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The impact of *SPARC* on age-related cardiac dysfunction and fibrosis in *Drosophila*

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ABSTRACT

Tissue fibrosis, an accumulation of extracellular matrix proteins such as collagen, accompanies cardiac ageing in humans and this is linked to an increased risk of cardiac failure. The mechanisms driving age-related tissue fibrosis and cardiac dysfunction are unclear, yet clinically important. *Drosophila* is amenable to the study of cardiac ageing as well as collagen deposition; however it is unclear whether collagen accumulates in the ageing *Drosophila* heart.

This work examined collagen deposition and cardiac function in ageing *Drosophila*, in the context of reduced expression of collagen-interacting protein *SPARC* (Secreted Protein Acidic and Rich in Cysteine) an evolutionarily conserved protein linked with fibrosis. Heart function was measured using high frame rate videomicroscopy. Collagen deposition was monitored using a fluorescently-tagged collagen IV reporter (encoded by the *Viking* gene) and staining of the cardiac collagen, Pericardin.

The *Drosophila* heart accumulated collagen IV and Pericardin as flies aged. Associated with this was a decline in cardiac function. *SPARC* heterozygous flies lived longer than controls and showed little to no age-related cardiac dysfunction. As flies of both genotypes aged, cardiac levels of collagen IV (Viking) and Pericardin increased similarly. Over-expression of *SPARC* caused cardiomyopathy and increased Pericardin deposition.

The findings demonstrate that, like humans, the *Drosophila* heart develops a fibrosis-like phenotype as it ages. Although having no gross impact on collagen accumulation, reduced *SPARC* expression extended *Drosophila* lifespan and cardiac health span. It is proposed that cardiac fibrosis in humans may develop due to the activation of conserved mechanisms and that *SPARC* may mediate cardiac ageing by mechanisms more subtle than gross accumulation of collagen.

1. Introduction

Collagens are crucial for the development of form and function in multicellular animals. In mammals, increased collagen deposition occurs in response to injury as part of the tissue repair process (Wynn, 2008). Collagen accumulation in humans, typically referred to as tissue fibrosis, is a clinically important feature of chronic disease states as well as ageing. In both humans and mammalian models, tissue fibrosis is linked to organ dysfunction and an increased risk of mortality. Understanding the molecular genetics of collagen deposition and fibrosis is therefore an important step towards developing anti-fibrotic therapies. Fibrosis-related proteins and the signalling pathways leading to fibrosis show a high degree of genetic and functional conservation in the animal kingdom (Volk et al., 2014; Hartley et al., 2016; Sessions et al., 2016). The fruit or vinegar fly, *Drosophila melanogaster* expresses several collagen genes, as well as matricellular proteins required for the assembly of extracellular matrices (Yasothornsrikul et al., 1997; Martinek et al., 2008). Collagens and associated matricellular proteins are important mediators of cardiac development in *Drosophila* (Hartley et al., 2016; Chartier et al., 2002; Drechsler et al., 2013). Despite *Drosophila* being highly amenable to studies of age-related cardiac decline (Wessells et al., 2004; Cannon et al., 2017; Klassen et al., 2017; Lee et al., 2010; Nishimura et al., 2014; Monnier et al., 2012), there are currently no studies examining collagen deposition in the ageing heart.

SPARC (Secreted Protein Acidic and Rich in Cysteine) is a well-characterised collagen binding matricellular protein involved in tissue fibrosis (Weaver et al., 2008; Bradshaw, 2012). SPARC is evolutionarily and functionally conserved and known to mediate collagen deposition in *Drosophila* embryos (Martinek et al., 2008). *SPARC* expression is increased in a number of clinically important settings and accumulates (along with other extracellular matrix (ECM) proteins) in the ageing mammalian heart (Bradshaw et al., 2010; de Castro Bras et al., 2014),

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suggesting it may play a role in cardiac dysfunction in human ageing. Recent findings indicate that reduced *SPARC* expression can correct cardiomyopathy in *Drosophila* (Hartley et al., 2016). In addition, reduced expression of the ECM proteins Laminin, Viking and Pericardin in the *Drosophila* heart can impede age-related cardiac dysfunction (Sessions et al., 2016). Despite this knowledge, there is no data on whether ECM proteins accumulate within the ageing *Drosophila* heart.

Embryonic and larval development of *Drosophila* is dependent on the expression of type-IV collagen α2 and α1 chains encoded by *Viking* and *Cg25C*, as well as Multiplexin (an ortholog of human *COL18A1*) and the heart specific type-IV-like collagen, Pericardin; the latter two proteins being integral to cardiac development (Chartier et al., 2002; Harpaz et al., 2013). Although Pericardin was initially described as a matrix forming type-IV-like collagen, it also adopts prominent fibre-like structures and acts as a 'tendenous' bridge between the heart and alary muscles. How these collagenous fibres are formed and maintained remains largely uncharacterised.

In humans and mammalian models, fibrosis is regarded as an accumulation of extracellular matrix in a given tissue. Most attention has focused on fibril forming collagens, however fibrosis is complex and outcomes in terms of organ function are affected not only by the gross accumulation of collagen subtypes and other ECM proteins but also by the ratios of different collagens and ECM proteins (Karsdal et al., 2017). Experimental evidence indicates that *Drosophila* may be a tractable model with which to study the mechanisms leading to tissue fibrosis in humans. For example, an accumulation of collagen in and around *Drosophila* adipocytes alters innate immunity (Zang et al., 2015) whereas diet-dependent changes to *Drosophila* heart function are associated with cardiac fibrosis (Na et al., 2013). These findings make *Drosophila* a valuable tool with which to understand and identify mechanisms regulating collagen deposition and its impact on organ function. Given that collagen turnover (i.e. the expression, deposition and degradation of collagen) as well as the ageing process are evolutionarily conserved, it was predicted that collagen may accumulate as part of the ageing process in the *Drosophila* heart model and that *SPARC* may mediate this process.

In this report we describe the accumulation of collagen in the ageing *Drosophila* heart and show that this accompanies the well-described age-dependent functional decline of the fly's heart. It is also shown that *SPARC* heterozygous flies have a longer lifespan as well as extended cardiac health span. Despite this, the accumulation of collagen around the heart does not seem to be affected by reduced *SPARC* expression; whereas, SPARC over-expression led to cardiomyopathy and Pericardin accumulation. The findings support the idea that age-related fibrosis in mammals is an evolutionarily conserved process which can be studied in simpler, genetically tractable models.

2. Materials and methods

2.1. Stock chemicals and fly husbandry

Picrosirius red and all stock chemicals were from Sigma (Poole, Dorset, UK). The *Canton Special*, w^{1118}, *yw*, $SPARC^{MI00329}$ (with a MiMIC insertion in the 5-prime region of the *SPARC* locus; described in (Venken et al., 2011)) and *Viking-GFP* strains were obtained from the Bloomington Drosophila Stock Centre. The *Dorothy-Gal4* line was described previously (Kimbrell et al., 2002) and is used to drive the expression of genes downstream of a UAS (upstream activation sequence) element. The *UAS-SPARC* line was described in (Martinek et al., 2008). Flies were reared and maintained on a standard cornmeal-yeast-agar diet under 12 h:12 h light:dark cycles at 25 °C. $SPARC^{MI00329}$ were backcrossed to a *yw* background for > 6 generations and then outcrossed once to the w^{1118} line to generate heterozygous flies for phenotyping and lifespan studies.

2.2. Lifespan studies

Ten male or female flies within 1 day of eclosion were collected and transferred to fresh vials with standard diet. Five to ten vials per trial were prepared. At least two trials were conducted for each genotype. Vials were maintained on their side and flies tipped to fresh food twice per week for the duration of the lifespan studies. All vials were maintained under 12 h:12 h light:dark cycles at 25 °C. Lifespan data is presented as Kaplan-Meier plots as well as the mean of the median (the point at which 50% of the population had died) and maximum lifespan (± SEM).

2.3. Histochemistry, imaging and qPCR

Adult flies were anaesthetised by brief exposure to CO_2, hearts were exposed by dissection and fixed using 1% formaldehyde for 15 min. Picrosirius red dye was then added for 5 min at ambient temperature and rinsed from dissected hearts using acidified water (1% acetic acid in water). Picrosirius red comprises sulphonic acid and stains the basic amine residues of most proteins but preferentially stains collagens because of their abundance and the large number of glycine residues. Colour phase images were captured on a Leica DMLB fitted with a Leica DC300 colour camera, images were taken using μManager software (Edelstein et al., 2010). Pericardin was imaged after staining dissected hearts with the mouse-anti Pericardin monoclonal antibody clone EC11 (Zaffran et al., 1995) (Developmental Studies Hybridoma Bank, University of Iowa) followed by a fluorescently-tagged secondary antibody. Fluorescence images were taken using a Zeiss LSM 710 confocal microscope and images quantified using ImageJ by drawing a line across a region of interest and recoding the maximum fluorescence signal.

2.4. Analysis of cardiac Pericardin

Hearts stained with anti-Pericardin antibodies were imaged by confocal microscopy and z-projections of stacks analysed in ImageJ. The same number of z-projected stacks was sued for each genotype and age. All images were of the distal region of the heart comprising approximately 2/3 of the total heart, where the majority of anti-Pericardin staining is found. Images were thresholded and the percentage of Pericardin positive signal quantified as a percentage of the total image. For measurement of Pericardin fibre thickness, single confocal image stacks were used and at least six fibre measurements were taken from four different flies. Data are presented as the mean fibre thickness (± SEM).

2.5. Heart function analysis

Adult flies were anaesthetised by brief exposure to Triethylamine vapour (20 μL of a 50% solution made in 50% ethanol/water, placed into the underside of a fly vial's cotton cap). Flies were dissected as described previously (Catterson et al., 2013) and imaged using a Zeiss Axiolab microscope fitted with a water immersion 10 × objective, linked to a Myocam S (Ionoptix Ltd., Ireland) high speed video camera. The beating heart was imaged for 10–20 s at 120 frames per second (fps) and heart function analysed using SOHA (Fink et al., 2009). For heart analysis involving SPARC over-expression, video capture was performed with a Ximea XDi camera and a frame rate of 25 fps.

2.6. Western Blotting

For each lane 10 female hearts were harvested and directly lysed in 10 μL Ripa buffer (Sigma, Poole, UK). Polypeptides were separated on a 4–20% SDS-page gel and transferred to a PVDF membrane. The membrane was then probed with anti-pericardin (DSHB, EC11) and anti-actin (DSHB JLA20) antibodies followed by secondary antibodies conjugated to alkaline phosphatase. Secondary antibodies were detected