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Lumican deficiency results in cardiomyocyte hypertrophy with altered collagen assembly

Loren E. Dupuis [a], Matthew G. Berger [b], Samuel Feldman [b], Lorna Doucette [b], Vennece Fowlkes [a], Shukti Chakravarti [c], Sarah Thibaudeau [a], Nicolas E. Alcala [b], Amy D. Bradshaw [d], Christine B. Kern [a,*]

[a] Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425, United States
[b] Honors College, College of Charleston, Charleston, SC 29401, United States
[c] Department of Medicine, Johns Hopkins School of Medicine, Baltimore, MD 21205, United States
[d] Gazes Cardiac Research Institute, Medical University of South Carolina, Charleston, SC 29425, United States

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ABSTRACT

The ability of the heart to adapt to increased stress is dependent on the modification of its extracellular matrix (ECM) architecture that is established during postnatal development as cardiomyocytes differentiate, a process that is poorly understood. We hypothesized that the small leucine-rich proteoglycan (SLRP) lumican (LUM), which binds collagen and facilitates collagen assembly in other tissues, may play a critical role in establishing the postnatal murine myocardial ECM. Although previous studies suggest that LUM deficient mice ($lum^{-/-}$) exhibit skin anomalies consistent with Ehlers–Danlos syndrome, $lum^{-/-}$ hearts have not been evaluated. These studies show that LUM was immunolocalized to non-cardiomyocytes of the cardiac ventricles and its expression increased throughout development. Lumican deficiency resulted in significant (50%) perinatal death and further examination of the $lum^{-/-}$ neonatal hearts revealed an increase in myocardial tissue without a significant increase in cell proliferation. However cardiomyocytes from surviving postnatal day 0 (P0), 1 month (1 mo) and adult (4 mo) $lum^{-/-}$ hearts were significantly larger than their wild type (WT) littermates. Immunohistochemistry revealed that the increased cardiomyocyte size in the $lum^{-/-}$ hearts correlated with alteration of the cardiomyocyte pericellular ECM components collagenα1(I) and the class I SLRP decorin (DCN). Western blot analysis demonstrated that the ratio of glycosaminoglycan (GAG) decorated DCN to core DCN was reduced in P0 and 1 mo $lum^{-/-}$ hearts. There was also a reduction in the β and γ forms of collagenα1(I) in $lum^{-/-}$ hearts. While the total insoluble collagen content was significantly reduced, the fibril size was increased in $lum^{-/-}$ hearts, indicating that LUM may play a role in collagen fiber stability and lateral fibril assembly. These results suggest that LUM controls cardiomyocyte growth by regulating the pericellular ECM and also indicates that LUM may coordinate multiple factors of collagen assembly in the murine heart. Further investigation into the role of LUM may yield novel therapeutic targets and/or biomarkers for patients with cardiovascular disease.

1. Introduction

Cardiac hypertrophy is generally defined as a reactive increase in cardiac size and myocardial mass in response to hemodynamic stress [1,2]. In pathophysiological conditions, cardiomyocytes enlarge and it's the enlargement of these contractile cells of the heart that results in an increase in organ size referred to as cardiac hypertrophy. However cardiomyocytes also expand in size during postnatal development where they switch from a stellate appearance to their characteristic rod-shaped mature phenotype. In an effort not to confuse the developmental cardiomyocyte cell enlargement after birth with pathological cell enlargement referred to as 'hypertrophy' we refer to the normal postnatal cardiac growth as eutrophy which, remains a relatively understudied developmental process by which cardiomyocytes enlarge as they mature which in turn causes the heart to expand, without significant cell proliferation [1].

In recent years there has been considerable investigation into defining the molecular markers involved in the specification of the cardiomyocyte progenitors. In addition, the examination of cell lineages, largely through CRE-lineage tracing, has established multiple origins of cardiomyocytes that comprise the fully formed mammalian heart. Significantly less is known about the maturation of cardiomyocytes from fetal stages onward [3]. Importantly, after birth cardiomyocytes

* Corresponding author at: 171 Ashley Avenue, BSB 647, Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425 United States.
E-mail address: kernc@musc.edu (C.B. Kern).

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switch from proliferative to eutrophic growth [4]. This transition from proliferative progenitors to differentiated cardiomyocytes requires exquisite timing to down-regulate developmental pathways and to upregulate terminal differentiation gene programs. Several pathways that promote proliferation actively work to prevent differentiation therefore maintaining the balance of pathways is important for normal cardiomyocyte differentiation and eutrophic growth [3].

Coincident with cardiomyocyte differentiation is the maturation of the extracellular matrix (ECM) architecture within the ventricles of the postnatal heart. In fact, the ECM structure is responsible for the rod-shaped phenotype of the adult cardiomyocytes [5,6]. Although ECM maturation often mirrors cardiovascular cell differentiation, the interconnection between ECM architecture and cell signaling programs that balance growth and differentiation remain poorly understood. There is mounting evidence that both the provisional and mature ECM are generated by the cardiac fibroblasts. Since collagen is required to maintain the integrity and biomechanical properties of the mature four-chambered heart [7–9] one of the major roles of the embryonic and adult cardiac fibroblasts is the adequate assembly of collagen fibers. In the ventricular myocardium the organization of the mature collagen network is established within the first months of postnatal life and maintained in the adult. Although the collagens comprise a major part of the cardiac ECM it is becoming increasingly evident that not all patients with collagen-related disorders have mutations in collagen genes or genes encoding enzymes that directly modify collagen. Therefore other classes of molecules that are integral for correct collagen assembly may also play a role in establishing and maintaining the cardiovascular ECM. The small leucine rich proteoglycans (SLRPs) are a class of ECM molecules that contain leucine rich repeats (LRR) and bind directly to collagen fibers to modify collagen assembly and growth [10–13]. We speculate that postnatal cardiac development is an important period to investigate the role of SLRPs and other factors responsible for the assembly of the collagen-rich matrix that is coincident with cardiomyocyte differentiation and eutrophy.

In this study we examined for the first time, the role of the class II SLRP lumican (LUM) in the developing murine heart. Previous studies using mouse models of LUM deficiency have determined its requirement for normal collagen fiber assembly in the skeletal muscle, [14] tendon [15] and cornea [16,17]. We evaluated the spatiotemporal localization of LUM throughout cardiac development and using a LUM deficient mouse model determined a potentially critical role for LUM in murine cardiac development.

2. Materials and methods

2.1. Mice

All mouse experiments were done under protocols approved by the Medical University of South Carolina IACUC. The lumican deficient mice ($lum^{-/-}$) used in this study were received from S. Chakravarti [18] and were bred into C57BL/6 (>10 generations).

2.2. Histology and immunohistochemistry

Standard histological procedures were used [19]. The lumican antibodies used included a gift from Dr. A. Oldberg, Lund University and a lumican antibody against mouse from R & D Systems™ (AF2846); laminin antibody was purchased from Abcam (ab11575). Collagenα1(I) was purchased from mdbioproducts (203002) and decorin was obtained from R & D systems™ (RF1060). Antibodies to α sarcomeric actin (Sigma, A2172), and α smooth muscle actin (Sigma, A 5228) were also utilized in this study to identify myocardial and smooth muscle cells respectively. Fluor-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Antibodies were used in murine tissues fixed in Amsterdam [20] and 4% paraformaldehyde as well as cryopreserved sections. All 4% paraformaldehyde tissue was also paired with citric acid antigen unmasking (H-3300, Vector laboratories, Burlingame, CA). Imaging was performed on the Leica TCS SP5 AOBS Confocal Microscope System (Leica Microsystems Inc., Exton, PA).

2.3. Myocardial area and heart size measurements

Histological sections (four sections/heart) from wild type (n = 8) and $lum^{-/-}$ (n = 8) P0 mice, over a 20 μm depth concomitant with the aortic valve, were used to determine muscle area in Amira™. The average ventricular area was measured in positive pixels in wild type and $lum^{-/-}$ littermates from two different litters. A set rectangular area was placed over the widest part of the right ventricle, septum and left ventricle and used for area measurements. Four sections were used per heart for pixel area.

The heart size was analyzed in wild type and $lum^{-/-}$ littermates at P0 and 1 month. The width and length of the hearts were quantified using the Olympus BX40 light microscope and accompanying software. Student's t-test, followed by Anova was utilized to determine statistical significance. P0 hearts; WT (n = 8), $lum^{-/-}$ (n = 8). 1 month; WT (n = 10), $lum^{-/-}$ (n = 8).

2.4. Amira™ three-dimensional reconstructions

Using approximately 250, 5 μm thick frontal sections, from each heart were stained with hematoxylin and eosin and used for three-dimensional (3D) reconstructions (n = 2 WT; n = 3 $lum^{-/-}$) at postnatal (P0). All of the heart sections that encompassed the ventricular lumens of the LV and RV from the wt and *lum* −/− were used. The positive pixels that represent cardiac tissue are identified using transparent purple and negative pixels on the internal area of the muscle were denoted in cyan and designate RV and LV lumen space.

2.5. Myocardial cell size measurement

The width of cardiomyocytes, defined as nucleated α-sarcomeric actin positive cells, was measured using the ruler tool in Photoshop™. Cardiomyocyte cell borders were delineated using IHC of the basement membrane component laminin. To control for differences in cardiomyocyte cell size within different regions of the murine heart, measurements were taken at the depth of the aortic valve. Cross sections of cardiomyocytes were also grouped and analyzed according to their location within the working myocardium i.e. right ventricle, septum or left ventricle. A total of n ≥ 4 was used for each genotype (WT/$lum^{-/-}$) and represented three aged-matched litters for each time point P0, 1 month (that exhibited increased myocardial area) and n = 3 each for adult (4 month-old) WT and *lum* −/−. Student's t-test, followed by Anova was utilized to determine statistical significance.

2.6. Electron microscopy

Sections of hearts preserved in Karnovsky's fixative were generated from 3 wt and 5 $lum^{-/-}$ mice at 1 month of age and processed for transmission EM. Collagen fibril diameters were measured in scanned images generated from electron micrographs with NIH image software. Collagen fibrils in at least 3 fields derived from sections of hearts from each mouse were quantified. EM images taken at ×20,000 magnification and were scanned into Adobe Photoshop (Fremont, WA) on an Epson flat bed scanner. A minimum contribution of 200 fibrils from each mouse was counted. Measurement of fibril diameter was performed with the NIH image software program, and data were transferred to the Microsoft Excel program (Redmond, WA) to generate average lengths and distributions of diameters.