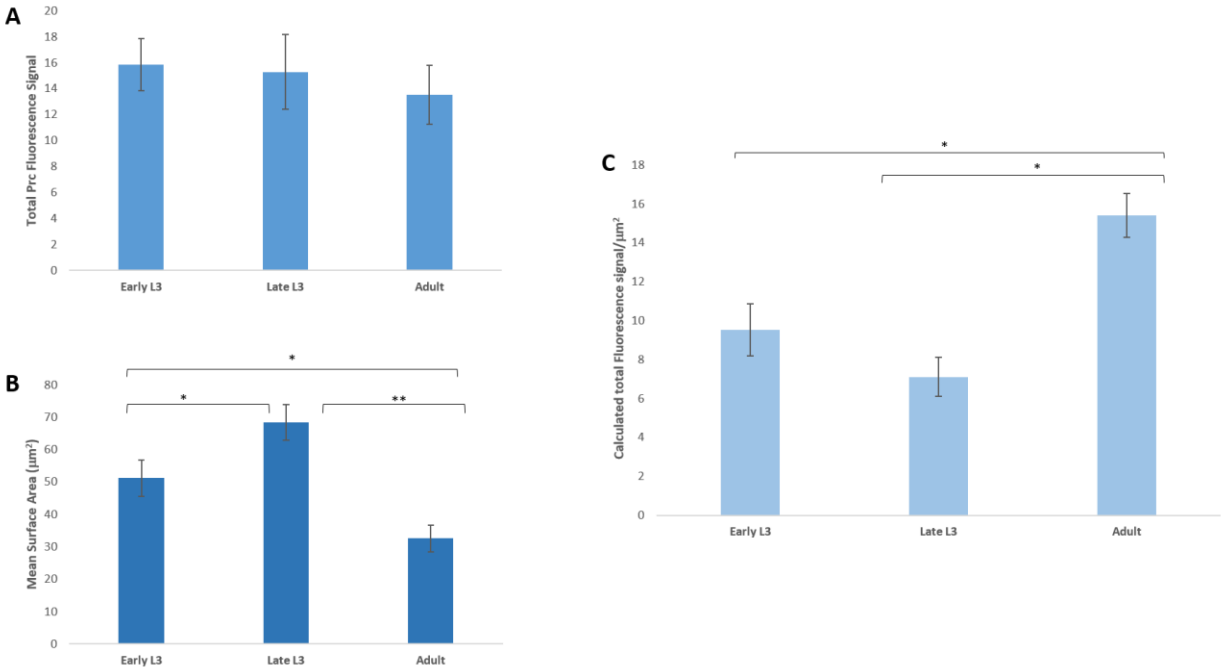


Figure 3.3B: Quantification of total Pericardin fluorescence signal, mean surface area, and calculated density of Pericardin per µm^2 at *Drosophila* dorsal vessel throughout development. (A) Quantification of total αPrc antibody fluorescence signal at abdominal segment A5 of the dorsal vessel in larval and adult developmental stages. The total αPrc fluorescence signal did not change significantly from L3 to adult development. (B) Quantification of the surface area of the dorsal vessel at abdominal segment A5 from early L3 to adulthood. The luminal diameter of the heart chamber increased significantly in sizes from early L3 to late L3, and significantly decreased in adulthood. (C) The calculated density of Pericardin per µm^2 was significantly greater in adult development compared to L3. Error bars: SEM. Sample size: Early L3 (n=5), Late L3 (n=7), Adult (n=8). F-test, followed by two-tailed T-test, P< 0.05 (*), P<0.001(**).

3.4: Pericardial cells do not contribute to extracellular matrix protein levels at the dorsal vessel

Identifying the cell types responsible for producing and secreting ECM proteins at the dorsal vessel throughout post-embryonic development, remains largely unexplored. Pericardial cells are implicated in dorsal vessel ECM production during embryogenesis, making them candidate cells for ECM production in later developmental stages. In order to test this, *vkg*-GFP; UAS-GFPRNAi and UAS-*lanA*RNAi were used to specifically knockdown GFP- tagged Type-IV Collagen production, and LamininA production respectively.

vkg-GFP;UAS-GFPRNAi/+ was used as a method control for GFP fluorescence detection in order to compare Type IV Collagen ECM protein levels at the dorsal vessel in late L3 development (Figure 3.4a(A)). GFP-tagged Type IV Collagen was specifically knocked down in pericardial cells, using *dot*Gal4, pericardial cell specific driver (Figure 3.4a(B)). *Daughterless (da)* Gal4 driver was used to ubiquitously express *vkg*-GFP;UAS-GFPRNAi acting as a manipulation control for RNAi depletion using a ubiquitous driver. *da*Gal4/ Vkg-GFP;UAS-GFPRNAi/+ successfully reduced levels of GFP-tagged Type-IV Collagen as seen in Figure 3.4a(C). Cross sectional images (Figure 3.4a(D,E,F)), show depletion of GFP- tagged Type IV Collagen around circumference of the dorsal vessel although quantification analysis revealed that transcript knockdown targeted to the pericardial cells does not significantly reduce Vkg-GFP levels (Figure 3.4a(G)). Quantification of surface area revealed no significant decrease in *vkg*-GFP;UAS-GFPRNAi/+ compared to experimental group (*dot*Gal4/Vkg-GFP; UAS-GFPRNAi/+).

UAS-*lanA*RNAi/+ was used as a method for GFP labeled antibody fluorescence detection in order to compare LamininA ECM protein levels in late L3 larva (Figure 3.4b(A)). LamininA was specifically knocked down in pericardial cells, using the pericardial cell specific driver, *dot*Gal4 (Figure 3.4b(B)). *Daughterless (da)* Gal4 driver was used to ubiquitously express UAS-*lanA*RNAi in order acting as a manipulation control for RNAi depletion using a ubiquitous driver. *da*Gal4/+;UAS-*lanA*RNAi was able to deplete LamininA (3.4b(C) but relatively less effectively compared to the ubiquitous depletion shown in Figure 3.4a(C). Cross sectional images (Figure 3.4b(D,E,F)) show reduction of LamininA protein through visualizing αLanA antibody fluorescence around circumference of the heart. This contradicts quantification analysis which revealed that the depletion was not significantly different from the control (Figure 3.4a(G)). Quantification of surface area detected no significant decrease in experimental and positive control groups. A comparable test of Pericardin production by the pericardial cells was not attempted because an effective RNAi transgene was not available. The available data suggests that pericardial cells do not play a direct role in post- embryonic ECM production or secretion at abdominal segment A5 of the dorsal vessel.

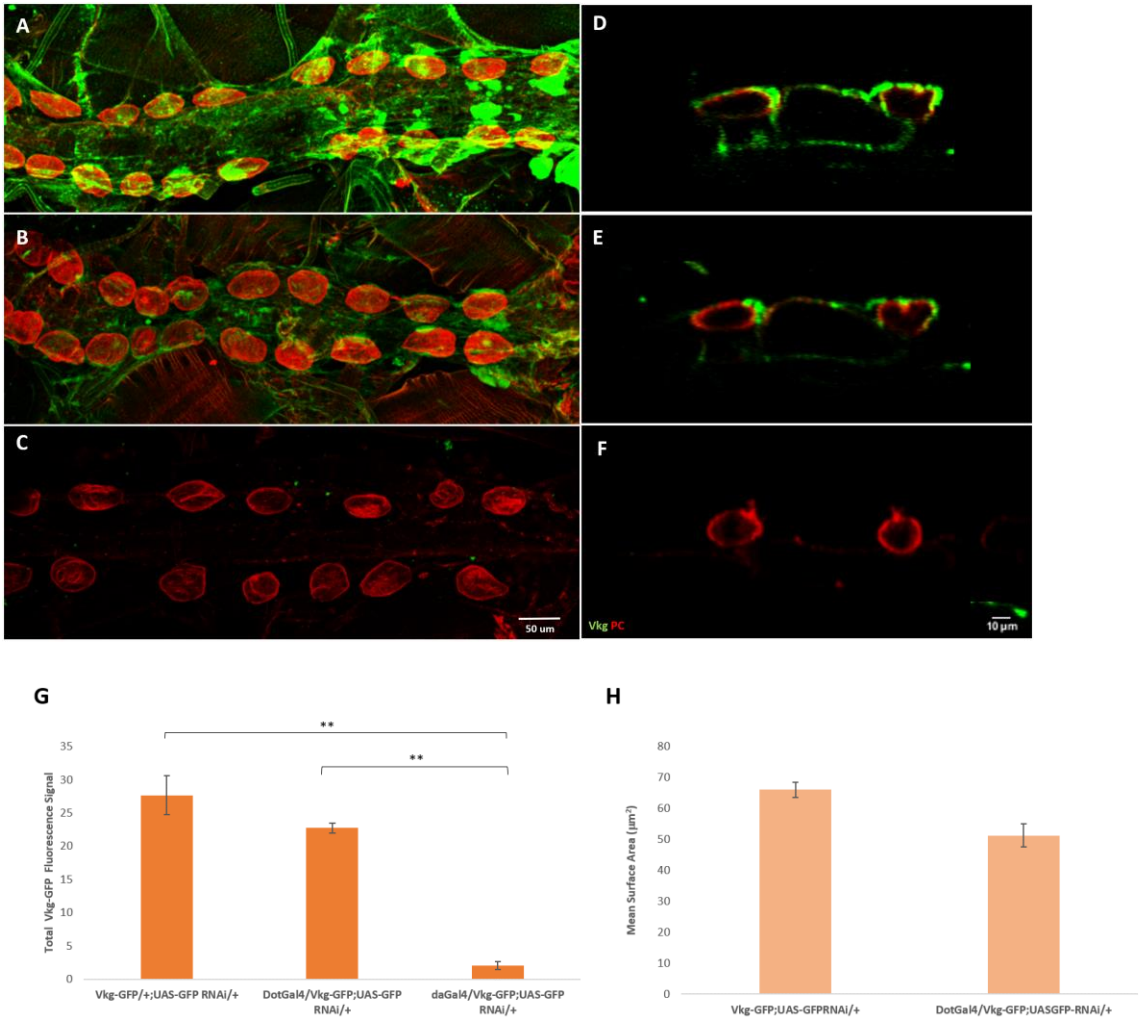


Figure 3.4a: **Pericardial cells likely do not play a direct role in secreting Type IV Collagen at the dorsal vessel during larval development.** (A) Method control for GFP fluorescence detection (*vkg*-GFP/+;UAS-GFPRNAi/+), late L3 dissection. Endogenous Type IV Collagen is visualized by *vkg*-GFP (green) at dorsal vessel. Pericardial cells (red) are visualized by αBPS labeling. (B) *dot*Gal4/*vkg*-GFP;UAS-GFPRNAi/+, late L3 dissection. (C) Manipulation control for RNAi depletion using ubiquitous driver (*daughterless*Gal4/*vkg*-GFP;UAS-GFPRNAi/+), late L3 dissection. (D,E,F) Cross sectional views do not detect Vkg-GFP fluorescence in pericardial cell cytoplasm (G) Total GFP fluorescence signal. (H) Mean surface area quantified at abdominal segment A5 of the dorsal vessel. SEM error bars. T-test, $P<0.05$ (**).

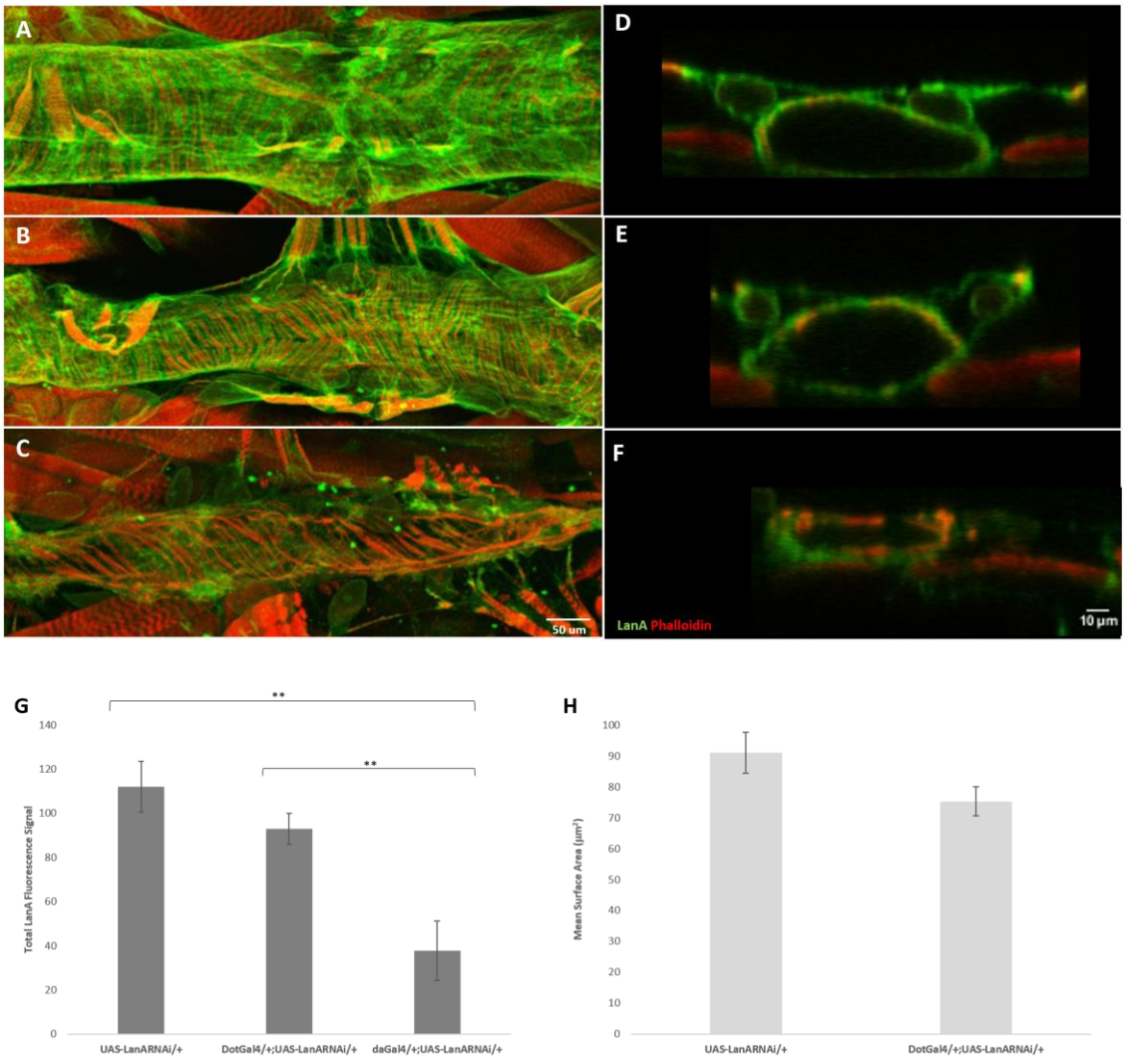


Figure 3.4b: Pericardial cells likely do not play a direct role in secreting LamininA protein at the dorsal vessel during larval development. (A) Method control αLanA fluorescence (UAS-*lanA*RNAi/+), late L3 dissection. αLanA visualized in green. Heart tube is visualized by labeling for F-actin using Alexa 543 conjugated Phalloidin. (B) *dot*Gal4/+;UAS-*lanA*RNAi/+ late L3 dissection. (C) Manipulation control for RNAi depletion using ubiquitous driver (*da*Gal4/+;UAS-LanARNAi/+), late L3 dissection. (D,E,F) Cross sectional views did not detect αLanA fluorescence within in pericardial cells (G) Total αLanA fluorescence signal. (H) Mean surface area quantified. SEM error bars. T-test, $P<0.05$ (**).

3.5: In a *KLF15* mutant background, Pericardial cells undergo degradation in late L3 development and are completely absent in adults.

In order to examine whether pericardial cells are required for proper ECM localization and deposition at the dorsal vessel, I first needed to determine a way to investigated ECM remodelling in the absence of pericardial cells. A *klf15* mutant background provided the solution, and the validity of the mutant strain was assessed. *klf15* is required for pericardial cell differentiation and functional endocytosis (Ivy et al 2015). In a *klf15* mutant background, pericardial cells are unable to differentiate into mature cells, resulting in pericardial cell degradation during late larval development, yielding adult flies completely absent of pericardial cells (Ivy et al 2015). To verify this, flies were dissected in a *klf15* mutant background at late L3 and adult developmental stages (Figure 3.5). Dorsal vessel musculature was visualized using and Alexa 543 conjugated Phalloidin (labeling F-actin), and B- Integrin antibody (αBPS) was used to visualize pericardial cells. Pericardial cell degradation was observed in *klf15* mutant L3 larva (arrow: Figure 3.5 and associated cross section). Other cells, labeled with αBPS appear to cluster around degrading pericardial cells in late L3. Pericardial cells were completely absent in adults in a *klf15* mutants background compared to corresponding control image.

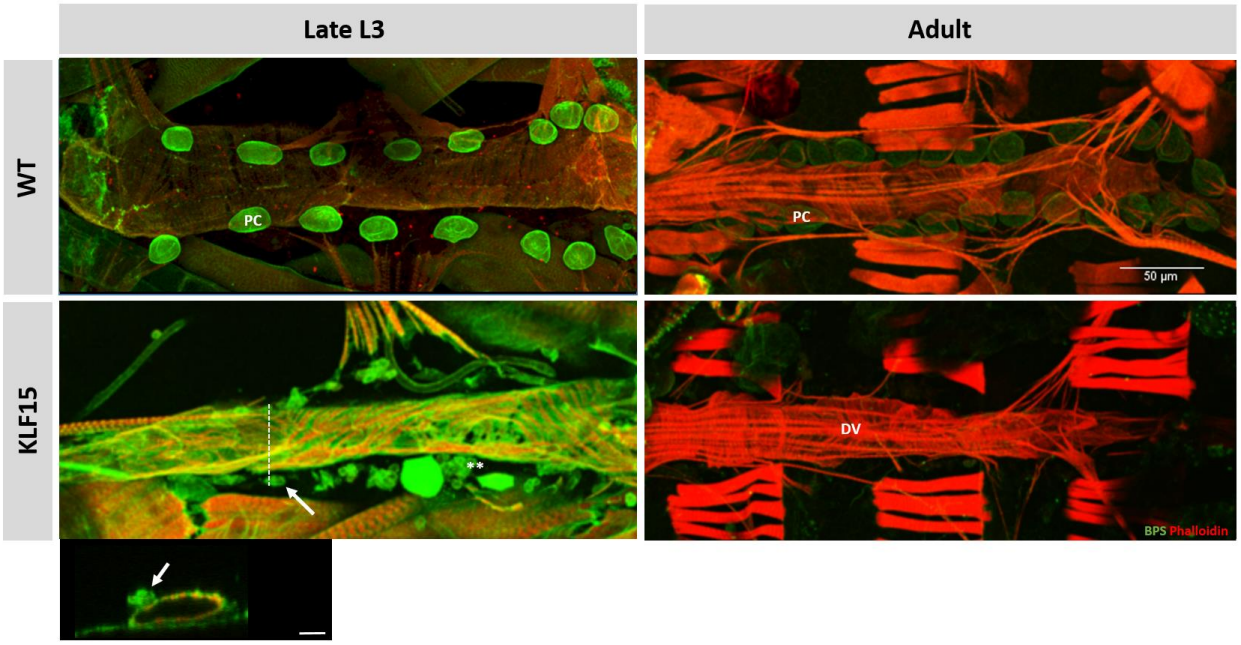


Figure 3.5: In a *klf15* mutant background, Pericardial cells undergo degradation in late L3 development and are completely absent in adults. Pericardial cells undergo degradation in *klf15* mutant background in late L3 which eclose into adults completely absent of pericardial cells. Monoclonal beta-Integrin antibody (αBPS; green) was used to visualize pericardial cells flanking the dorsal vessel. F-actin (Alexa 543 conjugated Phalloidin) was used to visualize dorsal vessel muscle fibers and visualize dorsal vessel stucture. Arrow: pericardial cell undergoing degradation. Scale bar: Cross section: 20um, Z-projection: 50 um.

3.6: In a *klf15* mutant background, hemocytes are recruited to engulf degrading pericardial cells at late L3 development.

Hemocyte clusters associate with the dorsal vessel, specifically around pericardial cells in late L3 development (Cevic 2016). Hemocytes are key players in immune response and are capable of engulfing large molecules as well as bacteria (Ghosh et al 2015). In a *klf15* mutant background, pericardial cells appear as "poly-cellular" clusters in late L3 development which were no longer present after metamorphosis (Ivy et al 2015). In Figure 3.5, *klf15* late L3 mutant larva, display a "poly-cellular" degradation phenotype and small cells were observed clustering around the associated area degrading area. I hypothesized that the small cells were hemocytes possibly recruited to engulf the pericardial cell debris. Hemocytes were then investigated as candidate cells to sites of pericardial cell degradation. *klf15*/y;*dot*GAL4/HmldsRed;UAS-LivActinGFP/+ line was created, dissected as late L3 and labeled with αBPS monoclonal antibody. Pericardial cell specific promoter, DotGAL4 was used to drive LifeActin-GFP to pericardial cells; and hemocyte specific fusion protein, HmldsRed, was used to visualize hemocytes. *klf15*/x: *dot*GAL4,HmldsRed/+;UAS-LiveActin-GFP/+ was used as a control method to confirm DotGAL4 specificity and observe wild-type hemocyte localization at late 3rd instar larva (Figure 3.6A). Cross sectional data of control (Figure 3.6A) verifies LifeActin-GFP localization to pericardial cells and there appeared to be no overlapping fluorescence signal for hemocytes (dsRed) and LifeActin-GFP. Figure 3.6B showed *klf15*/y;*dot*GAL4/HmldsRed;UAS-LivActinGFP/+ late L3, where pericardial cells undergo degradation in a *klf15* mutant background. The resolution was increased in Figure 3.6B by using a higher magnification, where hemocytes appear to cluster around

degrading pericardial cells (Figure 3.6C). HmldsRed and GFP fluorescence signal overlap was can be observed in both Z- projection and cross sectional views (Figure 3.6C,D). This evidence suggests that hemocytes are recruited to areas of pericardial cell degradation, and potentially engulf the debris in late L3 *klf15* mutant background.

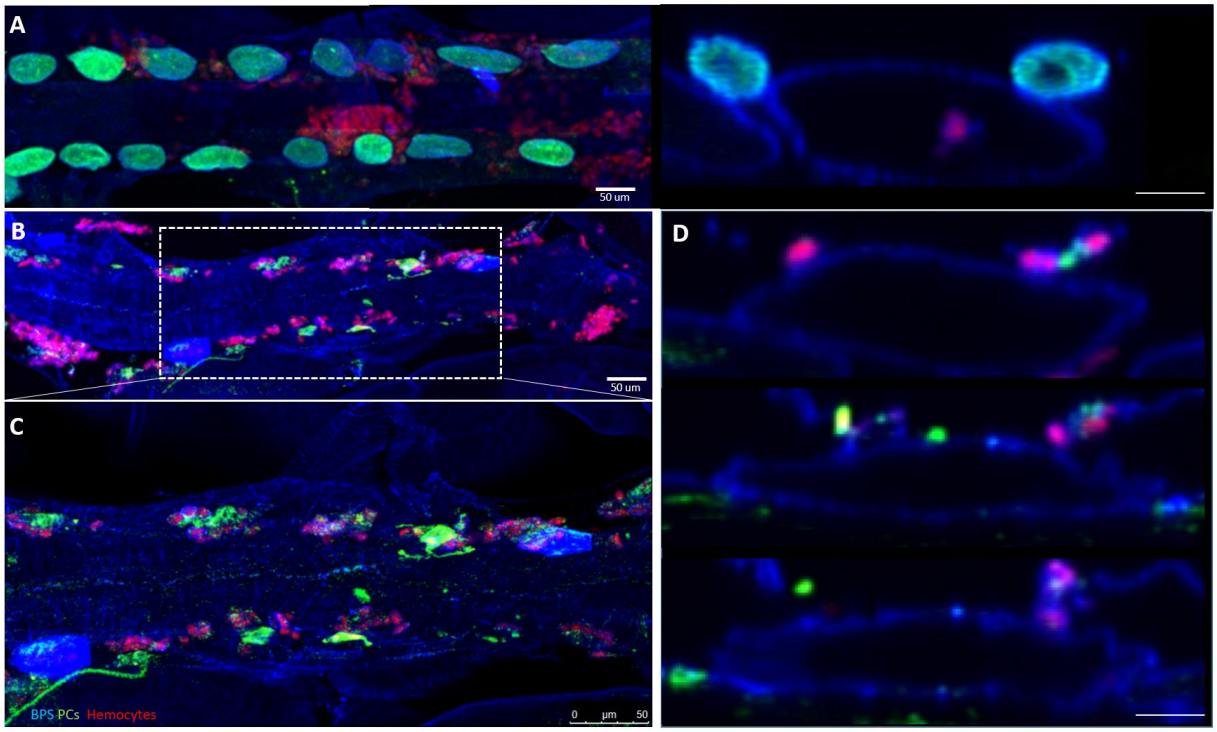


Figure 3.6: **In a *klf15* mutant background, hemocytes are recruited to degrading pericardial cells during late L3 development.** Late L3 dissected and imaged at confocal microscope. Dissections labeled with αBPS. (A) *klf15*/x: *dot*GAL4,hmldsRED/+;UAS-LifeActin-GFP/+ maximum projection of late L3, visualizing LifeActin-GFP driven to pericardial cells. Cross sectional view of 3.6A visualized LifeActin-GFP within pericardial cells, labeled with αBPS (Blue). Hemocytes are visualized in red. (B) *klf15*/y;*dot*GAL4,HmldsRed;UAS-LifeActinGFP maximum projection of late L3 visualizing hemocytes associating with degrading pericardial cells. (C) Enlarged projection of boxed segment in B. Hemocytes associate specifically at clusters containing LifeActin-GFP. (D) Cross section of late 3rd instar larva, examining hemocyte recruitment and pericardial cell degradation. Hemocytes recruited to areas where pericardial cells were degrading. Hemocytes (red) and LifeActin-GFP (green) fluorescence signal overlap is observed in cross sectional images. Ventral view of Z-stack maximum projection. Cross sectional images taken at abdominal segment A5. Cross sectional scale bars: 20um.

3.7: Extracellular matrix protein levels and localization is impaired in the absence of pericardial cells at late 3rd instar larva and adults.

Although pericardial cells do not appear to contribute to the dorsal vessel ECM network by directly producing and secreting ECM proteins after embryogenesis, the requirement of pericardial cells for proper dorsal vessel ECM deposition and localization has not yet been investigated.

ECM protein levels in late L3 and adults was observed by visualizing Type IV Collagen, LamininA and Pericardin protein expression in a *klf15* mutant background (3.7a). Adults in a *klf15* mutant background displayed substantially reduced total ECM fluorescence signal in comparison to late L3 (3.7a). Total fluorescence signal in Figure 3.7b assessed the amount of ECM protein localized to abdominal segment A5 of the dorsal vessel at both late L3 and adult developmental stages. Type IV Collagen, LamininA and Pericardin protein levels appeared significantly reduced in adult compared to late L3 in a *klf15* mutant background (Figure 3.7b). Cross sectional data showed degrading pericardial cells in late L3 and uniform ECM protein levels around dorsal vessel lumen and around pericardial cell pockets (Figure 3.7c). Two cross sectional snapshots of the adult dorsal vessel are shown in Figure 3.7c. Left adult cross sections show V4 valve cells and associating cellular cavities. There appeared to be Type IV Collagen and LamininA accumulation within these cavities at the luminal surface of the dorsal vessel, while Pericardin accumulation appears to be restricted to outer circumference of the dorsal vessel (Figure 3.7c). Images on the right show cross sectional images taken within abdominal segment A5 of dorsal vessel (Figure 3.7c). Type IV Collagen accumulation was detected at both the luminal and abluminal surface of the dorsal vessel at segment

A5, although higher magnification images would be required to validate this observation (Figure 3.7c). The surface area at abdominal segment A5 of the dorsal vessel was quantified for late L3 larva and adult in a *klf15* mutant background (Figure 3.7d). These results were compared directly to the calculated surface area data collected in Supplementary Figure 1. The surface area of the dorsal vessel in a of the *klf15* mutant background was significantly reduced compared to their WT counterparts (Figure 3.7d).

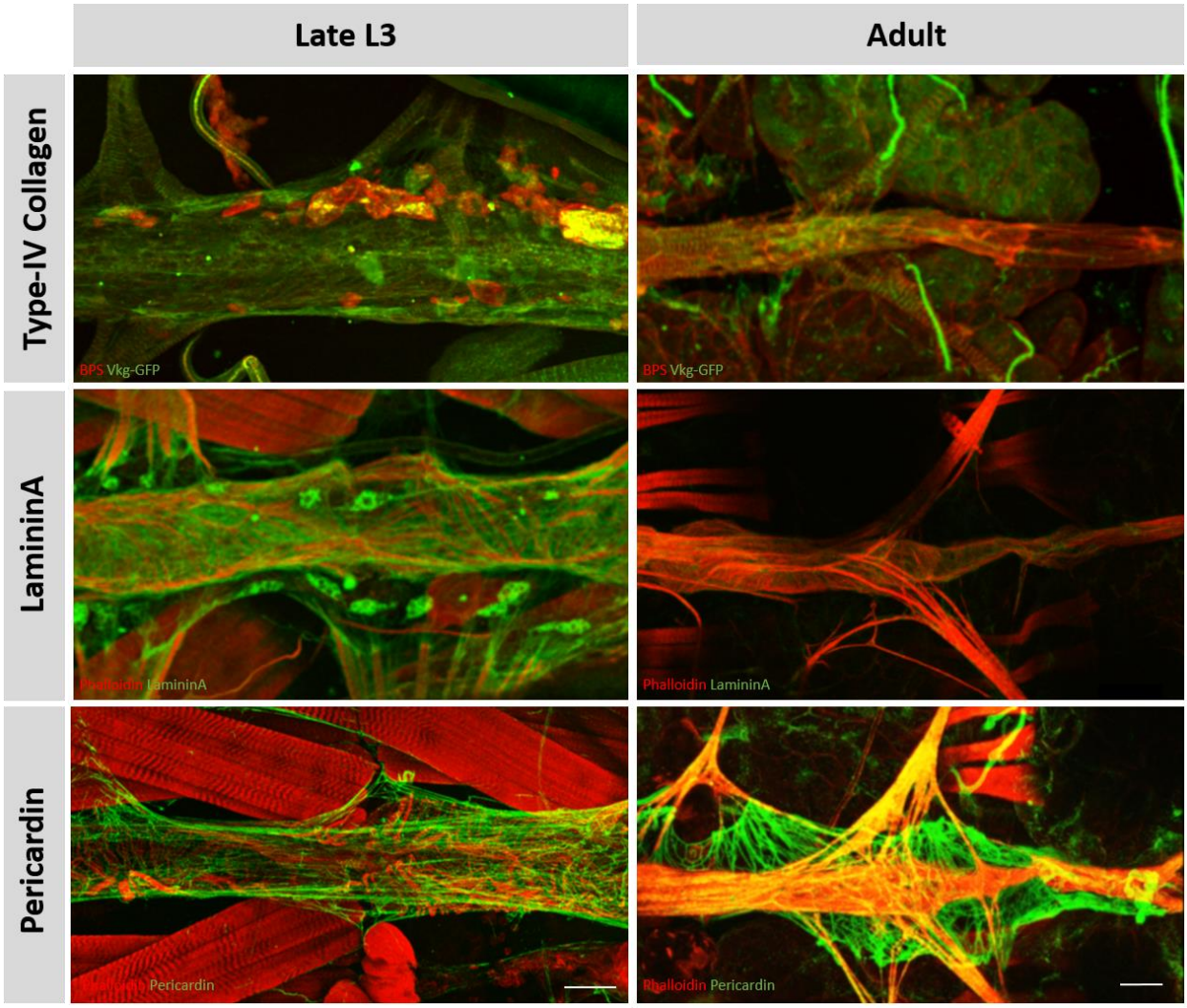


Figure 3.7a: ECM protein localization at the dorsal vessel in late L3 and adult *klf15* mutants. Type-IV Collagen, LamininA and Pericardin dorsal vessel ECM proteins were imaged in late L3 and adult *klf15* mutants. ECM proteins are visualized in green for all images. Scale bar: 50um. Alexa 543 conjugated Phalloidin was used to label F-actin (red) for LamininA and Pericardin. αBPS was used in Type IV Collagen experiments.

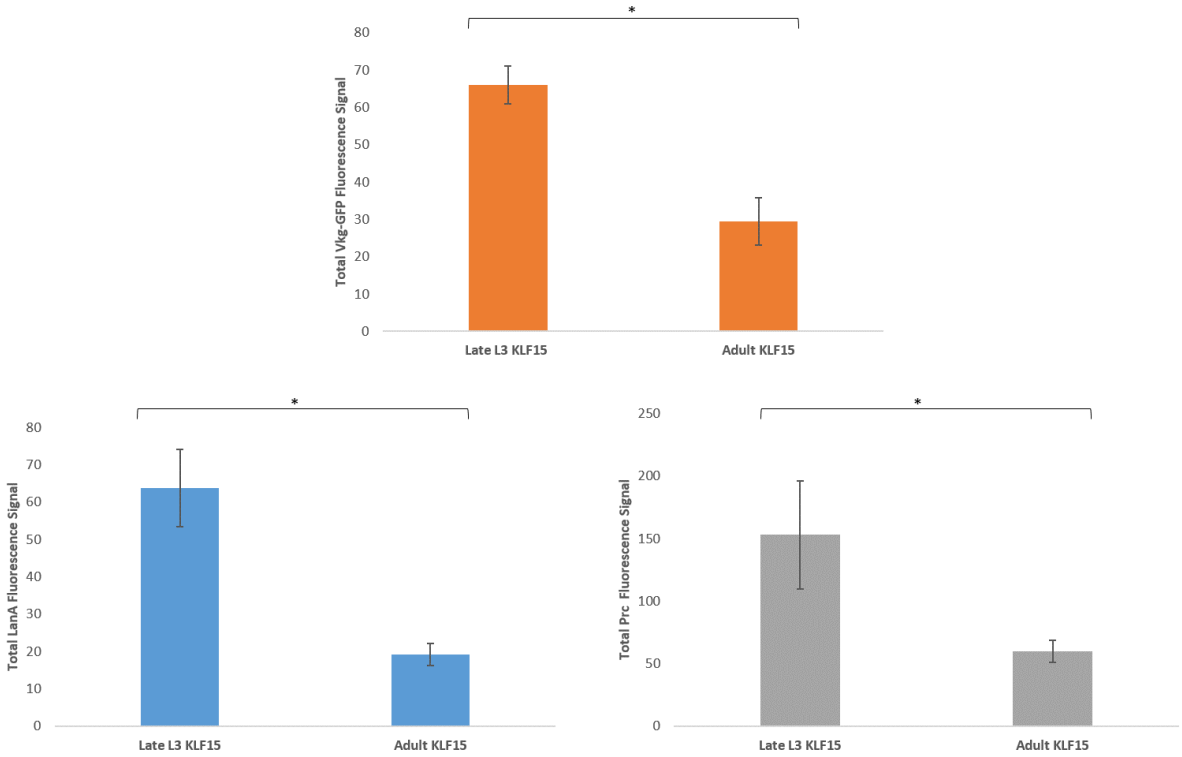


Figure 3.7b: Total ECM protein fluorescence signal in *klf15* mutant background at late L3 and adult developmental stages. In the absence of pericardial cells, total fluorescence signal corresponding to each ECM protein at abdominal segment A5 of the dorsal vessel declined significantly in adults when compared to late L3. SEM error bars. F-test followed by two- tailed T-test: $P<0.05$ (*).

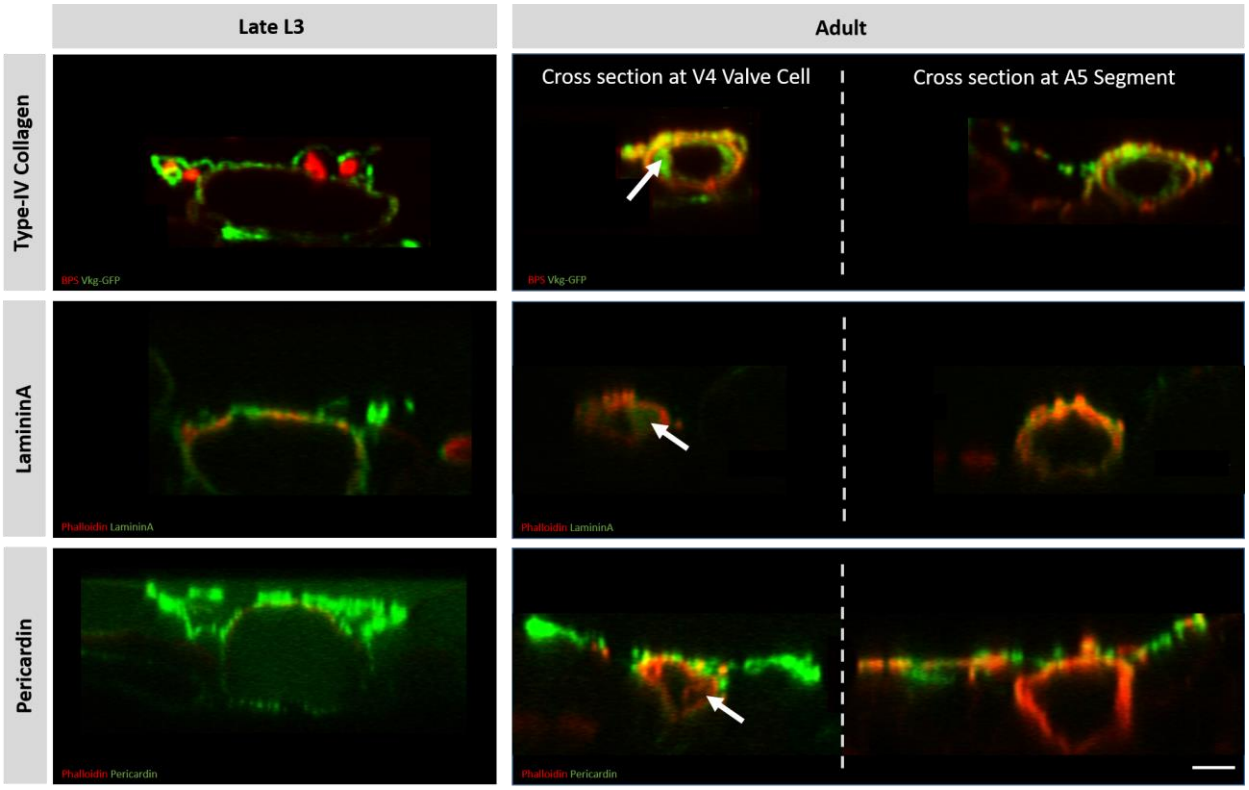


Figure 3.7c: Cross sectional views of late L3 and adults in a *klf15* mutant background, visualizing ECM protein localization at the dorsal vessel. Type IV Collagen, LamininA and Pericardin dorsal vessel ECM proteins were imaged in late L3 and adult *klf15* mutants. ECM proteins were visualized in green for all images. Arrow: Cellular cavities around V4 valve cells. Scale bar: 20um.

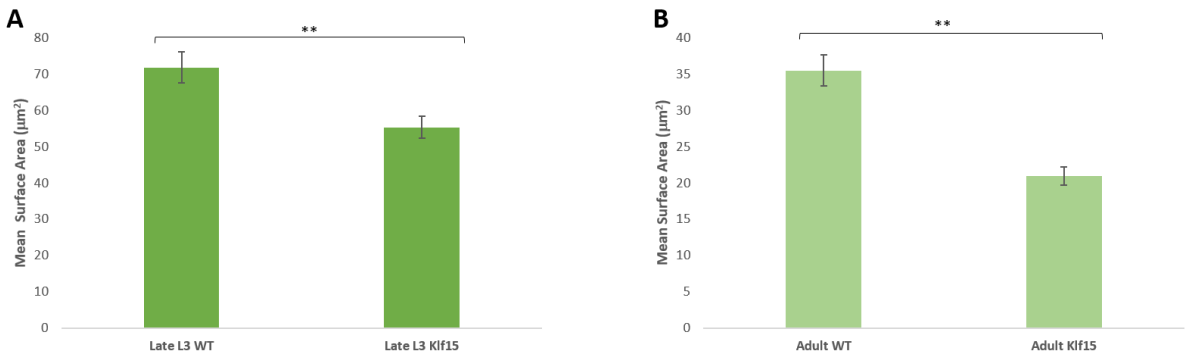


Figure 3.7d: Quantification of surface area at abdominal segment A5 of dorsal vessel in late L3 and adult *klf15* mutants. The surface area was significantly smaller in *klf15* mutant background at larval and adult stages of development compared to the control. SEM error bars. F-test followed by Two-Tailed T-test: Late L3, Adult, $P<0.001$ (**).

Chapter 4.0: Discussion and Future Directions:

The production crew is the backbone of a play. The crew provides support to the actors, and remodels the stage as the play unfolds. The extracellular matrix acts as the production crew adapting to growth and physiological changes occurring throughout cardiac development. The actors, the pericardial cells, are embedded in the extracellular matrix network and differentiate into nephrocyte- like cells that can regulate cardiac function through largely unexplored mechanisms. The interplay between actors and production crew is essential to a seamless performance. This relationship has been explored throughout this thesis and the specific implications that pericardial cells may have on extracellular matrix regulation and localization will be discussed in more detail within this section.

4.1: Pericardial cells remain embedded in the dynamic extracellular matrix environment of the dorsal vessel throughout development

This thesis investigates three extracellular matrix proteins: Type IV Collagen, LamininA, and Pericardin. The deposition and localization of these three proteins will be discussed below with respect to how the ECM is remodelled throughout post-embryonic development, and why I chose to focus on abdominal segment A5 of the dorsal vessel.

Type IV Collagen

Assessing Type IV Collagen expression can provide insight into ECM deposition and remodelling throughout morphological and physiological changes that occur during post-embryonic development. Type IV Collagen is the most predominant ECM protein localized to both the basement membrane and interstitial ECM environment surrounding the dorsal vessel and associated pericardial cells. Through immunohistochemistry techniques, coupled with confocal microscopy, I was able to visualize Type IV Collagen protein levels and localization around the dorsal vessel and pericardial cells from early L3 to adult developmental stages (Figure 3.1A). Type IV Collagen expression has been reported in cardiac tissue throughout early development; more specifically, *Drosophila* embryonic mRNA in situ- hybridization and enhancer trap experiments detect high Type IV Collagen expression in mesodermal derived tissues, including expression of alpha2 chain of Type IV Collagen in pericardial cells (Parco et al. 1986; Hollfelder et al. 2014). More specifically, while cross- sectional qualitative analysis does not detect Type IV Collagen protein within pericardial cells, Type IV Collagen is visualized around the pericardial cells and dorsal vessel circumference throughout L3 and adult development (Figure 3.1A(D,E,F)).

Pericardial cells appear tightly associated with the dorsal vessel throughout L3 development whereas this association appears to a lesser degree in adult cross sections (Figure 3.1A(D,E,F)). Perhaps this association between pericardial cells and the dorsal vessel is mediated by Type IV Collagen, anchoring the pericardial cells to the dorsal vessel to different degrees throughout post- embryonic development. It is widely accepted

that throughout development and differentiation, morphological changes are often accompanied by mechanical changes. For example, the vertebrate left ventricle of the heart develops increased collagen deposition/concentration with age, leading to increased cardiac stiffness and consequently less elasticity (Hyo-Bum Kwak 2013).

Qualitative analysis of dorsal vessel dissections shows the diameter of the dorsal vessel lumen increasing from early L3 to late L3 development, and this increase in size appears to accompany changes in Type IV Collagen-GFP tagged protein level (Figure 3.1A). To quantify this observation, the total fluorescence signal of Type IV Collagen-GFP was measured over a consistent region of interest (ROI) at the dorsal vessel cardiac chamber throughout post-embryonic development (Figure 3.1B(A)). The total fluorescence signal increased significantly from early L3 to late L3, then remained consistent from late L3 to adult development. This suggests that more Type IV Collagen is incorporated in the dorsal vessel ECM after early L3 (Figure 3.1B(A)). In conjunction with changes in total fluorescence signal, I also quantified changes in dorsal vessel surface area (Figure 3.1B(B)). Abdominal segment A5 of the dorsal vessel increases in size significantly from early L3 to late L3 development followed by a decrease in size after metamorphosis (Figure 3.1B(B)). To determine if Type IV Collagen protein levevls changes proportionally to dorsal vessel growth, I calculated the mean surface area of the abdominal segment A5 of the dorsal vessel throughout development (Figure 3.1B(C)). The changes in mean surface area appeared consistent throughout all wild-type dissections assessed in the first aim of my results, which are compiled in Supplementary Figure 1 of my appendix. This data, in combination with the total fluorescence signal,

was used to determine the calculated density of protein per um2 to examine accompanied changes in dorsal vessel surface area throughout development. Therefore, as the dorsal vessel undergoes structural changes throughout post-embryonic development, an increased density of Type IV Collagen protein appears at the dorsal vessel. The increase in Type IV Collagen protein at the dorsal vessel ECM is likely required to accommodate the dorsal vessel's change in surface area throughout larval development (Molina and Cripps 2001; Sellin et al. 2006). In mice, Type IV Collagen accumulation increases with age, correlating with an increased thickening of the cardiac basement membrane (Castro Brás, et al. 2014). To extend these findings, EM images of the dorsal vessel at specific developmental stages will allow us to measure BM thickness with age, and if so, suggest specific quantity and density of Type IV Collagen deposition with age.

LamininA

LamininA is restricted to the basement membrane of the ECM, playing an essential role in basement membrane assembly, composition, and ECM-cell adhesion in both humans and *Drosophila* systems (Yarnitzky and Volk 1995; Haag et al. 1999; Reim and Frasch 2010). During embryogenesis, LamininA is detected at the apical and basal cell surface of cardioblasts, and its deposition is localized between the cardioblast and pericardial cell surface (Yarnitzky and Volk 1995). I dissected and labeled the dorsal vessel of larva and adult flies for LamininA protein to visualize protein localization and quantify post-embryonic deposition. LamininA protein is seen surrounding pericardial cells and the dorsal vessel surface (Figure 3.2A), suggesting that it is constitutively expressed at the dorsal vessel ECM throughout post- embryonic development.

The total LamininA fluorescence signal suggests that the total amount of LamininA localized at the dorsal vessel ECM increases throughout larval development and remains consistent from late L3 to adult development (Figure 3.2B). An increase in LamininA protein at the dorsal vessel is likely to compensate for the growth occurring during L3 development. This is indicative when calculating the change in LamininA protein density throughout development (Figure 3.2B). The LamininA protein density is not significantly different from early L3 to late L3 development, suggesting that the increase in LamininA protein localization to the dorsal vessel ECM is likely to compensate for the growing heart chamber. In adults, the reduction in dorsal vessel surface area is accompanied by an increase in LamininA protein density (Figure 3.2B). This suggests that in L3 development, as the heart chamber undergoes cardiac remodelling corresponding to an increased luminal diameter, more LamininA protein is localized at the dorsal vessel to compensate for this growth and maintain a consistent LamininA density. After metamorphosis, there appears to be a shift in LamininA protein: as the surface area reduces, the total protein visualized at the dorsal vessel does not proportionally reduce to maintain a consistent protein density. Rather, LamininA protein density increases, suggesting either an increase in LamininA is required at the dorsal vessel in adult development, or LamininA protein regulation acts independently of the structural changes occurring at the heart chamber after metamorphosis. This data provides insight into BM remodelling throughout development, and would suggest a more LamininA- dense ECM throughout different stages of development. This can be explored through EM images of the dorsal vessel using ROI's at different segments throughout development.

Pericardin

Pericardin is a novel *Drosophila* protein, localized to the abluminal ECM of the dorsal vessel. Pericardin surrounds the dorsal vessel and associating pericardial cells throughout larval and adult development, unique in its web-like orientation and localization (Figure 3.3A). During embryogenesis, Pericardin protein expression is detected at the periphery of pericardial cells and at the basal surface of cardioblasts (Zaffran et al 1995; Chartier et al 2002). Pericardin protein concentration increases throughout embryonic development and remains abundant through larval and adult development (Chartier et al 2002). Total Pericardin fluorescence signal suggests that the amount of Pericardin protein localized at the dorsal vessel ECM remains consistent throughout larval and adult development. These findings align with western blot analysis of total Pericardin protein extract revealing a consistent level of protein expression in L3 and adult developmental stages (Drechsler et al 2013). Although Pericardin protein localization remains consistent, the density of Pericardin protein is significantly greater in adult development due to the reduction in DV surface area at abdominal segment A5 (Figure 3.2B). This suggests that irrespective of cardiac remodelling and changes in luminal diameter, a consistent amount of Pericardin protein is expressed in the dorsal vessel ECM in L3 larva and adult development.

The remodelling of the extracellular matrix in abdominal segment A5

I quantified extracellular matrix protein levels at the A5 abdominal segment of the posterior heart at a consistent region of interest (ROI). The A5 abdominal segment was

used as an ROI throughout all experiments because it persists as a segment representing the posterior heart throughout all developmental stages (Monier et al 2005; Zeitouni et al 2007). Changes in ECM protein localization and deposition can be monitored from embryogenesis through to adult development because segment A5 does not undergo programmed cell death during metamorphosis (Monier et al 2005; Zeitouni et al 2007). Pericardial cells remain consistent in their localization throughout development at the A5 segment and no changes to morphology or ultrastructure have been noted after embryonic development (Mills and King 1965; Zeitouni et al 2007; Lehmacher et al 2012; Curtis et al 1999; Molina and Cripps 2001). Although abdominal segment A5 of the dorsal vessel does not undergo histolysis during metamorphosis, morphological changes do occur and should be noted when interpreting the data presented in this thesis. Abdominal segment A5 trans-differentiates into the terminal heart chamber of the adult dorsal vessel. The terminal heart chamber yields a narrower luminal diameter, and cardiomyocytes differentiate from contractile muscle cells to non-contracting cells (Molina and Cripps 2001; Monier et al 2005). This thesis examines ECM remodelling in the post-embryonic dorsal vessel. By examining abdominal segment A5, I was able to explore changes in ECM remodelling under conditions of growth and increased cardiac load during L3 development. I was also able to explore subsequent changes in the ECM environment after metamorphosis where the luminal domain of the posterior heart is substantially reduced. Abdominal segment A5 of the dorsal vessel allowed me to maintain as much consistency as possible upon examining ECM remodelling throughout dorsal vessel

growth and development while allowing me to assess ECM regulation under different structural and physiological conditions.

4.2: The mean surface area of the dorsal vessel at abdominal segment A5 changes throughout development

I discussed earlier how *Drosophila* undergoes massive growth accompanying physiological changes after embryogenesis. As you would expect, the dorsal vessel undergoes structural and physiological changes to compensate. To explore ECM remodelling in the context of growth and subsequent shrinkage, I set out to calculate the surface area of the heart chamber at abdominal segment A5 throughout L3 and adult development. The surface area of the dorsal vessel at abdominal segment A5 is ~1.5 times greater in late L3 compared to early L3 larva (Supplementary Figure 1). After metamorphosis, the surface area decreases to approximately half the size compared to late L3 (Supplementary Figure 1). Although the growth of the dorsal vessel during L3 development has not been quantified in previous studies, expansion of the luminal domain during larval development has been noted in the literature (Bogatan et al 2015). While, abdominal segment A5 of the posterior heart is consistently measured throughout all experiments in this thesis, in adult development segment A5 might not be a generalizable representation of the cardiac tube diameter due to the trans-differentiation occurring during metamorphosis. Nevertheless, abdominal segment A5 allows us to look at ECM remodelling and growth during L3 development, and then subsequent remodelling followed by a reduction in dorsal vessel luminal diameter during adult

development. To specifically address changes in cardiac tube diameter throughout development, abdominal segment A3 might better represent the luminal diameter of the adult heart chamber. Future study of the transformation of A3 from aorta to heart during metamorphosis may also provide a stage to view ECM remodelling in another direction.

4.3: Pericardial cells may play a subsidiary role contributing to Type IV Collagen or LamininA at the dorsal vessel ECM

To investigate if pericardial cells produce ECM proteins at the dorsal vessel, fluorescence quantification measurements of GFP tagged Type IV Collagen and immunolabelled LamininA ECM proteins were measured following protein specific RNAi knockdown in pericardial cells. It has been previously demonstrated that dorsal vessel specific ECM protein, Pericardin, is no longer secreted by pericardial cells following embryogenesis and is mainly secreted by the larval fat body (Drechsler et al 2013); therefore, the role of post-embryonic pericardial cells in Pericardin ECM production will not be explored in this section. This section also canvasses future experiments to substantiate pericardial cells' subsidiary role in ECM production.

Type IV Collagen

Ubiquitous depletion of Vkg-GFP with dsRNA targeted to GFP, reduced fluorescence at the dorsal vessel almost entirely (Figure 3.4A). Compared to Vkg-GFP depletion in the control group, mRNA depletion specifically targeting pericardial cells did not significantly reduce protein fluorescence (Figure 3.4A). This suggests that pericardial

cells do not secrete Type IV Collagen at the dorsal vessel ECM. Duygu Cevic (2016) investigated this question looking at Type IV Collagen secretion from hemocytes and the fat body. Her results suggest that during L3 development the fat body is responsible for producing the majority of Type IV Collagen localized to the ECM of the dorsal vessel. Cevic (2016) also implicates hemocytes contributing to Type IV Collagen at the dorsal vessel. In accordance with her findings, Paster-Pareja and Xu (2011) note that the fat body appears to take over the majority of ECM production and secretion after embryonic development. Specifically, fat body specific knockdown of Tango1 expression, required for proper secretion of Type IV Collagen in *Drosophila,* revealed accumulation of intracellular Type IV Collagen during larval development (Saito et al. 2009; Paster-Pareja and Xu 2011). To affirm that pericardial cells are not involved in the secretion or production of Type IV Collagen, observing internal accumulation upon knockdown of CopII cargo adapter Tango1, or CopII coat component, Sar1 would help confirm this finding at larval and adult developmental stages.

LamininA

Ubiquitous depletion of LanA with dsRNA targeted to *lanA* mRNA, reduced LanA targeted antibody fluorescence at the dorsal vessel but not to the same extent as dsRNA mediated depletion of Vkg (Figure 3.4b). Compared to the total fluorescence signal in the control group, pericardial cell specific depletion of LanA was not significantly reduced (Figure 3.4B). This suggests that the pericardial cells do not substantially contribute to production of LamininA at the dorsal vessel ECM (Figure 3.4B). Pericardial cell restricted knockdown of CopII coat component, Sar1 would help confirm this finding at

larval and adult developmental stages by assessing internal accumulation of LamininA in the pericardial cells.

Future experiments

Future experiments can be conducted to substantiate a potential subsidiary role of pericardial cells in dorsal vessel ECM production. Although not significantly different from the control, the total fluorescence signal appears reduced in pericardial cell- specific depletion of Type IV Collagen and LamininA (Figure 3.4a(G), 3.4b(G)). This suggests that perhaps post- embryonic pericardial cells play a subsidiary role in dorsal vessel ECM production. Perhaps increasing the sample size or doing experiments at 29 degrees would sensitize the assay to subtle contributions from the pericardial cells. Although all experiments were done alongside a positive and negative control group, using a secondary positive control measuring the total fluorescence signal for pericardial cell driver would negate a possible ECM associated phenotype resulting from that specific driver. While pericardial cells may not play a substantial role in secreting LamininA or Type IV Collagen to the dorsal vessel ECM after embryogenesis, they may play an instructive role in ECM localization at the dorsal vessel.

4.4: Hemocytes are recruited to areas of pericardial cell degradation and trauma in late L3 KLF15 mutant larva.

I wanted to observe dorsal vessel associated cell types that interact with pericardial cells or respond to pericardial cell specific signals. Post- embryonic hemocytes cluster around the dorsal vessel in late L3 development, often localizing to the pockets adjacent to pericardial cells (Cevic 2016) (Figure 3.6A). This localization is found to be enhanced upon a triggered immunological response (Cevic 2016; Stofanko et al 2010). In late L3 *klf15* mutant larva, pericardial cells degenerate and hemocytes cluster around the degrading cells (Figure 3.6B,C). The localization of hemocytes appears specific to the regions of degrading pericardial cells compared to wildtype association at the dorsal vessel (Figure 3.6A,B,C). As discussed previously, post embryonic hemocytes function to clear infection from invading microorganisms and clear debris from apoptotic cells through phagocytosis (Sampson et al 2013; Lanot et al 2001). Specifically, Lanot and colleagues (2001) suggest, with transmission electron micrograph evidence, late L3 development plasmatocytes contain engulfed apoptotic neighbouring cells within their phagocytic vacuoles. In a *klf15* mutant background, I used a pericardial cell specific promoter to drive LifeActin-GFP to pericardial cells, and a hemocyte specific fusion protein, HmldsRed, to visualize hemocyte localization (Figure 3.6). When analyzing confocal images taken at the dorsal vessel of late L3 larva, hemocyte fluorescence appears to overlap with the GFP fluorescence driven to the pericardial cells (Figure 3.6 B,C,D). Both cross sectional and z- projections suggest hemocyte localization to degrading pericardial cells, and GFP fluorescence within the pericardial cell associated hemocytes (Figure 3.6 B,C,D). Higher magnification cross- sectional confocal images

coupled with transmission electron micrograph evidence would help confirm this observation. While I assume the hemocytes clustering around and engulfing the degraded pericardial cells are plasmatocytes, using a plasmatocyte- specific antibody would be able to confirm the hemocyte specific cell identify. Additional experiments blocking hemocyte- specific endocytosis using temperature sensitive, UAS-shi(ts)RNAi in a *klf15* mutant background would give further insight into pericardial cell- hemocyte interplay upon pericardial degradation in late L3 larva.

4.5: Pericardial cells are required for proper ECM localization and deposition at the dorsal vessel.

In a *klf15* mutant background, pericardial cells undergo degradation during L3 development and are completely absent in adults after metamorphosis (Ivy et al 2015). To investigate an instructive role for pericardial cells in dorsal vessel ECM deposition, I used immunolabelling techniques, coupled with confocal microscopy, to calculate the total fluorescence signal corresponding to each ECM protein of interest in the absence of pericardial cells.

In a *klf15* mutant background, there was a significant decline in total fluorescence signal corresponding to each ECM protein of interest at the dorsal vessel from late L3 to adult development (Figure 3.7A,B). This data contrasts previous wild-type results showing consistent the fluorescence signal from late L3 to adult development with regards to Type IV Collagen, LamininA, and Pericardin levels at the dorsal vessel ECM (Figure 3.1B,3.2B,3.3B). A decline in total fluorescence signal suggests that less protein is localized to the heart chamber within abdominal segment A5 of the dorsal vessel in the

absence of pericardial cells. Although my previous results suggest that pericardial cells are not directly involved in secreting ECM proteins after embryonic development at the dorsal vessel, perhaps pericardial cells influence ECM deposition and localization through other regulatory means.

Recently, Hartley and colleagues (2016) found that SPARC proteins accumulate in the circulating hemolymph of late L3 *klf15* mutant larva and adults. SPARC promotes Type IV Collagen localization to the basement membrane during embryogenesis, and is required for proper Type IV Collagen secretion from fat body cells (Martinek et al 2008; Bradshaw 2009; Volk et al 2014; Hartley et al 2016). Although the role of SPARC in post-embryonic development remains largely unexplored, Hartley and colleagues (2016) suggest pericardial cells play a novel role in regulating SPARC accumulation within the circulating hemolymph. Perhaps an increase in circulating SPARC in *klf15* mutant adult flies results in decreased ECM deposition at the dorsal vessel. Future experiments driving SPARC expression to pericardial cells in a *SPARC* mutant background and subsequently measuring a change in ECM protein deposition, could address the notion that pericardial cells influence dorsal vessel ECM deposition through SPARC protein regulation.

Upon pericardial cell degradation in late L3 development, empty pockets that once occupied pericardial cells are surrounded by ECM proteins (Figure 3.7C). This suggests that in late L3 development, ECM remodelling does not occur to compensate for the gaps in matrix, once occupied by pericardial cells. In contrast, after metamorphosis, the ECM environment around the adult dorsal vessel is remodelled and there are no longer empty pockets of matrix coinciding with pericardial cell localization (Figure 3.7C). Perhaps

hemocytes which appear to be recruited to areas of pericardial cell degradation at late L3 development, and persist through metamorphosis, are also responsible for ECM remodelling at the dorsal vessel.

Irregular accumulation of Type IV Collagen and LamininA at the luminal surface of abdominal segment A4 of *klf15* mutant adult dorsal vessels was observed through cross-sectional analysis (Figure 3.7C). No luminal accumulation of Pericardin was observed which is consistent with normal Pericardin localization restricted to the abluminal ECM (Figure 3.7C). Perhaps post- pericardial cells play an instructive role in ECM localization at the luminal surface of the dorsal vessel. Higher magnification cross sectional images would help confirm these findings.

ECM protein fluorescence at abdominal segment A5 of the dorsal vessel was assessed throughout these experiments to assess changes in ECM protein levels and localization at the dorsal vessel. I observed irregular localization of Type IV Collagen and LamininA at the luminal surface in abdominal segment A4; therefore, the decline in total fluorescence signal at abdominal segment A5 may not necessarily represent the amount of ECM protein levels at the dorsal vessel because pericardial cells might have an instructive role in protein localization at the dorsal vessel. Future experiments can look at multiple ROI's at the dorsal vessel in a *klf15* mutant background. Further analysis of total protein deposition was limited by the fact that my settings at the confocal microscope differed in *klf15* mutant experiments compared to wild-type analysis.

4.6: In the absence of pericardial cells, mean surface area of dorsal vessel at abdominal segment A5 is significantly decreased in *klf15* mutant background.

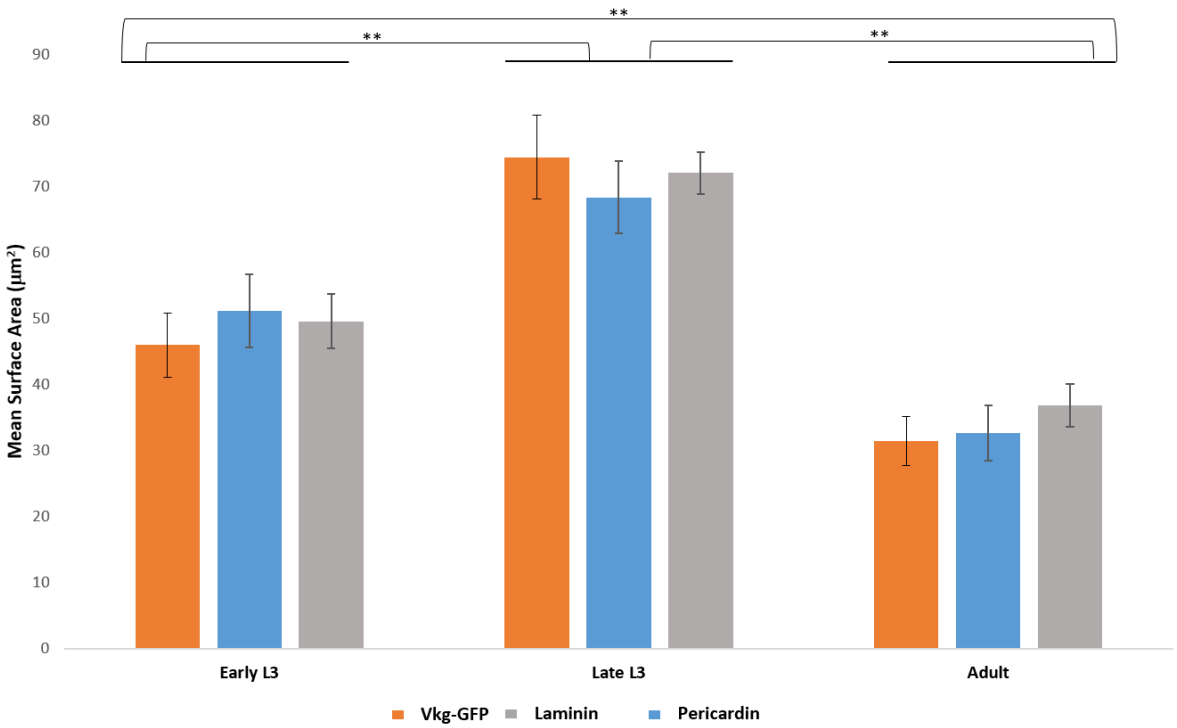
It is still up for debate in the literature if post-embryonic pericardial cells are required for proper cardiac function. Das et al. (2008) argues that post-embryonic pericardial cells are not required to maintain normal heart rate following post-embryonic removal of pericardial cells by induced cell death. Das et al. (2008) also argues that pericardial cells have no affect on cardiac remodelling during metamorphosis. Recently, Hartley and colleagues (2016) assessed cardiac function in *klf15* mutant background and pericardial cell specific *klf15* knockdown flies. They found that cardiac function was impaired as evidenced by long diastolic intervals (Hartley et al 2016). I found the mean surface area to be significantly smaller in larva and adult in *klf15* mutant background compared to the wildtype flies (Figure 3.7d). Perhaps the heart is smaller in size because it experiences less cardiac load; or Pericardial cells play a role in dorsal vessel growth.

4.7 Future Directions:

This thesis characterized ECM remodelling at the dorsal vessel throughout post-embryonic development, focusing on a regulatory role for pericardial cells in ECM regulation and production. My initial objective sought to characterize extracellular matrix deposition and localization at the dorsal vessel throughout post- embryonic development; while doing so, I was able to quantify novel changes to dorsal vessel structure and morphology throughout post- embryonic development. I was also able to examine the total protein localized to the dorsal vessel and how that deposition changes in accordance to growth throughout development. To further contribute to this area of research, examining an alternative abdominal segment at the dorsal vessel may provide insight into ECM remodelling under different morphological conditions throughout development. The second objective of my thesis asked whether pericardial cells produce ECM proteins at the dorsal vessel in post- embryonic development. Although evidence does not implicate pericardial cells as major contributors to the dorsal vessel ECM in post- embryonic development, perhaps they play a subsidiary role in protein deposition. In order to examine this subsidiary role, replicating experiments at 29 degrees could potentially sensitize the assay to subtle contributions from the pericardial cells. My final objective was to assess whether pericardial cells are required for proper ECM localization and deposition at the dorsal vessel. Although their role in ECM protein deposition at the dorsal vessel may appear to be subsidiary, my results suggest a novel role for pericardial cells in proper ECM localization in adult development. Future experiments assessing the regulatory role of pericardial cells on circulating SPARC could address the notion that

pericardial cells influence dorsal vessel ECM deposition through SPARC protein regulation. Finally, examining the potential interaction between pericardial cells and hemocytes in post- embryonic development could address novel cellular roles in ECM remodelling and cardiac function. The interplay between pericardial cells and the associating dorsal vessel ECM provides insight into a novel role for post-embryonic pericardial cells, and a deeper understanding of the intrinsic and dynamic extracellular environment.

Supplementary Figures:



Supplementary Figure 1: **Mean surface area of the dorsal vessel at abdominal segment A5.** Mean surface area of the dorsal vessel at abdominal segment A5 is significantly different in early L3, late L3 and adult developmental stages. Surface area measurements represented in Figures 3.1B,3.2B and 3.3B are not statistically different from each other for each developmental stage. SEM error bars. T-test, $P<0.05$ (**). Orange: Vkg-GFP, Grey: LamininA, Blue: Pericardin.

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