The Fibroblast Specific α11β1 Integrin is Important for Postnatal Cardiomyocyte Growth, Alignment and Function

by

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Abstract

Integrins are transmembrane adhesion and signaling receptors that interact with the extracellular matrix to mediate diverse cellular and developmental processes. Recent work has demonstrated cardiac fibroblasts mediate cardiomyocyte development through integrins. However, the predominant integrins involved and the role of integrins in cardiac development is poorly defined. Thus, we investigated the contribution of the α11β1 integrin (α11), which is expressed by fibroblasts and binds preferentially to type I collagen fibers, in mediating postnatal cardiomyocyte development and function. Utilizing a germline mutation of the α11 gene in mice, we found that deletion resulted in growth and structural abnormalities in cardiomyocyte development and reductions in myocardial collagen, which was associated with impairments in both active and passive diastolic function. These results suggest that the α11 integrin is a key component of the cardiac fibroblast that is required for the normal development of cardiomyocytes and maintenance of cardiac function.
Acknowledgments

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Contributions

Ilana Talior-Volodarsky contributed to images in Figure 15 (C, D), Figure 17 (C, D) and preparation of tissue for transmission electron micrography analysis (Figure 14).

Jean-François Desjardins and Dr. Golam Kabir performed the echocardiography and cardiac catheterization procedures, as well as contributed to the echocardiography and pressure-volume loop analysis (Table 3 and 4, Figure 18).

Melissa Mitchell and Jennifer Switzer contributed to breeding of the α11 null mice and overall conductance of the study.

Reynaldo Interior (SickKids) performed the hydroxyproline analysis (Figure 18).

Dr. Kim Connelly, Dr. Christopher McCulloch and Dr. Andras Kapus provided tremendous support for overall concept of experiments, analysis of results and drafting and revisions.

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List of Abbreviations

\( \alpha_{11} \) \( \alpha_{11}\beta_1 \) integrin

\( \alpha_{11} \) KO\(^{-/-}\) \( \alpha_{11} \) homozygous knockout

\( \alpha \)-SMA \( \alpha \)-smooth muscle actin

\( \alpha_{11} \) WT\(^{+/+}\) \( \alpha_{11} \) littermate wildtype control

1° HF Primary heart field

2° HF Secondary heart field

AU Arbitrary units

BAC Bacterial artificial chromosomes

BW Body weight

\( \text{Ca}^{2+} \) Calcium

DDR2 Discoidin domain receptor 2

d\( P/\text{d}t_{\text{max}} \) Maximum rate of pressure change

d\( P/\text{d}t_{\text{min}} \) Minimum rate of pressure change

E Embryonic day

ECM Extracellular matrix

EDP End-diastolic pressure

EDPVR End-diastolic pressure volume relationship

EF Ejection Fraction

EGF Epidermal growth factor
<table>
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<td>ESPVR</td>
<td>End-systolic pressure volume relationship</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FGF</td>
<td>Fibroblast-growth factor</td>
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<tr>
<td>HBEGF</td>
<td>Heparin-binding EGF-like growth factor</td>
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<tr>
<td>hESCs</td>
<td>Human embryonic stem cells</td>
</tr>
<tr>
<td>HFREF</td>
<td>Heart failure with reduced ejection fraction</td>
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<tr>
<td>HFpEF</td>
<td>Heart failure with preserved ejection fraction</td>
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<tr>
<td>HF</td>
<td>Heart failure</td>
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<td>HW</td>
<td>Heart weight</td>
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<td>ITGA11</td>
<td>The α11 integrin gene</td>
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<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
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<td>KO</td>
<td>Knockout</td>
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<td>LV</td>
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<td>MGO</td>
<td>Methylglyoxal</td>
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<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<td>PSI</td>
<td>Plexin-sempahorin-integrin</td>
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<td>PV Loop</td>
<td>Pressure-volume loop</td>
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<td>RGD</td>
<td>Arg-Gly-Asp</td>
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<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
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<td>PSR</td>
<td>Picrosirius red</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>SERCA</td>
<td>Sarcoplasmic reticulum Ca(^{2+}) ATPase</td>
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<td>SVT</td>
<td>Supraventricular tachycardia</td>
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<td>TAC</td>
<td>Transverse aortic constriction</td>
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<td>TIMPs</td>
<td>Tissue inhibitors of the MMPs</td>
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<td>TL</td>
<td>Tibial length</td>
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<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>WGA</td>
<td>Wheat germ agglutinin</td>
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Chapter 1

Introduction

Integrins are a broad family of heterodimeric adhesion receptors comprised of α and β subunits that dynamically interact with the extracellular matrix (ECM) to regulate diverse cellular and developmental processes including cell growth, differentiation and survival (1-3). Currently, twenty-four integrin receptors have been identified that are organized into four main families based on receptor-ligand interaction. Broadly, integrins function to mediate cell adhesion to the ECM as well as intracellular signal transduction (4). With the development of integrin-specific knockout (KO) approaches, integrins have been shown to play essential roles in tissue and organ development in numerous physiological systems (5). Currently however, the role of integrins in mediating cardiac development is not well defined.

During fetal development and in early adulthood, heart growth and function is critically dependent on the synthesis and remodeling of the ECM and the adhesion of cardiomyocytes to the ECM (6-8). These dynamic processes contribute to proper cardiac development by coordinating the arrangement of contractile elements, the cytoskeleton, endoplasmic reticulum and costameres, which are specialized domains in the sarcolemma (7). As integrins contribute to ECM remodeling in many tissues, these adhesion molecules are critical mediators of the structural organization of the cardiac ECM and influence the assembly of discrete structural components of the cardiomyocyte. Notably, integrins can regulate gap junction formation in the heart as shown by earlier observations showing that the expression levels of the α5β1 and β1
integrin influence the abundance of connexin 43, the major connexin found in the myocardium (9,10). Integrins contribute to normal myofibrillar organization and alignment since disruption of the α1β1 integrin inhibits the normal tissue-like pattern of cultured cardiomyocytes (11). Further, integrins play a key role in the transmission of mechanical forces between the cardiomyocyte cytoskeleton and the ECM (12,13). Integrin linkage to the Z-disk of the sacromeres via actin binding proteins including talin, vinculin, desmin, focal adhesion kinase (FAK) and α-actinin is critical for cardiac mechanotransduction (13). Maintenance of the mechanical integrity of the ECM and the transmission of mechanical signals from the ECM to cardiomyocytes is particularly important for cardiomyocyte maturation (14). Collectively, these studies indicate that integrins are important for coordinating the development of the cardiomyocyte.

The cardiac fibroblast is the most abundant cell in the myocardium (30-50% of the cell number) (15) but in spite of their numbers, the role of the fibroblast in cardiac development is not well defined. Cardiac fibroblasts are responsible for the production of collagen in the heart, a critical ECM protein necessary for myocardial integrity and function (16). Moreover, recent studies indicate that the cardiac fibroblast contributes to normal cardiac structure and function through paracrine signaling and intercellular interactions with cardiomyocytes (17). Dynamic interactions between fibroblasts and cardiomyocytes may involve integrin-mediated intercellular adhesion and signaling as the expression of the β1 integrin by embryonic cardiac fibroblasts directly influences cardiomyocyte proliferation (18). This work, while instrumental in demonstrating a novel relationship, assessed only the β1 integrin, the most common β subunit, making it unclear which are the predominant integrins involved in fibroblast-cardiomyocyte developmental processes.
In this study we sought to examine the contribution of the α11β1 (α11) integrin in mediating cardiac structure and function in mice. The α11 integrin binds preferentially to fibrillar type I collagen (19,20), the most abundant ECM cardiac protein (21). Further, while earlier analyses of human embryos demonstrated that the expression of the α11 integrin was restricted to non-muscle mesenchymal cells (22), more recent data show that the α11 integrin is expressed by adult human and rat cardiac fibroblasts (23) and was shown to be the predominant integrin expressed by fibroblasts (19). Accordingly, the purpose of the current study was three-fold: 1) to examine if other cells in the heart express the α11 integrin or whether it is cardiac fibroblast-specific, 2) to determine whether deletion of the α11 integrin in mice disrupts postnatal cardiac development and impairs cardiac function and lastly, 3) to assess whether the α11 integrin plays an important role in collagen deposition in the heart. Overall, the study findings provided here offer increased insight into the role of integrins in mediating cardiac developmental processes and continue to expand our understanding of the relationship between the ECM, integrins, fibroblasts and cardiomyocytes. Understanding the ways in which ECM-integrin interactions contribute to changes in the fibroblast and the cardiomyocyte may be critical for furthering our understanding of normal cardiac physiology, and ultimately, may be vital for improving therapeutic strategies.
Chapter 2

Literature Review

2.0 Cardiac Development

2.0.1 Embryology

Embryonic development of the heart is a complex process requiring precise spatiotemporal coordination. During mouse embryogenesis, formation of the heart and the cells that make up the heart is coordinated by three sets of cardiac progenitor cells arising from a common progenitor at gastrulation: 1) proepicardial, 2) primary heart field (1° HF) and 3) secondary heart field (2° HF) (24-26). Proepicardial progenitors were shown to give rise to smooth muscle cells and endothelial cells, which mainly migrate to and form the upper layer of the heart, known as the epicardium (27). Proepicardial progenitors may also give rise to fibroblasts and cardiomyocytes that migrate into the myocardium (28). The 1° HF progenitors give rise to cardiomyocytes, smooth muscle cells, conduction cells and endothelial cells, which migrate to and form the left ventricle (LV) and contribute to both the right and left atria (26). The last set of progenitors, 2° HF progenitors, give rise to identical cardiac cells as 1° HF progenitor cells, which migrate to and form the right ventricle and outflow tract and contribute to both the right and left atria (26). These cells first begin to form an organized 3-dimensional structure at embryonic day 7.5 (E7.5), where they become the 1° and 2° HF, together known as the cardiac crescent. By E8.0, cells from the cardiac crescent migrate and form the beginning of the linear heart tube, composed of both arterial and venous poles. The
linear heart tube continues to remodel and by E8.5 it begins to loop forming the primitive ventricles and atria and the outflow tract to the lungs. Finally, by E10.5 the four chambers of the heart are formed and are fully mature by E15 with the formation of the septum and valves (24).

2.0.2 Postnatal Changes

Following birth, the heart undergoes substantial changes to accommodate for significant alterations in arterial blood pressure, heart rate, tension and cell growth, processes that may be completely distinct from fetal development. Indeed, soon after birth, the rapid proliferation of cardiomyocytes during fetal life is thought to cease as cells become terminally differentiated, after which the predominant form of growth shifts from hyperplasia to hypertrophy (29,30). This is accompanied by metabolic changes (glycolysis shifts to fatty acid oxidation), increased ECM production as well as changes in the expression and organization of sarcomeric proteins (31). Moreover, the expression of genes related to cardiac developmental processes such as cell cycle progression, growth factors, transcriptional regulation, structural proteins and stress response factors are differentially expressed postnatally (32,33). One study demonstrated that of genes examined, 76% of embryonic genes and 22% of neonatal genes were differentially expressed in adult rat hearts (33). Another demonstrated that genes related to cell cycle and growth factors were reduced, while structural proteins and stress-related factors were increased in postnatal mouse hearts (32). Uniquely, the regulation of genes during the fetal to postnatal transition may involve epigenetic modifications. One study demonstrated that DNA methylation is important in regulating the expression of genes involved in heart growth and differentiation postnatally (34).
Together, current work suggests that significant changes occur in the heart after birth that require coordinated adaptation and that postnatal cardiac development processes may be distinct from embryonic processes.

2.0.3 The Extracellular Matrix

The interaction of cells with the ECM is essential for regulating cell and tissue function, organization, development and repair (35). In the heart, early embryonic development is characterized by rapid perturbations in cellular homeostasis, requiring precise cell-ECM coordination and remodeling. The ECM is critical for stabilizing the developing myocardium, providing a framework for normal muscular contraction and is important in the formation of valves and the cardiac skeleton (36). Furthermore, the ECM, once thought of as static, is now increasingly being recognized as playing an important role in tightly regulating cell migration, organization, differentiation and proliferation during formation of the embryo and anatomical structures of the heart. Indeed, several studies have shown that disruption of ECM components, ECM signaling or signaling that regulates ECM production can lead to cardiac developmental malformations (37-39).

The ECM and ECM remodeling is also critical in postnatal maintenance of heart structure and function. Immediately after birth, substantial changes in the hemodynamic load on the heart require significant alternations in the ECM to accommodate. As such, ECM production and degradation must be carefully regulated in order to maintain healthy cardiac tissue and normal function (40). Supporting this, alternations of ECM components, including collagens and fibronectins, has been demonstrated as an underlying mechanism contributing to heart failure in both humans and animals (41,42).
Due to the extensive amount of ECM in the heart, including collagens, fibronectin, laminins and other glycoproteins, the process of ECM remodeling and the contribution of ECM components to cardiovascular development is numerous and complex. To simplify this, the following paragraphs will describe only the contribution and importance of collagen, the main ECM protein in the heart (~85% of all ECM proteins), during cardiac development.

2.0.4 Importance of Collagen

In the developing heart several collagens are expressed. Collagen types I, III, V and VI are found in the ventricular myocardium, AV valves and the chordae tendineae (39,43). In addition, collagen II, IV, XI and XIII are found in developing valve structures and the tendinous apparatus and collagen XIV, XV and XVIII are also expressed in the developing and adult heart (37,44-46). The production of collagen is regulated mainly by fibroblasts, contributing to both synthesis and degradation, with turnover rate of collagen in the heart estimated to be between 80 to 120 days (47). Careful regulation of collagen homeostasis, as mentioned earlier, is critical for normal cardiac function by providing a structural framework for myocardial contraction and relaxation. While much is being investigated regarding the contribution of collagen to heart disease manifestation, relatively fewer studies have examined the role of collagen in the early stages of cardiac development.

Collagen type I is the main component of fibrils, providing tissues with tensile strength and serving as an anchorage for cells. Collagen type I has been shown to be important for AV valve development and maintenance of the elasticity and integrity of
the aortic vessel wall (39, 43). Mice that lack part of the collagen type I gene survive to adulthood but have increased incidence of aortic dissection and rupture due in part to fewer collagen fibrils and less compacted, irregular elastic lamellae (38). Moreover, complete knockout of the collagen type I gene is embryonically lethal due to rupture of developing vessels (48). Collagen type III also contributes to the formation of fibrils and may modulate the size of type I collagen fibrils, as co-localization with collagen type I was demonstrated in some tissues (49). Collagen type III is expressed by developing vessels, with deletion resulting in a similar phenotype to collagen type I, with rupture of blood vessels late in adulthood (50). Moreover, mice lacked proper development of collagen type I fibrils, suggesting it is critical for mediating fibrillogenesis in the heart. Interestingly, mutations in the collagen type III gene have been shown in humans with type IV Ehlers-Danlos syndrome, a disease leading to sudden aortic rupture (51).

Lastly, collagen type V is a minor component of fibrillogenesis, suggested to regulate fiber diameter and nucleation (52). Collagen type V is expressed throughout the mitral valve, as well as the annulus fibrosus, the fibrous skeleton of the heart (39). Deletion of the collagen type V gene in mice results in significantly decreased formation of collagen fibrils and is embryonic lethal due to cardiovascular abnormalities (53). Overall, it appears that the fibrillar collagens are critical for maintaining the structural integrity of blood vessels during cardiovascular development and that multiple collagens are necessary for the formation of collagen fibrils in the heart.

While sparse, studies have also shown collagen is important for the developing myocardium. Expression of collagen type VI, an important collagen for the formation of the microfibrillar extracellular network, was demonstrated in the myocardium of the developing heart (54). Its expression is thought to be important for providing tensile
strength to the surrounding tissue. Further, collagen type XIV, a fibril-associated collagen that predominately interacts with and adheres to collagen type I to promote fiber assembly (55), was shown to be expressed within the cardiac interstitium of the developing myocardium (46). Interestingly, mice lacking collagen type XIV demonstrate impairments in collagen fibril organization, defects in ventricular morphogenesis, cardiomyocyte and fibroblast development, which was associated with reductions in cardiac function (46). These studies highlight the importance of the collagen network for normal myocardial cell development and function.

2.0.5 Coordination of ECM Remodeling

Coordination of ECM homeostasis is regulated by a diverse family of proteolytic enzymes, known as matrix metalloproteinases (MMPs), and their inhibitors, the tissue inhibitors of the MMPs (TIMPs) (56,57). To date, >20 different MMPs have been identified and are broadly classified into two types: 1) those that are secreted into the extracellular space and 2) those that are membrane bound. Majority of MMPs are secreted into the extracellular space, binding to specific ECM proteins and once activated initiate ECM degradation. For example, MMP-1 and MMP-13 bind preferentially to and degrade the fibrillar collagens I-III (56). Degradation of ECM proteins is mediated by TIMPs, with four species currently identified that bind to and regulate MMP activity. TIMPs bind to the catalytic domain of active MMPs, preventing access to substrates and thus inhibiting proteolysis (57). MMPs and TIMPs expression is critical for mediating ECM remodeling in the heart, with studies demonstrating an importance in both developmental and disease processes (58,59). It was shown that mice deficient of TIMP-3 demonstrated evidence of dilated cardiomyopathy at 21
months of age in association with disrupted interstitial matrix deposition (59).
Furthermore, altered expression of MMPs and TIMPs was shown in human patients with dilated cardiomyopathy, suggesting a critical role in mediating adverse ventricular remodeling (58).

2.0.6 Role of the Cardiac Fibroblast

Cardiac fibroblasts account for 30-50% of the cells in the myocardium and about two-thirds of tissue volume (16). During embryonic development, cardiac fibroblasts originate from mesenchymal cells, whereas multiple sources give rise to cardiac fibroblasts in the neonatal heart, including epithelial-mesenchymal transformation, fibrocytes and bone-marrow derived cells (16). As mentioned earlier, cardiac fibroblasts are the main cells responsible for ECM production in the heart as well as influence ECM breakdown via the MMPs (57). As such, cardiac fibroblasts are crucial cells in the heart that participate in ECM regulation. Given that a balance between ECM synthesis and degradation is vital for maintaining proper myocyte and myofibrillar arrangement and function, the cardiac fibroblast has received major attention in mediating heart failure progression (21). A substantial amount of work has shown that the cardiac fibroblast contributes to worsening cardiac function via contributions to excess collagen deposition and differentiation into pro-fibrotic, highly contractile, myofibroblasts (15,21). Yet, as discussed in length above, regulation of ECM proteins is also critical during development of the heart. Despite this, our understanding regarding the contribution and importance of fibroblast-mediated ECM remodeling in cardiovascular development remains unclear.
Cardiac fibroblasts are ‘sense’ cells, responding rapidly to changes in the composition of the ECM and in response to mechanical cues. Situated alongside cardiomyocytes, cardiac fibroblasts are able to sense the physical properties of the extracellular environment and adjust to match the perceived stiffness of the surrounding tissue (60). For example, in response to pathological stimuli, such as pressure-overload, fibroblasts differentiate into highly contractile, stiff myofibroblasts (61). It has also been demonstrated that fibroblasts have a favoured level of mechanical load, as altering stiffness of fibroblasts grown on polyacrylamide gels changed proliferative responses (60). The interaction with the ECM has also been shown to influence the degree of cardiac fibroblast’s ability to adhere, spread and migrate in vitro (62). Furthermore, cardiac fibroblasts can transduce paracrine signals to neighbouring cells, including cardiomyocytes, to alter phenotypic information. Production of fibroblast-growth factor (FGF) by cardiac fibroblasts is critical for the regulation of proliferating cardiomyocytes during the early embryonic stages of cardiogenesis (63).

Expanding on this, Ieda et al. (2009) were one of the first to demonstrate that cardiac fibroblasts could induce changes in the adult cardiomyocyte and that this was dependent on the stage of fibroblast development (18). The authors demonstrated that embryonic cardiac fibroblasts co-cultured with cardiomyocytes induced cardiomyocyte proliferation via fibroblast-specific paracrine factors. In contrast, when the authors utilized adult cardiac fibroblasts instead of embryonic and repeated the co-culture, cardiomyocytes grew in size by not in number. Intriguingly, microarray analysis demonstrated unique gene profiles between embryonic and adult cardiac fibroblasts, with higher expression levels of ECM genes, including several members of the collagen family, found in embryonic cardiac fibroblasts. Together, results suggest that cardiac
fibroblasts are important mediators of cardiomyocyte development, and that changes are dependent on the physiological processes occurring in the heart during particular periods.

Other studies, while limited, have also demonstrated that cardiac fibroblasts may play an important role in mediating cardiac development. Molecules produced by fibroblasts, such as FGFs, have been shown to induce the expression of early cardiac transcription factors as well as ventricular specific markers (64,65). For example, FGF1 was shown to be required for the differentiation of multipotent precursor cells toward a cardiac myocyte lineage (65). Direct cardiac fibroblast-cardiomyocyte interactions via connexin 43 gap junctions may also be critical for intercellular communication (66,67).

In support of this, movement of membrane-impermeant dyes and calcium fluxes has been shown between fibroblasts and cardiomyocytes (68). Moreover, fibroblasts may be involved in electrical communication with cardiomyocytes. Interestingly, cardiac fibroblasts were shown in culture to mediate myocyte electrical synchrony and automaticity by altering depolarization (69-71). Further, cardiac fibroblasts were shown to modulate the degree of ion conductance and cation flux following mechanical stretch (72). It also appears that the relationship between cardiac fibroblasts and cardiomyocytes works in reverse, with studies showing cardiomyocytes can actively influence cardiac fibroblast fate. Angiotensin II secretion by cardiomyocytes following mechanical stretch was shown to induce cardiac fibroblast proliferation and increased collagen deposition (73,74). In conclusion, current work suggests that cardiac fibroblasts can modulate cardiomyocyte development through both signaling molecules as well as direct cell-to-cell communication, and that this process may work in reverse, highlighting the dynamic cellular interplay involved in cardiac development.
2.1 Heart Structure and Function

2.1.1 Structure of the Cardiac Sarcomere

The sarcomere is the basic unit of cardiac muscle responsible for contraction of the myocardium. The cardiac sarcomere is a highly organized cytoskeletal structure composed of myosin (thick filaments) and actin (thin filaments), which are involved in cross-bridge activity and muscular contraction, as well as a vast number of regulatory proteins (75). A sarcomere unit is defined as a segment between two neighbouring Z-disks, which cross-link thin filaments via tight interactions with α-actinin. Thick filaments, on the other hand, sit in the middle of the sarcomere and are attached to Z-disks via the protein titin. Once thought of as just a simple mechanical link, the Z-disk is now being understood as a vastly complex area of protein-rich structures that sense and respond to mechanical stress via signal reception and transduction, respectively (75). An increasing number of molecules have been identified on or in the vicinity of the cardiac Z-disk, some of which have been shown to be critical for normal cardiac function and maintenance. For example, loss of MLP, a specific cytoplasmic LIM protein, results in severe cardiac dysfunction and ventricular remodeling indicative of human dilated cardiomyopathy (76). Titin is also an important protein that mediates the assembly and passive mechanical properties of the sarcomere. Mice lacking the titin gene develop diastolic dysfunction, as evident by reduced sarcomeric length and increased passive tension of cardiomyocytes (77). Changes in the ratio of titin isoforms has also been noted as an underlying mechanism responsible for diastolic dysfunction (78). In addition, intermediate filaments, including desmin, are organized densely along the Z-
disk of the sarcomere (12). Intermediate filaments play a critical role in the alignment of myofilaments by organizing the way Z-disks line up as well as provide a support network for muscular contraction and relaxation. Mice lacking desmin demonstrate evidence of cardiomyopathy, with increased diastolic pressure and lower active force generation, indicating an essential role in the heart (79).

### 2.1.2 Systolic and Diastolic Heart Function and Failure

Cardiac function is a tightly regulated process that is made up of two phases: 1) systole (contraction) and 2) diastole (relaxation). Systole occurs when an action potential from the sinoatrial node propagates to the apex of the heart and back up, initiating an influx of calcium (Ca$^{2+}$) that binds to troponin C causing tropomyosin to reveal actin-binding sites, thus allowing myosin to bind to actin and initiate cross-bridge activity. Regulation of systole function is complex, requiring a balance of ECM proteins, myocardial structure, neurohumoral input and gene expression (80). A well-known cause of systolic dysfunction is myocardial infarction, which results in loss of blood flow to an area of the myocardium leading to death of cardiomyocytes and changes in the ECM, ultimately resulting in alterations in size, shape and function of the myocardium. In diastole, Ca$^{2+}$ is taken back up by the sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA), causing tropomyosin to change its conformation back, which blocks actin-binding sites and ceases cross-bridge activity. Myocardial relaxation is therefore dependent on cross-bridge detachment and removal of Ca$^{2+}$ from the cytosol. Supporting this, alterations in SERCA and its modulator phospholamban have been implicated in contributing to altered Ca$^{2+}$ transient and delayed cardiac relaxation (81,82). Furthermore, inability of the myocardium to elastically recall after contraction is also thought to reduce cardiac
relaxation, which may be due to alterations in the structural or biochemical features of the ECM (77,83,84).

Despite similar signs, symptoms and prognosis clinically, heart failure with reduced ejection fraction (HFREF), also referred to as systolic heart failure, and heart failure with preserved ejection fraction (HFpEF), also referred to as diastolic heart failure, are characteristically distinct. HFREF results from impaired contractile or pump function, whereas HFpEF is associated with disrupted ventricular relaxation, compliance or filling (85). Left ventricular autopsies from HFREF patients demonstrated markedly increased dilation with no increase in wall thickness, while in HFpEF the cavity size is normal or decreased and wall thickness is markedly increased (86). Biologically, HFREF hearts are characterized by a lengthening myocyte and an increase in myocyte length/width ratio, whereas HFpEF hearts are described by an increase in myocyte cross-sectional area with little or no change in length/width ratio (78,87). Both are characterized by changes in collagen, mainly increases, however HFREF is also linked to a disruption and degradation of fibrillar collagen, whereas HFpEF has been mainly attributed to increases in fibrillar collagen (88). This is associated with increases in MMPs in HFREF and decreases in HFpEF (89).

Functionally, echocardiography is used as a non-invasive clinical tool to distinguish between HFREF and HFpEF through the measurement of ejection fraction (EF). However, a major limitation of EF is it cannot be interpreted as a reflection of contractility in the absence of knowledge about preload and afterload, which is referred to as being load-dependent (90). As such, pressure-volume hemodynamic relations have been developed an alternative approach to assess ventricular performance
independent from loading conditions (Figure 1). In HFREF, a downward and rightward shift of the end-systolic-pressure-volume relationship (ESPVR) indicates reductions in contractile function, which is associated with a decrease in stroke volume and reduced ejection fraction. In HFpEF, an upward and leftward shift of the end-diastolic-pressure volume relationship (EDPVR) indicates ventricular stiffness and reduced compliance, characterized by a disproportionate and greater increase in pressure for any increase in volume (90).

Figure 1. Pressure-volume loops demonstrating changes associated with systolic and diastolic dysfunction. (Left) Systolic dysfunction. The slope of the ESPVR (top line), which is the slope of the maximum pressure that can develop for a given volume, becomes less steep and shifts to the right as contractility decreases. (Right) Diastolic dysfunction. The slope of EDPVR (bottom line), which is the minimum pressure that can develop for a given volume, becomes steeper and shifts to the left as ventricular compliance decreases and stiffness increases. (Middle) Normal, for comparison. LV, left ventricular. Adapted from (90).
2.2 Integrins

Integrins are a family of transmembrane adhesion receptors composed of non-covalently bonded α and β subunits (Figure 2) (2,91,92). The α subunit is composed of a head (most commonly the I domain) linked to a seven-bladed β-propeller, which contains various active domains, connected to a thigh, a calf 1 and a calf 2 domain. The β subunit, which is characteristically distinct from the α subunit, is composed of a plexin-semaphorin-integrin (PSI) domain, a hybrid domain and a β1 domain connected to four cysteine-rich epidermal growth factor (EGF) repeats. The I domain of the α subunit is bound directly to and its conformation is regulated by the β subunit I-like domain (2,91,92). Collectively, the integrin family is comprised of 18 α and 8 β subunits, forming 24 different combinations, and can be grouped according to four types of receptors: (1) collagen, (2) leukocyte-specific, (3) laminin and (4) Arg-Gly-Asp (RGD; Figure 3) (2).

With the advent of integrin specific KO approaches, integrins have been shown to contribute to distinct and critical functions in diverse cellular and developmental processes, including cell growth, differentiation and survival, due to integrins dual function as cell adhesion molecules and intracellular signal transducers.
Figure 2. Schematic of an integrin receptor highlighting the components that comprise the α and β subunits. (Left) Bent integrin conformation (inactivated), which is unfolded to reveal the components that makeup the α and β subunits (Right). In most integrins, the α subunit is comprised of a calf 1 and calf 2 domain joined to a thigh domain and β-propeller, whereas the β subunit is made up of a β-tail domain connected to a PSI, hybrid and βA-domain. The ‘head’ of the heterodimeric complex is facilitated by the non-covalently bonded β-propeller of the α subunit with the βA-domain of the β subunit, which serves as an area for ligand binding. Both the α and β subunits contain a transmembrane and cytoplasmic domain, which attaches to the cytoskeleton of the cell. Permission granted from (108).
**Figure 3. Integrin Family.** Integrins are grouped into four main families: (1) collagen ($\alpha\beta_1$, $\alpha\beta_2$, $\alpha10\beta_1$ and $\alpha11\beta_1$), (2) leukocyte-specific ($\alpha4\beta_1$, $\alpha9\beta_1$, $\alphaL\beta_2$, $\alphaM\beta_2$, $\alphaX\beta_2$, $\alphaD\beta_1$, $\alpha4\beta7$ and $\alphaE\beta7$), (3) laminin ($\alpha3\beta_1$, $\alpha5\beta_1$, $\alpha6\beta_1$, $\alpha7\beta_1$ and $\alpha7\beta4$) and (4) RGD ($\alpha5\beta_1$, $\alpha8\beta_1$, $\alphaIl\beta3$, $\alphaV\beta3$, $\alphaV\beta5$, $\alphaV\beta6$ and $\alphaV\beta8$). Adapted from (110).

Cell adhesion with the ECM is formed by most cell types, many of which are mediated by integrins and the intracellular interaction with the actin cytoskeleton (91-93). Integrins are the primary adhesion receptors involved in the formation of focal adhesions, elongated areas of the cell periphery. Integrins mediate transmembrane linkage and regulation of focal adhesions by acting as a mechanosensor and transducing signals via FAK as well as the Rho and Rac family of GTPases (94). The adhesion of cells to the ECM is critical for determining cell migration and fate through...
mechanical cues and cell signaling. Adhesion mediated by the α6β1 and αvβ1 integrins were shown to influence the degree of stem cell differentiation (95). Further, fetal lung mesenchymal cells null of the α8β1 integrin fail to form stable adhesions and have increased migration (96). Studies have also demonstrated cell adhesion is important for maintaining cell and tissue morphogenesis by providing structural stability. Mice deficient of the α7β1 integrin develop progressive muscular dystrophy due to impairment of tendinous junctions, which are necessary structural components for proper muscular contraction (97). Furthermore, mice null of the α8β1 integrin demonstrate impairments in kidney morphogenesis (98). Lastly, in addition to their roles as ECM-cell adhesion molecules, integrins also participate in cell-to-cell adhesion. Ligand bridging was shown between resting and active αIIbβ3 platelets and between αIIbβ3 and other integrin bearing cells (99). However, it appears that only select integrins are capable of cell-to-cell adhesion, as it was discovered that the β1 integrins do not participate in direct homophilic or heterophilic interactions, despite prior reports (100).

Aside from cell-ECM and cell-to-cell adhesion, due to their transmembrane structure, integrins participate in diverse signaling pathways, referred to as ‘outside-in’ and ‘inside-out’ signaling (Figure 4). In ‘outside-in’ signaling, direct ligand binding, including collagens, laminins, fibronectin and other extracellular matrix proteins, initiates a cascade of intracellular signals that influences a large host of molecules, such as kinases (ERK, Akt, Raf, MEK, FAK, JnK), small GTPases (Rho, Rac, Ras, Cdc42) and cytoskeletal organizers (Paxillin) (101). In doing so, integrins can mediate a multitude of cellular processes. For example, α2β1 integrin binding was shown to activate Ras homolog gene family, member A (RhoA), whereas α3β1 binding inhibited RhoA, each
responsible for different effects on cell motility (102). Integrin-ligand binding also activates the production of MMPs, responsible for matrix reorganization (103). The second form of signaling, ‘inside-out’ signaling, allows other molecules and external stimuli to influence the degree of integrin activation and affinity for ligand binding. Here, external activating stimuli, including collagen binding, selectin ligation and cytokine binding to G-protein coupled receptors, as well as other protein families, such as transforming growth factor (TGF)-β, activate intracellular signaling cascades that facilitate the docking of focal adhesion components, such as Talin and kindlins (23,104-106). These changes lead to conformational modifications in the cytosolic and extracellular domain of the integrin complex, ultimately regulating integrin activation and ligand binding affinity. ‘Inside-out’ signaling is especially important in platelet adhesion involving the αIIbβ3 integrin, where activation facilitates and inhibition prevents platelet aggregation (107). As such, the αIIbβ3 integrin is tightly regulated and activated only when needed. In sum, as a result of both cell adhesion and signal transduction, integrins are involved in a dynamic array of cellular processes and are integral members of diverse cell systems.
**Figure 4. Schematic demonstrating dynamic integrin signaling.** (Right) Outside-in signaling occurs when ligands, including collagen and fibronectin, bind and activate integrins, inducing a cascade of intracellular signaling. Outside-in signaling influences a dynamic array of cellular and developmental processes, including cell polarity, survival and proliferation as well as cytoskeletal structural organization. (Middle) Bent conformation of an inactivated integrin. (Left) Inside-out signaling occurs when intracellular proteins, including talin and kindlins, bind to the tail of the β subunit, influencing integrin activation and affinity for ligand binding. Inside-out signaling is critical for cell adhesion and migration as well as ECM assembly. Permission granted from (109).
2.2.1 Collagen Binding Integrins

As shown in Figure 3, there are four integrins, α1β1, α2β1, α10β1 and α11β1, which recognize and bind to collagen (summarized in Table 1). Of note, α1β1, α2β1 and α11β1 integrins are expressed in fibroblasts, with the α11β1 integrin being the predominant integrin present (19,111,112). The α10β1 integrin is the only collagen integrin that is not found in fibroblasts, but rather is found in chondrocytes, specifically in the cartilaginous matrix in bone and other tissues (113). α1β1 and α2β1 integrin expression has also been demonstrated in cardiomyocytes (114-116). Regarding ligand binding, both the α2β1 and α11β1 integrins are docking sites for collagen type I, whereas the α1β1 and α10β1 integrins are predominately associated with collagen type IV (19,111) As such, the collagen binding integrins share similar, yet distinct biological functions. The α1β1, α2β1 and α11β1 integrins have been shown to participate in collagen remodeling and turnover, with α1β1 and α2β1 integrins important for maintaining the contractility of collagen (19,103,117-121). α1β1, α2β1 and α11β1 integrins also influence fibroblast-myofibroblast differentiation (23,117,119). α10β1 is distinct from the rest of the collagen integrins in that it has a major role in growth plate morphogenesis and function in bone (113).
Table 1. Characteristics of the collagen-binding integrins.

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligand / Recognition Sequence</th>
<th>Cell Type(s)</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1</td>
<td>Collagen IV &gt; Collagen I</td>
<td>Fibroblasts Cardiomyocytes</td>
<td>Collagen remodelling and contraction, myofibroblast differentiation, cardiomysocyte alignment, vascular plasticity and strength</td>
<td>(117,122)</td>
</tr>
<tr>
<td></td>
<td>(GFOGER); Collagen IX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2β1</td>
<td>Collagen I &gt; Collagen IV</td>
<td>Fibroblasts Cardiomyocytes</td>
<td>Collagen remodelling and contraction, myofibroblast differentiation, re-epithelization</td>
<td>(118,120,121)</td>
</tr>
<tr>
<td></td>
<td>(GFOGER); Collagen IX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α10β1</td>
<td>Collagen IV &gt; Collagen VI &gt;</td>
<td>Chondrocytes</td>
<td>Growth plate morphogenesis and function</td>
<td>(113)</td>
</tr>
<tr>
<td></td>
<td>Collagen II (GFOGER); Collagen IX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α11β1</td>
<td>Collagen I &gt; Collagen IV</td>
<td>Fibroblasts</td>
<td>Collagen remodelling and turnover, myofibroblast differentiation</td>
<td>(19,23,103,119)</td>
</tr>
<tr>
<td></td>
<td>(GFOGER); Collagen IX</td>
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</tbody>
</table>

2.3 Integrins in Normal Cardiac Physiology

Integrins are fundamental components in the interaction between the ECM and cardiac cells, orchestrating changes in remodeling of the cardiac ECM (3,7). During early cardiovascular development, the ability of cells to interact with the ECM through integrins is critical for normal cell migration and morphogenesis (7). Several studies have demonstrated that inhibiting or disrupting integrin-ECM interactions impairs normal cell migration, differentiation, proliferation as well as organization (18,123-126). For example, inhibition of the αvβ3 integrin impairs the endocardial to mesenchymal cell transition required for normal valve development (126). Interestingly, integrin expression
during cardiac development is not static, but tightly regulated, suggesting remodeling of the cardiac ECM by integrins occurs at critical periods. Studies examining the embryonic expression of integrins have noted upregulation during early embryonic development followed by a loss of expression during a critical cell-cell transformation and subsequent downregulation as the heart matures (115,116,127). Expression of α1β1 and α2β1 integrins, for example, has been demonstrated in early fetal and neonatal cardiomyocytes with no detectable expression in adult cardiomyocytes (114,115). Furthermore, different areas of the heart express different integrins and the expression of integrins in areas of the heart changes overtime (128). A good example of this is the α6β1 integrin, where early expressed was noted only in the myocardium, later in the both the endocardium and myocardium and eventually by birth the expression is limited only to the endocardium (92). While still an area of ongoing investigation, current results suggest that (1) integrins are essential for normal cardiovascular development, (2) integrin expression is dynamic and changes throughout cardiovascular development, (3) integrins involved in cardiovascular formation may be different and distinct from those involved in maintenance and lastly, (4) different cells and areas of the heart express different integrins. The following paragraphs will highlight the known roles integrins play in mediating cardiac fibroblast and cardiomyocyte interactions during development.

2.3.1 Collagen Remodeling via Fibroblasts

Cardiac fibroblasts play a necessary role in maintaining ECM homeostasis, through synthesis of ECM proteins as well as secretion of proteases, such as MMPs, involved in ECM degradation (57). Of ECM proteins, collagen type I and type III are functionally the
most prevalent in the heart (type I > type III), providing the necessary framework, mechanical support and chemical signals for normal cardiomyocyte arrangement and function (4,50). Numerous studies have shown that integrins are critical coordinators of collagen matrix organization. α1β1, α2β1 and α11β1 are necessary for normal fibroblast attachment to collagen type I, as well as contraction and anchoring to type I collagen matrices (19,112,118,129). Inhibition of α1β1 resulted in a 40% reduction of fibroblast attachment to collagen type I (112). Blocking α2β1 also resulted in reduced cellular processes and contraction in fibroblasts suspended in a matrix of type I collagen (118). Furthermore, α11β1 was shown to coordinate collagen matrix reorganization and anchoring during periods of mechanical strain by regulating ECM enzymes, including MMP-13 (103). While α1β1, α2β1 and α11β1 all appear to contribute to collagen remodeling via fibroblasts, differences in regional binding to collagen recognition sites amongst these integrins results in varied degrees of adhesion to fibroblasts and cell attachment to collagen, highlighting the complexity of this relationship (129). While not all of the above work was demonstrated in cardiac fibroblasts, these studies suggest that integrins may be critical coordinators of the cardiac ECM by mediating fibroblast-collagen processes.

2.3.2 Cardiomyocyte Development

The cardiac ECM is essential for normal cardiomyocyte function, specification as well as cell-cell communication and organization during cardiomyocyte development (3,11,112,130). Integrins act as critical linkages between the cardiac ECM and the cardiomyocyte and affect the organization of discrete structural components of the cardiomyocyte. α1β1 was shown to be required in order for neonatal cardiomyocytes
plated in collagen lattices to develop tissue-like organizational patterns (11). Blocking the α1β1 integrin inhibited the formation of the rod-like cell shape of plated neonatal cardiomyocytes as well as reduced myocyte adhesion to collagen and laminin. This study was the first to suggest that integrins actively communicate phenotypic information to the cardiomyocyte, which in turn is necessary for proper cellular organization and cell-cell alignment (131). Integrins may also regulate gap junction formation in the heart, as the α5β1 and β1 integrins were shown to influence connexin 43 expression, the major myocardial connexin (9,10). Further, integrins mediate mechanotransduction between the cardiomyocyte and the ECM, which is important for cardiomyocyte growth and maturation (13,14). Integrins also appear to be critical for normal cardiac development. Shai et al. (2002) demonstrated that ventricular myocyte-restricted deletion of the β1 integrin in mice led to profound increases in fibrosis, disrupted myocardial organization and cardiac function, ultimately resulting in heart failure (132). Similarly, Li et al. (2012) showed that cardiac-myocyte specific excision of the β1 integrin gene in the adult mouse impaired cardiomyocyte mechanotransduction and disrupted the response of cardiomyocytes to adrenergic stimulation (133). Together, these studies highlight the necessity of integrins for mediating developmental changes in the cardiomyocyte.

2.3.3 Fibroblast-Cardiomyocyte Proliferation

While the above studies demonstrate a diverse role of integrins in cardiac fibroblast-mediated ECM remodeling and cardiomyocyte development, our understanding of the ways in which the cardiac ECM signals through integrins to affect the combined nature of fibroblast-cardiomyocyte interactions remained unclear. Ieda et al. (2009) were the
first to demonstrate that cardiac fibroblasts could induce changes in the cardiomyocyte and that this was dependent on intercellular interactions with integrins, highlighting the importance of cell-to-cell and integrin-cell interactions in the heart (18). Embryonic cardiac fibroblasts co-cultured with cardiomyocytes were shown to induce cardiomyocyte proliferation through fibroblast-secreted ECM proteins fibronectin and collagen as well as heparin-binding EGF-like growth factor (HBEGF). Uniquely, cardiomyocyte proliferation was reduced following pre-treatment with an anti-β1 integrin antibody, suggesting β1 integrin signaling is important for this process. This affect was also noted in the presence of HBEGF as well as cell attachment to fibronectin or collagen, highlighting the necessity of the β1 integrin for propagation of cardiac ECM signaling. Cardiac specific deletion of the β1 integrin in mice also resulted in reduced cardiomyocyte proliferation and growth of the myocardium at embryonic day 12.5, coincident with the development of cardiac fibroblasts. In all, study findings suggest a dynamic relationship between the ECM-integrins-fibroblasts-cardiomyocytes that are necessary for normal communication and subsequent cardiac development (Figure 5).
Figure 5. Integrins mediate fibroblast-cardiomyocyte interactions during cardiac development. (Left) Integrins located on cardiac fibroblasts mediate both cell attachment to collagen as well as collagen remodeling, contributing to proper function of cardiac fibroblasts as well as coordination of ECM remodeling. (Right) Integrins located on cardiomyocytes contribute to transmission of mechanical signals required for growth and maturation, cell alignment of myofibrils as well as structural organization, which is necessary for normal cardiac development and function. (Middle) Together, integrins mediate ECM-signals (yellow arrow) from cardiac fibroblasts that induce cellular changes in the cardiomyocyte.

2.4 The α11β1 Integrin

Despite the historical characterization and knowledge of integrin molecules, the α11 integrin was discovered in 1995 by a group in Norway (134). Originally termed the αmt integrin, four years later the same group characterized this integrin, discovering it contained a β1 integrin subunit and re-classified it as the 11th α subunit (22). Since then,
the α11β1 integrin was shown to be a collagen receptor, binding more preferentially and mediating cell adhesion to type I collagen (19). Here, the α11 integrin was demonstrated to be important for collagen organization and cell migration. Not surprisingly, the α11 integrin was later discovered to be expressed predominately by collagen-producing fibroblast cells, with the earliest work demonstrating a unique function in fibroblast-mediated collagen remodeling of the periodontal ligament (20). The following paragraphs will highlight the current knowledge regarding the α11 integrin, including overview of the α11 gene, protein and promoter constructs, known expression in the body and in vitro and in vivo studies demonstrating its role in ECM remodeling.

2.4.1 Gene and Protein Structure

The α11 integrin gene (ITGA11), in both humans and mice, is compromised of 30 exons and 29 introns (135). In humans, the α11 integrin gene is localized on chromosome 15q23, spanning 130 kb, whereas the mouse α11 integrin gene is located on chromosome 9, spanning 106 kb. The α11 integrin is composed of 1166 amino acids, with a typical α chain structure (22,135). On the extracellular side, the α11 integrin contains an I domain (195 amino acids long) between FG-GAP repeats 2 and 3, with 7 repeats in total, connected to a thigh, a calf 1 and a calf 2 domain. Ligand binding occurs via coordination of the metal ion-dependent adhesion site motif and three potential divalent cation binding motifs located on the I domain. Intracellularly, the α11 integrin is composed of a short cytoplasmic tail of 24 amino acids containing a Gly-Phe-Phe-Arg-Ser motif. Linking the extracellular and intracellular domains is a 23 amino acid long transmembrane domain. Homology between the human and mouse α11 integrin protein structure was determined to be 89% in total protein structure and 97% with the I
domain, demonstrating that, while some differences do occur, the protein is quite conserved across species (136).

2.4.2 Promoter

The α11 integrin proximal promoter, important for transcriptional activity, was demonstrated to span nt -176 and +25 nt in the 3 kb ITGA11 promoter region (137). Recent characterization has shown that the α11 integrin proximal promoter contains two Sp1 sites and an Ets-1 site that are necessary for α11 gene expression, with conservation evident between human and mouse (138,139). Supporting this, inhibiting Sp1, via the Sp1 inhibitor mithramycin A, reduced α11 RNA and protein levels in primary fibroblasts (139). Moreover, mutation and deletion analyses using luciferase reporter assays demonstrated Sp1 binding sites together with Ets-1 were needed for α11 integrin proximal promoter activity in mesenchymal cells (139). Interestingly, cytokines have been also shown to be involved in regulation of α11 integrin expression. The α11 integrin was revealed to contain a novel Smad 2/3 binding site located between -809 and -1300 nt of the α11 integrin promoter (140). Further, TGF-β1 was also shown to drive α11 promoter activity and this response was discovered to be dependent on a Smad-binding element at nt -182/-176 and a Sp1-binding site at nt -140/-134 of the α11 promoter (138).

2.4.3 Expression in the Body

The α11 integrin was first discovered to be expressed in human fetal muscle cells (134). Later, human embryos were examined to determine the distribution of α11 integrin across all tissues during human embryonic development (19). α11 protein and mRNA
expression was shown in various mesenchymal cells in areas of highly organized interstitial collagen networks, including cartilage along the ribs and distal parts of the limbs, around forming vertebrae and intervertebral discs as well as the cornea (19). However, despite prior work, no expression of the α11 protein could be detected in myogenic cells, including skeletal muscle tissue and cardiac tissue. Later, it was shown that the α11 integrin is indeed expressed in human and rat cardiac fibroblasts (23). One of the possibilities for the discrepancy between results may be that α11 integrin expression changes at different times during development. As such, a more thorough analysis of multiple time points during embryonic development may be required to get an accurate picture of where the α11 integrin is expressed in vivo. Supporting this, as mentioned earlier, other integrins have shown varied expression throughout development and into adulthood. α11 integrin expression was also analyzed in mouse embryos, where it was shown to be expressed in fibroblasts of the periodontal ligament, in tendons and intestinal villi fibroblasts (136). Further, melanoma and lung carcinoma tumours abundantly express the α11 integrin (141,142). A thorough analysis of α11 integrin expression in adult tissue has yet to be performed.

2.4.4 In Vitro Extracellular Matrix Remodeling

As mentioned earlier, the α11 integrin mediates cell attachment, contraction and anchoring to collagen. In particular, it was discovered that the α11 integrin binds preferentially to collagen type I and also interacts with collagen types IV and IX with less affinity (19). Specifically, the I domain of the α11 integrin recognizes a special triple-helical sequence known as GFOGER present in collagen type I (143). Collagen type I binding to the α11 integrin enhances α11 integrin activity and mediates downstream
signaling (119). In addition, the α11 integrin may also participate in collagen remodeling. Fibroblasts deficient of the α11 integrin displayed downregulation of a number of genes involved in collagen remodeling, including MMP-13 and cathepsin K (103). Furthermore, fibroblast cells cultured in a 3D collagen gel induced MMP-13 expression in an α11 integrin-dependent manner (103). Overall, it appears not only is the α11 integrin important for cell attachment to collagen but it also may be critical for regulating collagen homeostasis, which has vast implications in both normal and pathological human physiology.

Indeed, the α11 integrin may play a role in pathological conditions. Multiple studies have demonstrated that the α11 integrin mediates fibroblast-myofibroblast differentiation (23,119,140). The expression of the α11 integrin was shown to be upregulated following myofibroblastic transformation in human cardiac fibroblasts treated with methylglyoxal (MGO) and in human corneal fibroblasts treated with TGF-β1 (23,119). Moreover, inhibiting the α11 integrin reduced myofibroblastic differentiation, as evident by reductions in α-smooth muscle actin (α-SMA) expression. This was shown to be dependent on TGF-β signaling. Inhibiting the α11 integrin in human cardiac fibroblasts prevented the associated increased expression of TGF-β2 following MGO treatment (23). Moreover, in the same model, inhibition of Smad3 signaling via the SIS3, a specific inhibitor of Smad3 phosphorylation, reduced the associated increase in α11 integrin expression. Further, as mentioned earlier, the α11 integrin was revealed to contain a novel Smad 2/3 binding site on the α11 integrin promoter and TGF-β1 was also shown to drive α11 integrin promoter activity at two specific binding sites (138,140). Together, studies suggest that the α11 integrin is important in regulating myofibroblast
formation and that this is dependent on TGF-β signaling, highlighting a novel role of the α11 integrin in pathological remodeling (Figure 6).

![Diagram](image)

**Figure 6. The different functions of the α11 integrin.** (A) The α11 integrin mediates fibroblast adhesion, contraction and anchorage to collagen type I matrices. (B) Through activation of MMPs, the α11 integrin regulates collagen turnover. (C) The α11 integrin is also an important regulator of myofibroblast differentiation through TGF-β signaling.

### 2.4.5 *In Vivo* Extracellular Matrix Remodeling

A group in Norway, headed by Dr. Donald Gullberg, was the first to generate mice deficient of the α11 integrin and subsequently explore phenotypic changes following loss of this integrin (20). Loss of the α11 integrin is not embryonic lethal, with mice born viable and fertile. However, α11 integrin-deficient mice were reported as smaller and had increased mortality in comparison to both heterozygous and wildtype mice. Further, mice displayed impaired periodontal ligament organization, an important ligament during
rodent incisor eruption. As such, mice had delayed incisor eruption and altered tooth shape. This was thought to be due to impairments in collagen remodeling and organization, as fibroblasts lacking the α11 integrin in vitro displayed reductions in cell adhesion and spreading to collagen type I, reduced ability to retract collagen lattices and reduced cell proliferation. This was associated again with reductions in MMPs, particularly MMP-13, in both mutant tissue and α11 integrin-deficient fibroblasts. Thus, the authors conclude by saying the α11 integrin is particularly important for collagen organization of the periodontal ligament and that the α11 integrin is crucial for tooth eruption. No other defects were reported.

Aside from this study by Popova et al. (2007), exploration of the contributions of the α11 integrin to ECM remodeling in vivo is limited. In the heart, determining the major players of ECM remodeling is critical for the potential discovery of therapeutic strategies aimed at reducing fibrosis development. To my knowledge, only one study has assessed the contribution of the α11 integrin in mediating fibrosis development in the heart. Talior-Volodarsky et al. (2012) demonstrated in a model of diabetic cardiomyopathy in rats that the α11 integrin was increased in association with increases in interstitial myocardial fibrosis (23). As demonstrated earlier, this study also showed that α11 integrin expression correlated with myofibroblastic differentiation in vitro through TGF-β mediated signaling, suggesting it may mediate fibrosis progression. While this study was instrumental in demonstrating this relationship, it remains unclear whether the α11 integrin is an important mediator of collagen deposition and myofibroblastic transformation outside of the diabetic setting. As such, exploring this association in other heart disease models, such as pressure-overload due to transverse
aortic constriction (TAC) or myocardial infarction, is needed to further our understanding of the biological and clinical importance of the α11 integrin.

2.5 Research Gap and Addressing

2.5.1 Role of the α11 Integrin in the Heart

The main finding from the first exploration of the α11 integrin null mice was in regards to the periodontal ligament organization and subsequent tooth eruption. Other organ systems were not studied in subsequent detail. It therefore remains unclear whether the α11 integrin is important for cardiac development. Utilizing a germline mutation of the α11 integrin, this study sought to determine whether the α11 integrin is an important integrin required for cardiac structure and function, which has previously been unexplored. In doing so, study findings will further our understanding of the contribution of integrins in postnatal cardiac development. Furthermore, it remains unclear what cells express the α11 integrin in the heart. Current studies have shown that the α11 integrin is expressed in cardiac fibroblasts with conflicting reports on whether it is also expressed by myogenic cells. Utilizing a magnetic isolation technique, study findings will help clarify what cells express the α11 integrin in the heart, which is vital for understanding its potential contribution to cardiac developmental processes.
2.5.2 Predominant Integrins Involved in Fibroblast-Cardiomyocyte Interactions

The paper by Ieda and colleagues (2009) was instrumental in showing that integrins may be involved in a complex dialogue between cardiac fibroblasts and cardiomyocytes during cardiac development (18). However, it remains unclear which are the predominant integrins involved in this relationship. The authors assessed only the contribution of the β1 integrin subunit, which, as illustrated in Figure 3, is the most common β subunit to a vast number of integrins. Therefore, examining the contribution of specific integrins to cardiac developmental processes is critical for furthering our knowledge of the relationship between integrins and cells in the heart. In this study, we focused on the contribution of the α11 integrin and examined postnatal developmental changes to the myocardium and functioning heart. Out of the collagen-binding integrins, the α11 integrin binds most preferentially to collagen type I, the main ECM protein in the heart, was shown to be expressed by cardiac fibroblasts and participates in ECM remodeling in the heart, suggesting the α11 integrin is a good candidate to play a role in cardiac development. Further, examining the role of the α11 integrin will allow for more finite exploration regarding the importance of integrins in the heart. Collectively, study findings may further our understanding of the dynamic interplay between the ECM, integrins, fibroblasts and cardiomyocytes, emphasizing the complex interactions that are required for normal cardiac development.
2.5.3 Fibroblast-mediated Collagen Remodeling via the α11 Integrin

The ECM is a critical component of cardiomyocyte development and function in both health and disease, yet our understanding of the critical mediators of ECM remodeling in the heart is unclear. The α11 integrin, given its affinity for collagen type I, may be an important regulator of collagen remodeling in the heart. Currently however, little is understood regarding the influence of the α11 integrin in mediating interstitial collagen deposition during both embryonic heart development and following disease progression. To address this, my thesis will determine whether collagen remodeling is impaired following deletion of the α11 integrin, and in reverse, whether the α11 integrin is enhanced following fibrotic development in the heart. If the α11 integrin is shown to play a major role, then study findings may open the door to future investigation examining whether it is possible to manipulate the α11 integrin or molecules involved in α11 integrin signaling to prevent fibrosis development in failing hearts. Moreover, these results will potentially further highlight the biological significance of the α11 integrin in both health and disease.
2.6 Research Aims and Hypotheses

1. To determine what cells express the α11 integrin in the heart

Current work has demonstrated the α11 integrin is expressed in human and rat cardiac fibroblasts (23). However, earlier work has shown that the α11 integrin is also expressed in human fetal myocytes, suggesting this integrin may also be expressed by myogenic cells (134). It is therefore unclear in mice what cells in the heart express the α11 integrin. Based on current work, it is hypothesized that the α11 integrin will be expressed specifically in cardiac fibroblasts and not in other cells in the heart.

2. To examine whether the absence of the α11 integrin disrupts cardiomyocyte development and impairs cardiac function postnatally

Studies have shown that various integrins are critical for cardiomyocyte development and cardiac function (3,11,130). Deletion of the β1 integrin lead to disrupted myocardial organization and impaired cardiac function, subsequently resulting in heart failure (132,133). The findings from Ieda et al. (2009) further demonstrate a role of the β1 integrin subunit in mediating paracrine signaling induced by fibroblasts, which is required for normal cardiomyocyte hyperplasia (18). Given that the α11 integrin binds to the most abundant and functionally prevalent collagen in the heart and if it is determined that the integrin is expressed solely on cardiac fibroblasts, then it is hypothesized that deletion of the α11 integrin may affect intercellular communication between cardiac fibroblasts and cardiomyocytes, leading to disrupted development of cardiomyocytes, and ultimately, impaired cardiac function.
3. To assess whether the α11 integrin is important for interstitial collagen deposition in the myocardium.

The α11 integrin was previously shown to participate in collagen type I remodeling \textit{in vitro} by regulating MMP expression (103,119). Furthermore, the α11 integrin was also shown to be important for normal fibroblast adhesion to collagen type I as well as the formation of collagen type I lattices (20). Given the role of the α11 integrin in collagen remodeling and cell attachment to collagen, it is hypothesized that: (1) interstitial collagen in the myocardium of mice null of the α11 integrin will be reduced and, (2) in reverse, fibrosis development in the heart will be associated with increases in α11 integrin expression. The ability of the α11 integrin to regulate collagen matrix formation in the myocardium may highlight a novel role of the α11 integrin in the heart and may explain the impairments in cardiac function in the mice null of the α11 integrin, if evident.
Chapter 3
Research Aim #1

3.0 Hypothesis and Aims

3.0.1 Hypothesis

It is hypothesized that the $\alpha_{11}$ integrin will be expressed specifically in cardiac fibroblasts and not in other cells in the heart.

3.0.2 Specific Aims

The specific aim of this chapter is to determine the expression of the $\alpha_{11}$ integrin in the heart by separating cardiac fibroblasts from other cells in the heart and then determining protein abundance of the $\alpha_{11}$ integrin. Determining whether the $\alpha_{11}$ integrin is cardiac fibroblast restricted is important for setting the framework of the subsequent chapters examining and discussing the potential role and function of the $\alpha_{11}$ integrin.
3.1 Methods

3.1.1 Mouse Cardiac Fibroblast Isolation

Cardiac fibroblasts were isolated from mouse hearts, as previously described (23). In brief, adult (~10 weeks of age) C57BL/6 mice ($n = 10$) were euthanized with CO$_2$ and hearts were excised immediately and placed in ice-cold PBS containing antibiotics. Ventricles were washed, minced (1 mm$^3$ pieces), and digested with trypsin and bacterial collagenase to produce cell suspensions that were used for purification of mouse cardiac fibroblasts. Cells were incubated with rat anti-mouse antibody to CD90 (recognizing Thy-1) coupled to magnetic microbeads (Miltenyi Biotec Inc., CA, USA) to purify fibroblasts (Figure 7). Concentrations of antibodies were used as recommended by the company protocol. This study was approved by the University of Toronto Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).
Adult C57 Mice (n=10)

Trypsin and Collagenase

1° Anti-mouse CD90 (Thy-1.1)
Magnetic Microbeads

2° Anti-mouse Magnetic Microbeads

Fibroblast

Magnet

Cardiomyocytes & Other Cells (NF)

Fibroblasts (F)

Miltenyi Biotech Magnetic Column

Figure 7. Illustration of the isolation of cardiac fibroblasts from adult C57BL/6 mice. Adult C57BL/6 hearts were isolated, minced (1 mm³ pieces), and digested with trypsin and bacterial collagenase. Cell suspensions were then incubated with primary anti-mouse to CD90 (recognizing Thy-1) magnetic microbeads, washed in PBS buffer and then coupled to secondary anti-mouse magnetic microbeads. Cells were once again washed in PBS buffer and then added to a Miltenyi Biotech Column attached to a magnetic base. All cells in which the magnetic microbeads did not attach to [cardiomyocytes and other cells (NF)] flow down the column and were collected in one tube, whereas the cells in which the magnetic microbeads attached to remained in the column [fibroblasts (F)]. The column was then removed and the remaining cells [fibroblasts (F)] were collected in a separate tube.
3.1.2 Immunoblotting

Isolated fibroblast and non-fibroblast cells were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, MO, USA) and extracts were generated by mechanically homogenizing samples. Protein content was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., ON, CA) and BSA, as standard. Lysates were resolved by 8% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 4% milk and immunoblotted for α11 integrin (1:500, R&D Systems, Inc., MN, USA), cardiac troponin T (1:1000, Abcam, Inc., ON, CA), vimentin (1:1000, Cell Signaling Technology, MA, USA), and GADPH (1:5000, Cell Signaling Technology, MA, USA) overnight at 4°, incubated with appropriate HRP-conjugated secondary antibodies and detected by the ECL System (Amersham, Buckinghamsire, UK).
3.2 Results

3.2.1 α11 Integrin is Exclusive to Cardiac Fibroblasts

To confirm successful isolation of cardiac fibroblasts, fibroblast and non-fibroblast lysates were immunoblotted for vimentin (fibroblast marker) and cardiac troponin T (cardiomyocyte marker). Vimentin expression was robust in the fibroblast fraction, with minimal expression in the non-fibroblast fraction (Figure 8A). Consistent with these results, cardiac troponin T was expressed only in the non-fibroblast fraction with no expression evident in the fibroblast fraction (Figure 8B). To examine whether the α11 integrin is cardiac fibroblast specific or is expressed in other cells in the heart, both the fibroblast and non-fibroblast fractions were immunoblotted for the α11 integrin. A band was detected only in the fibroblast cell fraction, suggesting the α11 integrin is restricted to cardiac fibroblasts (Figure 8C).
Figure 8. The $\alpha_{11}$ integrin is restricted to cardiac fibroblasts. Immunoblotting images of vimentin (A, ~57 kDa), cardiac troponin T (B, ~40 kDa) and $\alpha_{11}$ integrin (C, ~150 kDa), utilizing GAPDH as a loading control (~37 kDa). Fibroblast cell fraction (F) was compared to non-fibroblast cell fraction (NF).
3.3 Discussion

3.3.1 Isolation of Mouse Cardiac Fibroblasts

In the current study, magnetic microbeads were utilized with antibodies recognizing Thy 1 to isolate mouse cardiac fibroblasts. Isolated cells were vimentin positive and cardiac troponin T negative, indicative of successful cardiac fibroblast isolation. In mice, Thy 1 is also expressed by other cells, including peripheral T cells, epidermal cells and keratinocytes (144). Special care was taken to insure that all blood, fat and connective tissue was removed from dissected hearts prior to digestion with trypsin and collagenase so that only cells in the myocardium were extracted. While we cannot rule out non-specific binding of Thy 1 to other cells in the heart other than cardiac fibroblasts, previous work has demonstrated that Thy 1 is a good marker for cardiac fibroblast isolation. Ieda et al. (2009) demonstrated that Thy 1 positive cells expressed increased mRNA expression of fibroblast-markers including vimentin, discoidin domain receptor 2 (DDR2), periostin and fibroblast specific protein-1, while expression of cardiomyocyte markers such as Nkx2.5 and α-actinin 2 were low in comparison to Thy 1 negative cells (18). Furthermore, Thy 1 positive cells were correlated with vimentin and DDR2 positive cells during fibroblast development in utero (18). Moreover, another study demonstrated that cultured cardiac fibroblasts demonstrated positive expression of both DDR2 and Thy 1 when analyzed by flow cytometry (145). Results were also confirmed in ventricular sections and in cultured cardiac fibroblasts via immunohistochemistry (145). Therefore, current results suggest that Thy 1 is a valid marker for isolation of cardiac fibroblasts.
3.3.2 α11 Integrin is Expressed in Cardiac Fibroblasts in the Heart

After isolating mouse cardiac fibroblasts, immunoblotting determined that α11 integrin was expressed in cardiac fibroblasts in the heart and not in other cells, including cardiomyocytes. Previously, the α11 integrin was shown to be expressed in human and rat cardiac fibroblasts as well in whole hearts (23). Similar to this study, Talior-Volodarsky et al. (2012) also performed cardiac fibroblast isolation in rat hearts utilizing magnetic microbeads recognizing Thy 1 and demonstrated similar findings with α11 integrin expression evident only in the fibroblast cell fraction (23). Interestingly, prior work revealed that the α11 integrin was not expressed in human embryonic cardiac tissue analyzed at 4 and 8 weeks (19). It is thought that the discrepancy in results is due to differential expression of the α11 integrin during different stages of cardiac development. A thorough analysis of α11 integrin expression in embryonic development of the heart is necessary to determine whether this is the case. Overall, current results are in line with prior work in the heart, further supporting the notion that the α11 integrin is expressed specifically in cardiac fibroblasts across species.

3.3.3 Limitations

Given the α11 integrin was only recently characterized, there is a lack of commercially available products recognizing the α11 integrin. This impeded the ability to utilize alternative methods, such as immunofluorescence or flow cytometry, to further demonstrate that the α11 integrin is cardiac fibroblast specific. Furthermore, we did not examine or include major blood vessels, such as the aorta, in tissue preparation for cell digestion. We therefore cannot rule out that the α11 integrin may be expressed in cells
compromising the major blood vessels. Lastly, vimentin is also expressed in smooth muscle and endothelial cells in the heart and is not restricted solely to cardiac fibroblasts. Utilization of other markers, such as DDR2, may have been useful to confirm successful isolation of cardiac fibroblasts. However, majority of studies have shown and have used vimentin as a marker for cardiac fibroblasts (16). In addition, as mentioned above, isolation of cardiac fibroblasts utilizing Thy 1 has been demonstrated in multiple studies.
Chapter 4
Research Aim #2

4.0 Hypothesis and Aims

4.0.1 Hypothesis

Given that the α11 integrin binds to the most abundant and functionally prevalent collagen in the heart and is expressed solely on cardiac fibroblasts, it is hypothesized that deletion of the α11 integrin may affect intercellular communication between cardiac fibroblasts and cardiomyocytes, leading to disrupted development of cardiomyocytes, and ultimately, impaired cardiac function.

4.0.2 Specific Aims

The specific aims of this chapter are to utilize mice null of the α11 integrin to: (1) determine whether normal development, focused mainly on the heart, is impaired postnatally, (2) examine changes in postnatal cardiomyocyte growth, (3) determine whether the structural organization of cardiomyocytes is disrupted and lastly, (4) assess whether there are reductions in cardiac function. Ultimately, utilizing a germline mutation of the α11 integrin will help to establish the potential role of the α11 integrin in postnatal cardiac development and function.
4.1 Methods

4.1.1 α11 Integrin Null Mice

α11 heterozygotic mice were acquired from Dr. Gullberg (University of Bergen, Norway) as previously described (20). In brief, parts of the exon 3 and intron 3 of ITGA11 were replaced with an internal ribosome entry site, lacZ, and a PGK neo cassette by gene targeting. This was introduced into ES cells by electroporation, resistant colonies were injected into the blastocysts of C57BL/6J mice and chimeric males were mated with C57BL/6J mice to generate α11 heterozygotic mice. Acquired α11 heterozygotic mice were then bred to generate homozygous mice (KO−/−) and littermate controls (WT+/+), which were confirmed at 3 weeks of age by genotyping. DNA from ear notches were prepared (Sigma-Aldrich, MO, USA) and amplified using the following primers: forward: CCATCATGACAGGAGACGTATACAA; reverse: TGGTCAGTGGATGGGTTAGGAAG. Animals were housed at constant room temperature (21±1°C) with a 12-h light/dark cycle and were fed mashed chow and water ad libitum. To overcome the lack of periodontal ligament development observed earlier in α11 KO−/− mice (20) that affects their eating and growth, mice were fed mashed instead of hard chow to enable normal growth. Mice were sacrificed at 4 and 8 weeks to examine developmental changes in the myocardium. This study was approved by the St. Michael's Hospital Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).
4.1.2 Histology and Immunostaining

For overall morphology, whole hearts were isolated, immediately fixed in 10% formalin for 4-8 hours and embedded in paraffin. Paraffin-embedded longitudinal and transverse sections (4 µm thick) were prepared and stained with hematoxylin and eosin. For measurements of cross-sectional area, de-paraffinized sections were stained with wheat germ agglutinin (WGA; 1:100, Life Technologies, ON, CA) and nuclei were stained with DAPI (1:1000, Cell Signaling Technology, MA, USA). Ten, 40 x images at the level of the subendocardium of the LV were imaged and the cross-sectional area of >50 randomly chosen circular-shaped nucleated cardiomyocytes were measured (146) using AxioVision software (Version 4.8.2).

Immunofluorescence staining for desmin (1:50, Cell Signaling Technology, MA, USA) and staining for fluorescent WGA (1:100, Cell Signaling Technology, MA, USA) was used to assess intermediate filament localization and cardiomyocyte alignment, respectively. Whole hearts were embedded in OCT, frozen immediately in liquid nitrogen and stored at -80°C; 8 µm thick sections were cut and fixed in 4% PFA for 45 minutes, washed in 1x PBS and blocked with Protein Block Serum-Free for 1 hour (Dako Canada, ON, CA) before incubating with primary and fluorescent secondary antibodies. Immunostaining for connexin 43 was used to localize gap junctions (1:400, Cell Signaling Technology, MA, USA). Paraffin sections were dewaxed, boiled in citrate buffer (pH 6.2-6.5), washed in 1x PBS, blocked for 1 hour with Protein Block Serum-Free (Dako Canada, ON, CA) and incubated with primary and secondary antibodies.
4.1.3 Electron Microscopy

Dissected hearts were fixed in Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.3) at 4°C for 4 h, washed three times in 0.1 M sodium cacodylate buffer, post-fixed in 2% OsO₄ in 0.1 M sodium cacodylate for 90 min at room temperature (21°C), and washed three times in 0.1 M sodium cacodylate buffer. Samples were embedded in Epon 812 resin. Thin sections were placed on nickel grids, stained with uranyl acetate and lead citrate and examined with an electron microscope (Hitachi High Technologies America, Inc.).

4.1.4 Echocardiography

All echocardiographic assessments were done using the Vevo® 2100 system and a MS-400 Red probe (VisualSonics, Ontario, CA). Echocardiography assessments were performed at end-study at 4 and 8 weeks using the Vevo® 2100 Visual Sonics software. In brief, animals were placed on a heating pad to maintain a body temperature of 37 ± 1 °C and secured in the supine position. A MS-400 Red probe was then placed on the heart and both long axis and short axis images were taken by adjusting either the probe or heating pad or both.

4.1.5 Cardiac Catheterization

Cardiac catheterization was performed to examine pressure and volume changes at 8 weeks (end study), as previously published (147). In brief, animals were placed on a heating pad to maintain a body temperature of 37 ± 1 °C, intubated and placed on a respirator. Mice were secured in a recumbent position (sagittally) and the right jugular
vein was cannulated. Pressure was calibrated after warming the catheter (Model SPR-838; Millar Instruments, Texas, USA) in 0.9% NaCl at 37 °C for 30 minutes. The right internal carotid artery was then identified and ligated cranially. A 2F miniaturized combined conductance catheter-micro-manometer was inserted into the carotid artery to obtain aortic blood pressure, and then advanced into the LV until stable pressure–volume (PV) loops were obtained. The abdomen was opened and the inferior vena cava and portal vein identified. A cotton-tip applicator was pressed on these vessels to allow rapid reduction in cardiac preload. All loops were obtained with the respirator turned off for 5–10 s and the animal apneic. Data were then acquired under steady state conditions and during preload reduction. Calibration from relative volume units conductance signal to absolute volumes (in µl) was undertaken using a previously validated method of comparison to known volumes in Perspex wells (148). Using the pressure conductance data, a range of functional parameters was then calculated (Millar analysis software PVAN 3.4).

4.1.6 Statistical Analysis

An unpaired t-test was used to assess differences between α11 KO−/− and α11 WT+/+ at both 4 and 8 weeks. Statistical significance was accepted with a value of P less than 0.05. All statistical tests were performed using the software program GraphPad Prism (version 5, GraphPad Software, Inc.).
4.2 Results

4.2.1 α11 KO⁻/⁻ Mice Exhibit Delayed Development That Normalizes Towards Adulthood

Consistent with previous data, α11 KO⁻/⁻ mice were born normally and displayed no major developmental abnormalities (20). At 4 weeks, the α11 KO⁻/⁻ mice were slightly smaller in comparison to the α11 WT⁺/⁺ mice (Table 2). Body weight (BW) and tibial length (TL) were reduced in the α11 KO⁻/⁻ mice (both measures \(P<0.01\), Table 2). However, at 8 weeks, the α11 KO⁻/⁻ mice appeared to normalize, demonstrating no obvious phenotype in comparison to the α11 WT⁺/⁺ mice (Table 1). TL and BW were similar between mice \((P>0.05\), Table 2). Mortality was not observed in either group.

Table 2. Phenotypic characteristics of the α11 WT⁺/⁺ and α11 KO⁻/⁻ mice.

<table>
<thead>
<tr>
<th></th>
<th>4 weeks α11 WT⁺/⁺</th>
<th>4 weeks α11 KO⁻/⁻</th>
<th>8 weeks α11 WT⁺/⁺</th>
<th>8 weeks α11 KO⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>11</td>
<td>18</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>13.3 ± 1.2</td>
<td>11.7 ± 1.5 **</td>
<td>30.1 ± 7.3</td>
<td>32.2 ± 10.9</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>71.7 ± 5.6</td>
<td>62.9 ± 5.9 ***</td>
<td>131.9 ± 30.3</td>
<td>126.8 ± 28.8</td>
</tr>
<tr>
<td>Lung Weight (mg)</td>
<td>108.8 ± 18.6</td>
<td>100.9 ± 16.6</td>
<td>160.1 ± 27.8</td>
<td>165.7 ± 40.0</td>
</tr>
<tr>
<td>Tibial Length (mm)</td>
<td>14.0 ± 0.5</td>
<td>13.5 ± 0.7 *</td>
<td>14.9 ± 0.9</td>
<td>14.5 ± 0.9</td>
</tr>
<tr>
<td>HW: TL (mg: mm)</td>
<td>5.1 ± 0.3</td>
<td>4.7 ± 0.5 **</td>
<td>8.9 ± 2.3</td>
<td>8.8 ± 2.2</td>
</tr>
<tr>
<td>LW: TL (mg: mm)</td>
<td>7.7 ± 1.2</td>
<td>7.5 ± 1.2</td>
<td>10.8 ± 2.3</td>
<td>11.5 ± 3.1</td>
</tr>
</tbody>
</table>

\(N\), number per group; HW, heart weight; LW, lung weight; TL, tibial length; HW:TL and LW:TL indicates the ratio of wet heart and lung weight to tibial length, respectively. Data are presented as mean ± SD. \(^*P<0.05\) when compared to α11 WT⁺/⁺. \(^{**}P<0.01\) when compared to α11 WT⁺/⁺. \(^{***}P<0.001\) when compared to α11 WT⁺/⁺.
4.2.2 \( \alpha 11 \text{KO}^{/-} \) Hearts Normalize Towards Adulthood and Exhibit No Cardiac Defects

We next examined whether loss of the \( \alpha 11 \) integrin affects cardiac development. Gross morphological analysis revealed no major differences in the formation of the atrium, ventricles and interventricular septum at 4 and 8 weeks (Figure 9). Both longitudinal and cross-sectional sections of the heart revealed similar morphology between mice (Figure 9). Similar to the phenotypic data, hearts collected from the \( \alpha 11 \text{KO}^{/-} \) mice were smaller than the \( \alpha 11 \text{WT}^{+/+} \) mice at 4 weeks and by 8 weeks this difference was normalized. Heart weight (HW) and HW indexed to TL were reduced in the \( \alpha 11 \text{KO}^{/-} \) mice at 4 weeks \((P<0.001, P<0.01, \text{respectively, Table 2})\). In addition, structural assessment obtained by echocardiography revealed reductions of LV mass, LV wall thickness, end-diastolic area, end-diastolic volume as well as LV internal diameter in diastole at 4 weeks (all measures \(P<0.05, \text{Table 3})\). By 8 weeks, HW and HW indexed to TL as well as structural parameters obtained by echocardiography were not significantly different between mice \((P>0.05, \text{Table 2 and 3})\).
Table 3. Echocardiographic characteristics of the α11 WT<sup>+/−</sup> and α11 KO<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th></th>
<th>4 weeks</th>
<th>8 weeks</th>
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<tbody>
<tr>
<td></td>
<td>α11 WT&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>α11 KO&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>50.0 ± 8.2</td>
<td>42.9 ± 6.7</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.60 ± 0.07</td>
<td>0.55 ± 0.05&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.62 ± 0.05</td>
<td>0.57 ± 0.06&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.6 ± 0.2</td>
<td>3.4 ± 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.4 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>EDA (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>8.3 ± 0.7</td>
<td>7.4 ± 1.0&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>ESA (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>3.5 ± 1.0</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>53.3 ± 8.0</td>
<td>47.1 ± 6.7&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>20.0 ± 4.8</td>
<td>16.4 ± 5.1</td>
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LVM, left ventricular mass; LVAWd, left ventricular anterior wall thickness in diastole; LVPWd, left ventricular posterior wall thickness in diastole; LVIDd and LVIDs, left ventricular internal diameter in diastole and systole, respectively; EDA and ESA, end-diastolic and end-systolic endothelial area, respectively; EDV and ESV, end-diastolic and end-systolic volume, respectively. Values are means ± SD. <sup>*</sup>P<0.05 when compared to α11 WT<sup>+/−</sup>. 

Figure 9. Morphology of the heart is similar in the α11 KO−/− mice. H & E stained heart sections illustrating overall heart morphology in the α11 WT+/+ and α11 KO−/− mice in longitudinal (A-B) and cross-sectional orientation (C-D) at 4 and 8 weeks, respectively. LAA, left atrial appendage; RAA, right atrial appendage; LV, left ventricle; RV, Right Ventricle. Magnification 2x. Scale bar 250 µm.

4.2.3 Cardiomyocyte Size is Reduced in α11 KO−/− Mice

LV sections from α11 KO−/− and α11 WT+/+ mice were then co-stained with WGA and DAPI to determine whether the size of cardiomyocytes was affected by the expression of α11 integrin (Figure 10). At 4 and 8 weeks, cardiomyocyte cross-sectional area in the endocardium of the LV was decreased in the α11 KO−/− mice compared with the α11
WT\textsuperscript{+/−} mice (\(p<0.0001\) and \(p<0.05\), respectively; Figure 10C, F). Further, electron micrographs of LV sections from 8-week-old α11 KO\textsuperscript{−/−} and α11 WT\textsuperscript{+/−} mice showed reduced cardiomyocyte thickness in the α11 KO\textsuperscript{−/−} mice (\(P<0.05\); Figure 11C).

![Figure 10. Cardiomyocyte cross-sectional area is reduced in the α11 KO\textsuperscript{−/−} mice.](image)

WGA (structural, green) + DAPI (nuclei, blue) stained LV sections illustrating myocyte size in the α11 WT\textsuperscript{+/−} and α11 KO\textsuperscript{−/−} mice at 4 weeks (A-B) and 8 weeks (D-E). Quantification of cardiomyocyte cross-sectional area (\(\mu m^2\)) at 4 (C) and 8 weeks (F). Values are means ± SEM. *\(P<0.05\) when compared to α11 WT\textsuperscript{+/−}, ****\(P<0.0001\) when compared to α11 WT\textsuperscript{+/−}. Magnification 40x. Scale bar 50 \(\mu m\).
Figure 11. Impaired cardiomyocyte development in the α11 KO−/− mice at 8 weeks.

Representative transmission electron micrographs assessing cardiomyocyte morphology in the LV of the α11 WT+/+ mice (A) compared with the α11 KO−/− mice (B). Quantification of cardiomyocyte thickness (nm, C) and distance between Z-lines (nm, D). Values are means ± SEM. *P<0.05 when compared to α11 WT+/+. Magnification 9600x. Scale bar 500 nm.

4.2.4 Deletion of the α11 Integrin Impairs the Structural Organization of Cardiomyocytes

As integrins contribute to the maintenance of ECM integrity in cardiac tissues, we next assessed whether deletion of the α11 integrin affects the cytoskeletal organization of
cardiomyocytes. Compared with α11 WT^{+/+} mice, immunostaining for desmin in LV sections of α11 WT^{+/+} mice showed marked clustering of desmin intermediate filaments, particularly located at regions of intercalated disk formation (Figure 12). LV sections that were immunostained for connexin 43 also showed prominent clustering of, and reduced distance between, gap junctions in the α11 KO^{-/-} mice compared with the α11 WT^{+/+} mice (Figure 13). Further, WGA-stained LV sections showed impaired cardiomyocyte membrane organization in the cardiomyocytes of α11 KO^{-/-} mice, which was in contrast to the normal, aligned cardiomyocyte membranes of α11 WT^{+/+} mice (Figure 14). Lastly, electron micrographs of LV sections from 8-week-old α11 KO^{-/-} and α11 WT^{+/+} mice demonstrated changes in sarcomere length in the α11 KO^{-/-} mice (P<0.05; Figure 11D).
Figure 12. α11 KO−/− cardiomyocytes demonstrate intermediate filament clustering.

Desmin stained LV sections illustrating intermediate filaments (bright horizontal white lines) in the α11 WT+/+ and α11 KO−/− mice at 4 weeks (A-B) and 8 weeks (D-E). White arrows illustrate areas of intermediate filament clustering along the cardiomyocyte.

Magnification 40x (A-B); Magnification 25x (C-D). Scale bar 50 µm (A-B); Scale Bar 75 µm (D-E).
Figure 13. α11 KO−/− cardiomyocytes exhibit altered connexin 43 organization.

Connexin 43 stained LV sections illustrating gap junctions (brown) in the α11 WT+/+ and α11 KO−/− mice at 4 weeks (A-B) and 8 weeks (D-E). Black arrows illustrate clustering of gap junctions as well as reduced distance between gap junctions along the α11 KO−/− cardiomyocytes. Magnification 400x. Scale bar 50 µm.
Figure 14. $\alpha_{11}$ KO$^{-/-}$ cardiomyocytes display evidence of impaired membrane organization. WGA stained LV sections illustrating longitudinal cardiomyocyte structure (white) in the $\alpha_{11}$ WT$^{+/+}$ and $\alpha_{11}$ KO$^{-/-}$ mice at 4 weeks (A-B) and 8 weeks (D-E). (B, D) $\alpha_{11}$ KO$^{-/-}$ cardiomyocytes appear wavy and chaotic when compared to the normal, well-aligned cardiomyocytes of the $\alpha_{11}$ WT$^{+/+}$ mice (A, C). Magnification 40x (A-B); Magnification 25x (C-D). Scale bar 50 $\mu$m (A-B); Scale Bar 75 $\mu$m (D-E).
4.2.5 Loss of the α11 Integrin Impairs Diastolic Function

Finally, we examined whether the loss of the α11 integrin alters cardiac function. With the use of invasive cardiac catheterization, PV loop hemodynamic analysis revealed impairments in diastolic function in the α11 KO⁻/⁻ mice at 8 weeks (Table 4, Figure 15). Steady-state PV loop analysis showed increased end-diastolic pressure (EDP) in α11 KO⁻/⁻ mice (P<0.01; Table 4). Further, α11 KO⁻/⁻ mice exhibited reduced diastolic relaxation in the active phase, indicated by a 28% increase in the time constant of relaxation Tau (P<0.05; Figure 15D). α11 KO⁻/⁻ mice also showed evidence of compromised diastolic relaxation in the passive phase, as measured by the EDPVR, which was increased by ~150% in the α11 KO⁻/⁻ mice compared with the α11 WT⁺/⁺ mice (P<0.05; Figure 15C). Likewise, a load-sensitive measure of diastolic function, the minimum rate of pressure change (dP/dtₘᵢₙ), was increased (less negative) in the α11 KO⁻/⁻ mice compared with wild type (P<0.05; Table 4), further demonstrating reduced diastolic function. While there was a trend for measures of systolic function such as the maximum rate of pressure change (dP/dtₘₐₓ) and pre-load recruitable stroke work, these were not statistically significant (P>0.05; Table 4). Finally, there was no difference in end-systolic pressure, ESPVR, ejection fraction or heart rate between mice (P>0.05, Table 4). Due to the small weight of the mice at 4 weeks we could not perform PV loop analysis at this time point.
Table 4. Cardiac catheter acquired hemodynamics in the α11 WT^{+/+} and α11 KO^{-/-} mice at 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>α11 WT^{+/+}</th>
<th>α11 KO^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (BPM)</td>
<td>395 ± 60.9</td>
<td>374.7 ± 53.2</td>
</tr>
<tr>
<td>ESP (mm Hg)</td>
<td>92 ± 11.3</td>
<td>84.3 ± 9.2</td>
</tr>
<tr>
<td>EDP (mm Hg)</td>
<td>6.8 ± 1.7</td>
<td>13.6 ± 1.5**</td>
</tr>
<tr>
<td>dP/ dt_{max} (mm Hg/s)</td>
<td>7301.7 ± 1763.6</td>
<td>5558.3 ± 1466.4</td>
</tr>
<tr>
<td>dP / dt_{min} (mm Hg/s)</td>
<td>-6609.2 ± 1273.8</td>
<td>-4808.2 ± 966.4*</td>
</tr>
<tr>
<td>ESPVR (mmHg/µL)</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>PRSW (mm Hg)</td>
<td>70.2 ± 10.9</td>
<td>61.25 ± 33.5</td>
</tr>
<tr>
<td>EF (%)</td>
<td>60.2 ± 6.9</td>
<td>62.0 ± 12.2</td>
</tr>
</tbody>
</table>

BPM, beats per minute; EDP, end systolic pressure; ESP, end systolic pressure; dP/dt_{max}, the maximal rate of pressure change; dP/dt_{min}, the minimum rate of pressure change. PRSW, preload recruitable stroke work. EF, ejection fraction. Values are means ± SD. *P<0.05 when compared to α11 WT^{+/+}. **P<0.01 when compared to α11 WT^{+/+}. 
Figure 15. Diastolic function is impaired in the α11 KO<sup>−/−</sup> mice. Representative pressure-volume loops during preload reduction in the α11 WT<sup>+/+</sup> (A) and α11 KO<sup>−/−</sup> mice (B). The steeper slope of the EDPVR (bottom blue line) in the α11 KO<sup>−/−</sup> mice (B) compared with the α11 WT<sup>+/+</sup> (A) indicates reduced chamber compliance. Quantified values of the slope of the EDPVR show elevation in the α11 KO<sup>−/−</sup> mice when compared to the α11 WT<sup>+/+</sup> mice (C). α11 KO<sup>−/−</sup> mice also exhibited significantly reduced diastolic relaxation in the active phase, indicated by a 28% increase in the time constant of relaxation Tau (D). *P<0.05 when compared to α11 WT<sup>+/+</sup>. 
4.3 Discussion

4.3.1 A Fibroblast Specific Integrin is Required for Normal Cardiomyocyte Growth, Alignment and Function

Despite the abundance of fibroblasts in the myocardium, accounting for the greatest percentage of cell number (15), relatively few studies have examined the importance of the fibroblast in regulating normal development of the cardiomyocyte, both in vitro and even fewer in vivo. Here we show for the first time that a fibroblast specific integrin, the $\alpha_{11}\beta_1$ integrin, is required for normal cardiomyocyte structure, organization and growth, which in turn is necessary for normal cardiac function. Cardiomyocytes examined from the $\alpha_{11}$ KO-/- mice demonstrated reductions in both in cross-sectional diameter and width, suggesting the $\alpha_{11}$ integrin plays a role in mediating normal growth of cardiomyocytes during postnatal development. Loss of the $\alpha_{11}$ integrin also impaired normal organization of key components of the cardiomyocyte and disrupted normal cardiomyocyte alignment. We also found the loss of the $\alpha_{11}$ integrin impaired normal cardiac function, with invasive PV loop analysis demonstrating a reduced ability of the $\alpha_{11}$ KO-/- hearts to relax during diastole. Collectively, these results suggest that the $\alpha_{11}$ integrin is a key component of the cardiac fibroblast that is required for the normal development of the cardiomyocyte and for regulating structural and functional development of the heart. These findings suggest a novel mechanism by which interactions between fibroblasts and cardiomyocytes, mediated through integrins, are important for cardiac development.
4.3.2 The Loss of the α11 integrin Does Not Result in Developmental Delay or Noticeable Cardiac Defects

The α11 KO−/− mice were previously characterized as dwarf, demonstrating reduced body weight throughout development, as well as displayed increased mortality (20). Here, we show that the α11 KO−/− mice normalize by 8 weeks, with no observed mortality evident in these mice. Arising from the lack of periodontal ligament development and delay in incisor eruption (20), the α11 KO−/− mice were not as able to masticate conventional hard chow. To overcome this, the α11 KO−/− mice were fed a soft chow and after 4 weeks they appeared to develop normally. Accordingly, the loss of the α11 integrin does not necessarily impair normal development, as previously thought. Given the changes in incisor eruption occur as early as 2 weeks, it is possible that the reduced development evident in the α11 KO−/− mice at 4 weeks may also be due to impaired feeding habits, as mice are not weaned until they are 4 weeks of age.

Furthermore, the lack of the α11 integrin did not appear to result in the presence of cardiac defects postnatally. The ventricles, atrium and interventricular septum appeared to develop normally in the α11 KO−/− mice, with no gross abnormalities present. Furthermore, echocardiography revealed no major changes in ventricular mass or dilation at 8 weeks, further suggesting the lack of the α11 integrin does not lead to disruptions in the morphology of the heart. Prior work demonstrated that deletion of the β1 integrin, the most common β subunit to a vast number of integrins, lead to abnormal heart development. One study demonstrated that hearts of β1 integrin null mice were severely dilated and ventricular walls were thicker, which was associated with robust
fibrosis (132). β1 integrin null mice also displayed impairments in myofibrils and intercalated disks, suggesting the β1 integrin is required for maintenance of cardiomyocyte and myocardial integrity. Furthermore, it was also shown, in a separate study, that loss of the β1 integrin in mice lead to reductions in ventricular development and enlargement in some atria, which was again associated with accumulation of interstitial fibrosis (18). Given that there were no cardiac abnormalities or changes to cardiac morphology evident in the α11 null mice, these results suggest that other β1 integrins may be involved in the spatiotemporal development of the heart.

4.3.3 The α11 Integrin May Be Important for Fibroblast Induced Cardiomyocyte Growth

It was previously demonstrated that adult cardiac fibroblasts co-cultured with cardiomyocytes could induce cardiomyocyte growth, including enhanced sacromeric organization and increased cell size, through specific fibroblast-derived factors (18). Furthermore, it was shown that adult cardiac fibroblasts display distinct gene expression profiles in comparison to embryonic cardiac fibroblasts, with a higher induction of hypertrophic factors, including a 58-fold increase in interleukin 6, evident in adult cardiac fibroblasts (18). Therefore, cardiac fibroblasts may play different roles during different periods of cardiac development, with adult cardiac fibroblasts suggested to be important in postnatal growth of cardiomyocytes. In line with these results, we demonstrate that deletion of the fibroblast-specific α11 integrin results in impaired postnatal growth of cardiomyocytes, indicating the α11 integrin is important for this process. WGA + DAPI stained LV sections revealed reductions in cross-sectional area
of cardiomyocytes, with reduced cardiomyocyte thickness also evident upon electron micrograph analysis.

Integrins have demonstrated, in diverse physiological systems, the ability to induce complex signaling pathways following mechanical activation and ligand binding (6,17,149,150). Integrins have been shown to induce a cascade of intracellular signals that influences a large host of molecules (151). Some of these molecules, in particular Akt, Ras, mitogen-activated protein kinases and TGF-β, have been shown to be key signaling molecules involved in physiological cardiomyocyte growth, suggesting integrins may mediate this process (151). Furthermore, in the absence of particular integrins, intracellular signal transduction has been shown to be impaired, further highlighting the importance of integrins for signaling processes (152,153). Integrins have also been shown to be directly responsible for physiological growth of cardiomyocytes. Overexpression of the β1D integrin induced growth of neonatal ventricular myocytes, with inhibition reducing this transformation (154). Most recently, cardiac fibroblasts have also been shown to secrete a variety of paracrine factors, some that have been previously demonstrated to also be influenced by integrins, including TGF-β1 (17). A well-known body of literature has also shown that mechanical signals from the ECM to the cardiomyocyte are necessary for maturation of cardiomyocytes (14). Integrins, including the α11 integrin, are known to play a vital role in mechanotransduction (6,119,155). α11 integrin expression was shown to be upregulated following mechanical activation via cell attachment to collagen type I, suggesting it is induced in a mechanosensitive manner (119). While speculative, it may be that the α11 integrin is required for propagation of fibroblast-mediated signaling, possibly following ligand activation via collagen type I binding or via
mechanotransduction. As such, without the α11 integrin normal communication between fibroblasts and cardiomyocytes is impaired, disrupting normal postnatal growth of cardiomyocytes. Future studies are necessary to determine the precise mechanism by which the α11 integrin mediates cardiomyocyte growth through the cardiac fibroblast.

4.3.4 The α11 Integrin May Be An Important Mediator of the Structural Organization of Cardiomyocytes

Myofibrillogenesis, the process of cardiac muscle formation, is a complex process that requires the precise arrangement of sarcomeric components and coordination with cytoplasmic components, including the cytoskeleton and the ECM (3). Integrins are suggested to play a critical role in linking the cardiac ECM and the cardiomyocyte cytoskeleton in a manner essential for normal myofibril attachment (7). One study showed that addition of anti-β1 integrin antibodies blocked spreading of cultured cardiac myocytes and disrupted normal myofibrillogenesis (156). The group also demonstrated that myofibril assembly overtime involved co-localization of the β1 integrin with α-actinin at regions of the Z disk and focal adhesions, suggesting integrins play a direct role in cardiomyocyte adhesion (156). In another study using neonatal rat ventricular myocytes, β1 integrin expression was discovered early in myofibrillogenesis during the formation of focal adhesions and costameres (157). Furthermore, integrins can link directly to the Z-disk via other cytoskeletal proteins, including talin, FAK, desmin, filamin, tensin and vinculin (3,7). In this study we demonstrate that localization of desmin, an intermediate filament involved in maintaining sarcomere architecture, was abnormal with significant clustering of intercalated disks present in the α11 KO−/−.
cardiomyocytes. In addition, we show that sarcomeric membrane alignment, as evident by WGA staining, was disrupted and chaotic in the α11 KO−/− cardiomyocytes. Thus, results suggest that the α11 integrin may be a critical mediator of the structural organization of cardiomyocytes. Still, given that the α11 integrin is not found on cardiomyocytes, the precise way in which the loss of α11 integrin disrupts discrete structural components of the cardiomyocyte remains unclear. Fibroblasts have been suggested to coordinate deposition of collagen at special regions along the Z-disk in conjunction with myofibrillogenesis, with studies demonstrating integrins are also present at this time (7). While speculative, it may be that the α11 integrin is important for coordinating collagen attachment during myofibrillogenesis, such that deletion disrupts ECM organization, ultimately impairing structural organization of cardiomyocytes. Further investigation is necessary to determine the precise nature of this relationship.

Connexin 43 expression is the most abundant connexin in the heart and is important for establishing electric and metabolic coupling between cardiomyocytes (158). It was previously shown that other integrins could influence connexin 43 expression. Overexpression of the α5β1 decreased connexin 43 expression (159), whereas loss of the β1 integrin led to upregulation (9). Furthermore, in another study in osteocytes, the α5β1 was required for opening of the connexin 43 hemichannel (149). In this study, loss of the α11 integrin also disrupted gap junction formation, as evident by increased clustering and reduced distance between connexin 43 channels. Still, the exact mechanism by which the α11 integrin affects connexin 43 expression remains unclear.
4.3.5 Normal Diastolic Function is Dependent on the α11 Integrin

Diastolic relaxation and ventricular filling is a complex process primarily determined by both active (ATP requiring) and passive (determined by chamber compliance) phases (81). At the molecular level, altered calcium handling and impaired ATP synthesis remains the key mechanisms by which active relaxation is prolonged (160). Passive LV properties, determined by the viscoelastic properties of the ventricle are primarily determined by cellular stiffness along with interstitial fibrosis (78). Our data demonstrated a marked impairment of diastolic function in the α11 KO⁻/⁻ mice, in both the active and passive phases. The assessment of cardiomyocyte size using multiple complementary techniques in α11 KO⁻/⁻ mice showed a significant reduction in cardiomyocyte cross-sectional area and thickness and increased sarcomeric length, in association with impaired diastolic function. The mechanism for impaired diastolic function in α11 KO⁻/⁻ mice remains elusive, however it is likely due to increased passive tension of cardiomyocytes, secondary to alterations in myofilamentary and cytoskeletal proteins which were disrupted in the α11 KO⁻/⁻ mice (160). This is supported by work showing that changes in sarcomeric length can have a substantial effect on cardiac function (87,161). Thus, small changes in fibroblast remodeling of the cardiac interstitium may be critical during cardiac development for normal diastolic function, a well-described concept in the context of heart failure (15,23,160).

Furthermore, changes in cardiac function following deletion of integrins have been reported elsewhere. Ventricular myocyte-restricted deletion of the β1 integrin in mice resulted in abnormal cardiac function (132). In particular, mice demonstrated increases (less negative value) in dP / dt_min, an index of cardiac relaxation, which was
also demonstrated in our study. Furthermore, mice had significantly decreased \( \text{dP} / \text{dt}_{\text{max}} \), an index of cardiac contractility. Mice in our study demonstrated small, albeit non-significant, change in \( \text{dP} / \text{dt}_{\text{max}} \), possibly indicating different functions of different \( \beta_1 \) integrins for cardiac function. They also demonstrate increases in EDP in \( \beta_1 \) integrin knockout mice, albeit non-significant. Another study, in which the \( \beta_1 \) integrin gene was deleted in a cardiac-myocyte specific fashion in the adult mouse, demonstrated that the deletion impaired both short- and long-term cardiomyocyte mechanotransduction and disrupted the response of cardiomyocytes to adrenergic stimulation (133). In particular, isolated cardiomyocytes demonstrated reductions in kinase signaling, including FAK and integrin-linked kinase, as well as multiple signaling pathways, including p38, Akt and extracellular signal-regulated kinase 1/2, following mechanical stimulation. In addition, adrenergic-mediated signaling through extracellular signal-regulated kinase, p38 and Akt were also reduced in the \( \beta_1 \) integrin deficient mice. Collectively, results support the role of the \( \beta_1 \) integrin in mediating cardiac function, with some results supporting and in line with current study findings.

4.3.6 Limitations

The current study utilized a germ line mutation of the \( \alpha_{11} \) integrin, which is a global whole KO approach. As such, all tissues in the body are devoid of the \( \alpha_{11} \) integrin, not just the heart, which raises the possibility of off-target effects. To our knowledge, the only characterized defect present in the \( \alpha_{11} \) KO\(^{-/-}\) mice was the lack of the periodontal ligament development and impeded tooth eruption (20), which we overcame by feeding the mice soft versus hard chow. While \( \alpha_{11} \) KO\(^{-/-}\) mice normalized at around 8 weeks, they were slightly smaller when examined at 4 weeks. It is possible that early
developmental delay may have affected some of the examined parameters. However, given the relatively small changes apparent, this possibility seems unlikely. To overcome this limitation in the future, we are currently in the process of breeding a fibroblast-specific conditional KO of the α11 integrin. Furthermore, we did not examine the expression levels of the other collagen-binding integrins in order to determine whether or not there may be compensatory changes in expression potentially affecting the observed phenotype. However, it was previously shown that inhibition of the α11 integrin did not result in changes in the expression patterns of the other collagen-binding integrins in human cardiac fibroblasts, suggesting this may also be the case in vivo (20,23).

We also did not examine the contribution of the α11 integrin to cardiac development in utero. Embryonic cardiac fibroblasts were shown to induce proliferation of cardiomyocytes when co-cultured, which was inhibited by the deletion of the β1 integrin (18). Thus, it seems plausible that the loss of the α11 integrin could impair cardiomyocyte proliferation in utero. Furthermore, given the suggested importance of integrins in ECM remodeling during fetal development of the heart, it is also possible the α11 integrin may coordinate components of cardiac development during different embryonic stages. Moreover, it would be interesting to explore the expression pattern of the α11 integrin during embryonic heart development and how this correlates with changes occurring the heart at particular periods. Future studies are necessary to determine the contribution of the α11 integrin to embryonic development of the heart.

Study analysis was also limited mainly to the LV. We did not thoroughly examine other components of the heart in the α11 KO−/− mice. While no gross cardiac defects
were present, it is possible other compartments of the heart are affected by the loss of the α11 integrin. Further examination is necessary to determine the contribution of the α11 integrin to other areas of the heart.

Lastly, we did not determine a molecular mechanism that links loss of the α11 integrin to reductions in cardiomyocyte development and overall cardiac function. The best model to examine this relationship would be a co-culture system of cardiomyocytes with fibroblasts similar to the Ieda et al. (2009) paper (18). Unfortunately, co-culturing cardiomyocytes with fibroblasts proved technically challenging and difficult. As a result, we were unable to establish a co-culture system with reliable efficiency. Utilizing a co-culture system and manipulating α11 integrin expression and potential signaling pathways will be useful to determine a mechanistic link to explain study findings.
Chapter 5

Research Aim #3

5.0 Hypothesis and Aims

5.0.1 Hypothesis

Given the role of the α11 integrin in collagen remodeling and cell attachment to collagen, it is hypothesized that: (1) interstitial collagen in the myocardium of mice null of the α11 integrin will be reduced and, (2) in reverse, fibrosis development in the heart will be associated with increases in α11 integrin expression.

5.0.2 Specific Aims

The specific aims of this chapter are to: (1) determine whether there are differences in the amount of collagen in the α11 KO−/− mice compared to the α11 WT+/+ mice and, (2) examine α11 integrin expression following pathological-induced fibrotic remodeling of the myocardium in humans and mice. Assessing whether the α11 integrin contributes to collagen deposition in the heart is an important finding that may explain the functional impairments witnessed in the α11 integrin null mice. Furthermore, this may highlight a fundamental role of the α11 integrin for collagen matrix formation in the heart, which has important implications in both cardiac developmental and disease processes.
5.1 Methods

5.1.1 Transverse Aortic Constriction

~10-week-old male mice with C57BL/6 background (n = 35) were acquired for experimental purposes (Charles River, Quebec, Canada; The Jackson Laboratory, Maine, USA). Female mice were not examined, as the hormone estrogen is known to have cardioprotective effects that may result in different cardiac remodeling (163). Animals were housed at constant room temperature (21 ± 1 °C) with a 12-h light/dark cycle and were fed standard mouse chow and water ad libitum. Following a one-week acquisition period, mice were divided into two groups: 1) sham (n = 14) and 2) TAC (n = 13). Constriction of the transverse aorta was performed as previously described (164,165). In brief, mice were anesthetized, midline sternotomy was performed, the aorta was visualized, and 6.0 Prolene suture was placed around the aorta distal to the brachiocephalic artery. The suture was tightened around a blunt 25-26-gauge needle placed adjacent to the aorta. Needle size was selected based on the weight of the mice at time of surgery (20-24g – 26-gauge needle, 25-30g – 25-gauge needle) as recommended to induce a 0.4 mm diameter constriction (164). The needle was then removed inducing a narrowing of the transverse aorta. Sham animals underwent a similar procedure with midline sternotomy and aorta visualization without tightening of the suture around the aorta. At 6 weeks post, all animals were euthanized. Following euthanasia, cardiac tissue from the apex of the heart was immediately frozen in liquid nitrogen and stored in -80°C for RNA and protein analysis, whereas midline sections were immediately fixed in 10% formalin for histological analysis. Study was approved by
St. Michael’s Hospital Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

5.1.2 Histology and Immunostaining

To assess total fibrillar collagen differences, sections were stained for Picrosirius Red (PSR). In brief, LVs were isolated from α11 KO−/− and α11 WT+/+ (8 week old, n = 6 per group; see 4.11 for generation of mice) and sham and TAC mice, immediately fixed in 10% formalin for 4-8 hours and embedded in paraffin. Paraffin embedded sections (4 µm thick) were prepared and stained for PSR. Ten, 16 x images at the level of the subendocardium of the LV were imaged and areas of red (associated with collagen) were selected for utilizing ImageScope software (Version 8.0.39.1065). Calculation of the proportional area stained per image was then determined utilizing ImageScope software and an average was calculated from the 10 representative images per animal and then per group.

To specifically examine differences in collagen type I, immunostaining for collagen type I (1:100, MD bioproducts, MN, USA) was also performed in paraffin embedded heart sections from α11 KO−/− and α11 WT+/+ mice. Further, to assess myofibroblastic differentiation, immunostaining for α-SMA (1:3600, Sigma-Aldrich, ON, CA) was also performed in paraffin embedded heart sections from sham and TAC mice. Paraffin sections were dewaxed, boiled in citrate buffer (pH 6.2-6.5), washed in 1x PBS, blocked for 1 hour with Protein Block Serum-Free (Dako Canada, ON, CA) and incubated with primary and secondary antibodies. Similar to PSR, ten, 10 x images at the level of the subendocardium of the LV were imaged and areas of brown were
selected for utilizing ImageScope software (Version 8.0.39.1065). Calculation of the proportional area stained per image was done as described above.

5.1.3 Hydroxyproline Analysis

LVs from α11 KO⁻/⁻ and α11 WT⁺/⁺ mice (16 weeks old, \( n = 3 \) per group) were isolated and immediately placed in liquid nitrogen. Tissue samples were then lyophilized for 24 hours to remove all water and stored at -80°C until ready for analysis. Samples were then sent for hydroxyproline analysis (Amino Acids Analysis Facility, Advanced Protein Technology Centre, Dept. of Molecular Structure & Function, Hospital for Sick Children). In brief, 0.01 gm of LV tissue was weighted and hydrolyzed for 24 hours at 110°C using 6N HCl with 1% phenol. After hydrolysis, an aliquot was taken and derivatized with phenylisothiocyanate and then ran on high-pressure liquid chromatography. The data is expressed as µg hydroxproline / mg of dry tissue weight.

5.1.4 Human Heart Tissue Samples

Human normal LV lysates (\( n = 2 \)) were commercially purchased (BioChain Institute, Inc., ON, CA and Abcam Inc., ON, CA). Human end-stage heart failure samples were graciously donated (162). In brief, human studies were conducted with the approval of the University Health Network Research Ethics Board (Protocol 10-0703-TE). Informed written consent was obtained from patients with ischemic end-stage heart failure (HF, \( n = 5 \)) before the insertion of a LV assist device. At the time of the surgical procedure, a sample was obtained from the apex of the LV and snap frozen in liquid nitrogen. Samples were then stored at -80°C.
5.1.5 Immunoblotting

LVs were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, MO, USA) and extracts were generated by mechanically homogenizing tissue samples. Protein content was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., ON, CA) and BSA, as standard. Lysates were resolved by Novex® 4-20% Tris-Glycine Gels (Thermo Fisher Scientific Inc., MA, USA) and transferred to nitrocellulose membranes. Membranes were blocked with 4% milk and immunoblotted for α11 integrin (1:500, R&D Systems, Inc., MN, USA) and β-actin (1:5000, Abcam Inc., ON CA) or GADPH (1:5000, Cell Signaling Technology, MA, USA) overnight at 4°C, incubated with appropriate HRP-conjugated secondary antibodies and detected by the ECL System (Amersham, Buckinghamsire, UK). Quantification was performed utilizing ImageJ Software (Version 1.48).

5.1.6 RNA Isolation and Quantification

Total RNA was isolated from homogenized cardiac tissue using TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA (2 µg) was converted to cDNA (for a 20 µL reaction) using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Life Technologies, Grand Island, NY) and stored at -20°C until further analysis.

Measurement of gene expression was expressed relative to RPL13a, using MicroAmp® Optical 384-Well Reaction Plates with a ViiA™ 7 Real-Time PCR System (Life Technologies Inc., Burlington, Ontario). Experiments were performed in triplicate and data analysis was performed using Applied Biosystems Comparative CT method.
Primer sequences: $\alpha_{11}$ integrin forward GACAGCACCAGCAGACGAT; reverse GCTGTTTGCCCTTGACCTCGAA; RPL13a forward GCTCTCAAGGTGTCTGCTGA; reverse AGATCTGCTTCTTCTTCGATA.

5.1.7 Statistical Analysis

An unpaired $t$-test was used to assess differences between $\alpha_{11}$ KO$^{-/-}$ and $\alpha_{11}$ WT$^{+/-}$ mice and between sham and TAC mice. Statistical significance was accepted with a value of $P$ less than 0.05. All statistical tests were performed using the software program GraphPad Prism (version 5, GraphPad Software, Inc.).
5.2 Results

5.2.1 α11 Integrin Null Mice Demonstrate Reductions in Interstitial Collagen

Quantification of total fibrillar collagen (collagen I and III) utilizing PSR staining revealed significantly decreased interstitial collagen in the myocardium of α11 KO⁻/⁻ mice in comparison to the α11 WT⁺/⁺ mice (Figure 16A-C, \( P<0.05 \)). Similarly, collagen type I staining revealed reduced type I collagen deposition in the myocardium of the α11 KO⁻/⁻ mice in comparison to the α11 WT⁺/⁺ mice (Figure 16D-F, \( P<0.05 \)). In addition, the ratio of total collagen to type I collagen was significantly different in the α11 KO⁻/⁻ mice (Figure 17). A strong trend towards significant reduction was also demonstrated when assessing hydroxyproline content, a major component of collagen, in the α11 KO⁻/⁻ mice (Figure 18, \( P=0.0538 \)).
Figure 16. Myocardial interstitial collagen is reduced in α11 KO−/− mice at 8 weeks.

PSR stained LV sections illustrating collagen deposition (red) in the α11 WT+/* (A) and α11 KO−/− mice (B). Quantitation of percent positive red pixels per subendocardial area (C). Collagen type I stained LV sections (brown) in the α11 WT+/* (D) and α11 KO−/− mice (E). Quantitation of percent positive brown pixels per subendocardial area (F). Values are means ± SEM. *P < 0.05 when compared to α11 WT+/*; **P<0.005 when compared to α11 WT+/*. Magnification 160x (A-B), 100x (D-E). Scale bar 50 µm (A-B), 100 µm (D-E).
Figure 17. Ratio of total collagen to collagen type I is different in α11 KO−/− mice at 8 weeks. Total collagen and collagen type I quantification (% positive pixels/area) plotted for α11 WT+/+ mice (Blue) and α11 KO−/− mice (Red). Values are means ± SEM. *P<0.05 when compared to total collagen.

Figure 18. Hydroxyproline content is reduced in the α11 KO−/− mice. Comparison of hydroxyproline content between α11 WT+/+ mice and α11 KO−/− mice, demonstrating a reduction in the α11 KO−/− mice. P=0.0538 when compared to α11 WT+/+. Values are means ± SEM.
5.2.2 Fibrotic Remodeling is Associated with Increases in α11 Integrin Expression

Given the lack of the α11 integrin in the heart was associated with reductions in collagen, we next explored whether the reverse is also true, whether enhanced collagen deposition in the heart is associated with increases in α11 integrin expression. Indeed, α11 integrin expression correlated with collagen remodeling in both patients with heart failure (HF) and in experimentally induced HF in mice (Figure 19, 21-22). α11 integrin protein expression was notably, albeit non-significantly, upregulated in ischemic HF patients (Figure 19). Furthermore, mice undergoing pressure-overload induced fibrotic remodeling via TAC (Figure 20), revealed an associated ~0.4-fold increase and ~5-fold fold increase in the relative α11 integrin mRNA and protein levels, respectively, in comparison to sham animals (both $P<0.05$, Figure 21-22).
**Figure 19. α11 protein expression in human heart failure.** (A) Representative Western Blot image of α11 integrin protein expression demonstrating robust expression in healthy human controls as well as notable upregulation in ischemic HF samples. (B) Quantification of α11 integrin protein expression relative to β-actin (AU, Arbitrary Units). Expression is shown relative to control, set at 1. Data are the mean ± SEM.
**Figure 20. Fibrosis evident in TAC animals.** PSR stained LV sections illustrating collagen deposition (red) in sham (A) and TAC mice (B). Quantitation of percent positive red pixels per subendocardial area (C). α-SMA IHC stained LV sections illustrating myofibroblast differentiation (brown) in sham (D) and TAC mice (E). Quantitation of percent positive brown pixels per subendocardial area (F). Values are means ± SEM. †P<0.01 when compared to sham; ‡P<0.0001 when compared to sham. Magnification 200x (A-B), 100x (D-E). Scale bar 50 µm (A-B), 100 µm (D-E).
**Figure 21. α11 integrin mRNA expression is upregulated in TAC mice.** α11 integrin mRNA levels were increased in TAC mice compared to sham. C_T values were RPL13a-corrected (AU). Expression is shown relative to sham, set at 1. Data are the mean ± SEM. *P<0.05 when compared to sham.

**Figure 22. α11 integrin protein expression is upregulated in TAC mice.** (Top) Immunoblotting image of α11 integrin protein expression demonstrating upregulation in TAC mice. (Bottom) Quantification of α11 integrin protein expression relative to GAPDH (AU). Data are the mean ± SEM. *P<0.05 when compared to sham.
5.3 Discussion

5.3.1 Myocardial Collagen is Reduced in the α11 KO<sup>−</sup> mice

Overall, multiple assessments demonstrated that myocardial collagen is reduced in the hearts of the α11 KO<sup>−</sup> mice. These findings are novel as they suggest that the α11 integrin may participate in collagen deposition in the myocardium, which has previously not been shown. Moreover, these results are consistent with prior work demonstrating that the α11 integrin is a critical mediator of collagen matrix organization and remodeling. The α11 integrin was previously shown to be an important mediator of collagen organization in the periodontal ligament (20). The α11 integrin was also shown to mediate collagen matrix remodeling by regulating the expression of MMPs, in particular MMP-13, a protein involved in ECM degradation (103). Together, current results suggest that the α11 integrin may be important for collagen homeostasis in a diverse array of cell and tissue systems.

Interestingly, however, reductions in collagen levels in the α11 integrin null mice or in other integrin knockouts have not been previously reported. Periodontal ligament fibroblasts null of the α11 integrin displayed reductions in MMP-13 levels, which was associated with increases in collagen deposition (103). The discrepancy in the current study suggests that the α11 integrin may have different functions in different tissues. Furthermore, mice null of the β1 integrin were shown to develop robust myocardial fibrosis (18,132). Differences here are likely due to the utilization of a β1 integrin KO approach, which affects a vast number of integrins. As well, both studies utilized a cardiomyocyte specific deletion, which may explain disparities between results. Still, it is
unclear what is causing the reductions in collagen apparent in the hearts of the α11 integrin null mice. It would be interesting to explore whether the loss of the α11 integrin in the heart disrupts normal collagen interactions with fibroblasts such that this initiates collagen degradation. Inhibiting the α11 integrin in fibroblasts impairs normal attachment to collagen type I, as well as contraction and anchoring of fibroblasts to collagen type I matrices, suggesting that this may be a possibility (20,103). Further investigation is required to determine the precise mechanism by which collagen remodeling is impaired in the heart following deletion of the α11 integrin.

5.3.2 Shift in the Abundance of Fibrillar Collagen

The differences in total fibrillar collagen (types I and III) and collagen type I evident in the α11 KO−/− mice suggests that there are changes to the ratio of collagen type I to type III. In the heart, ~85% of total collagen is type I, thick fibers involved in tensile strength and resistance to stretch and deformation, with only ~11% of total collagen being type III, thin forms that increase resilience (15). A shift in the ratio of collagen types from type III to type I has been shown in various forms of heart disease, with the thought that this is partly responsible for the development of cardiac dysfunction (21). Collagen type I is much thicker and stiffer than collagen type III, suggesting that increases in collagen type I may result in increased chamber stiffness and reduced compliance of the heart. Here, differences in total collagen compared with collagen type I in the α11 KO−/− mice were associated with significant impairments in diastolic function as well as mild, albeit non-significant, decreases in systolic function. These results suggest that differences in the ratio of collagen type I and III may also work in reverse, such that increases in collagen type III in comparison to type I may lead impaired structural integrity of the myocardium.
While research in this area is minimal, studies have demonstrated increases in collagen type III corresponding to more severe phases of diastolic dysfunction in examined hypertensive human patients (166).

5.3.3 Differences in Interstitial and Total Collagen May Explain Functional Impairments in the α11 KO−/− mice

It is well-studied that alternations of ECM components, including collagen, is an underlying mechanism contributing to functional impairments in the heart, ultimately contributing to heart failure progression as the heart becomes stiffer and less compliant (41,42). However, the vast majority of studies have examined and focused on enhanced collagen remodeling leading to alterations in cardiac function. Thus, very little is known and understood regarding whether impaired collagen synthesis also results in disrupted cardiac function. Only one study to my knowledge has shown that reductions in interstitial collagen in the myocardium leads to alterations in cardiac function. Tao et al. (2012) demonstrated that mice null of collagen XIV, a fibril-associated collagen that predominately interacts with collagen I and is expressed in the developing myocardium, impaired normal cardiac function (46). In particular, by 12 months mouse hearts null of collagen XIV began to significantly dilate, in the absence of hypertrophy, as well as displayed significant declines in ejection fraction. This study, in conjunction with current results demonstrating that α11 integrin null mice show impairments in cardiac function, suggests that indeed a finite balance of collagen is necessary in the heart in order to for cardiomyocytes to function properly.
Interestingly, some work has demonstrated an association between impaired cardiac function and disruption and degradation of fibrillar collagen. Models of chronic supraventricular tachycardia (SVT), a cause of LV dysfunction and dilation, have demonstrated impairments in collagen synthesis (167,168). In particular, impairments in both systolic and diastolic function were associated with reductions in hydroxyproline content and collagen fibril diameter (168). Scanning electron microscopy also revealed disruption of collagen struts and thickening of collagen weaves between adjacent myocytes from SVT animals. Another study utilizing the same model demonstrated similar findings with collagen concentration decreasing by 22% in SVT animals (167). This study also demonstrated that SVT isolated cardiomyocytes had a 50% reduction in adhesion capacity to ECM components found in the basement membrane. These results suggest that changes within the ECM, in particular collagen homeostasis, may accompany systolic and diastolic dysfunction associated with human cardiomyopathies. Thus, disruptions in collagen remodeling in the α11 KO−/− mice may explain the impairments in diastolic function evident following PV loop analysis. While there were mild, non-statistical, reductions in systolic parameters, it is possible that if mice were followed for longer (i.e. greater than 8 weeks) these differences would be more pronounced. Nonetheless, study findings indicate that reduced myocardial collagen may also be an important underlying mechanism leading to cardiac dysfunction, highlighting the need for more work exploring this relationship.
5.3.4 The α11 Integrin May be a Critical Mediator of Collagen Deposition in the Heart

Study findings also demonstrated that α11 integrin expression was enhanced in human and mouse hearts where increased collagen deposition is evident. These findings further establish and support a role of the α11 integrin in mediating collagen deposition in the heart, highlighting its potential biological importance in both health and disease processes. Other integrins have also been shown to regulate collagen synthesis and remodeling. The collagen binding integrins, α1β1 and α2β1, have shown to be positive regulators of collagenases, in particular the family of MMPs (103,169). Integrins have also been shown to be upregulated in pathological heart conditions, in conjunction with changes in ventricular remodeling of collagen (23,114,170,171). In particular, increased expression of the α1β1 and α2β1 integrins was demonstrated following aortic constriction (114,170). In addition, increased expression of the α1β1 and α10β1 integrins was witnessed in examined LVs of patients with dilated cardiomyopathy (171). Lastly, in diabetic rats with associated increases in ventricular collagen deposition, upregulation of the α11 integrin was also noted (23). Therefore, these studies highlight the importance of integrins for mediating collagen deposition in the heart, with thesis findings further demonstrating a role of the α11 integrin in this area.

5.3.5 Mechanism of α11 Integrin Activation

The current study suggests that cells in the heart respond to pathological stimuli by increasing the production of the α11 integrin. While a mechanism was not demonstrated in this thesis, prior work has shown that TGF-β signaling may positively regulate α11
integrin expression. The α11 integrin promoter contains specific binding sites for Smad proteins, suggesting TGF-β signaling can drive α11 integrin promoter activity (138,140). However, an equally important consideration is that adverse remodeling promotes changes in cell number, which may also influence the degree of α11 integrin abundance. Ischemia and pressure-overload via TAC has been shown to result in the accumulation of cardiac fibroblasts (172). Thus, further investigation is necessary to determine whether the increases in α11 integrin are due to a biologically meaningful response (i.e. induction via TGF-β), are a result of changes in cardiac fibroblast abundance, or possibly a combination of both.

5.3.6 Limitations

It appears that loss of the α11 integrin results in reductions in myocardial collagen, suggesting the α11 integrin is important for collagen deposition in the heart. However, the precise mechanism by which the α11 integrin mediates myocardial collagen is unknown. In mouse periodontal ligament fibroblasts, the α11 integrin was shown to regulate collagen remodeling through MMP-13 and cathepsin K (103). It is plausible that similar mechanisms also exist in the heart. Further work is necessary to determine the key mediators of α11 integrin-dependent collagen remodeling in the heart.

In addition, while we demonstrate an association between fibrotic remodeling and the α11 integrin, it is possible that other cellular changes occurring in the hearts of ischemic HF patients and mice subject to TAC are influencing the degree of α11 integrin activation. However, it has been consistently shown both in vivo and in vitro that the α11 integrin is responsive to changes in collagen deposition as well as fibroblast-
myofibroblast differentiation (23,119). Furthermore, the demonstrated work also highlighting direct TGF-β-, the major pro-fibrotic cytokine, α11 integrin communication further supports a potential role of the α11 integrin in fibrosis development (138,140).
Chapter 6
General Discussion and Conclusion

6.0 Combining Research Aims

It was first hypothesized that the α11 integrin is cardiac fibroblast specific and not expressed in other cells in the heart. Utilizing a magnetic column isolation technique in mice, we confirmed that the α11 integrin was expressed only in the fibroblast cell fraction. A similar result was also demonstrated utilizing the same technique in rat cardiac fibroblasts (23). This study also demonstrated that the α11 integrin is expressed in human cardiac fibroblasts (23), suggesting the α11 integrin is expressed in cardiac fibroblasts across species.

It was then hypothesized that disruption of the α11 integrin in vivo would lead to impairments in cardiomyocyte development and function, as previous work demonstrated a critical role of integrins in mediating fibroblast-cardiomyocyte communication. Indeed, loss of the α11 integrin impaired cardiomyocyte growth and alignment and disrupted discrete structural components of cardiomyocytes, which was ultimately associated with impaired diastolic function. The finding that the α11 integrin is important for diastolic function, and not necessarily systolic function, was surprising as we had thought initially that cardiomyocyte contraction would be impaired. Prior work has demonstrated that loss of the β1 integrin in the heart resulted in significantly abnormal cardiac contractility in addition to impairments in relaxation (132). While there was a trend in reductions in systolic function evident, they were not significantly different between mice. It is possible if we followed the α11 integrin null mice for longer that
differences would have been more pronounced. We were also surprised by the degree of structural changes evident in the cardiomyocytes of α11 null integrin mice. Given that the α11 integrin is fibroblast-specific and not found on cardiomyocytes, it is unclear how the α11 integrin is involved in the coordination of cardiomyocyte structure.

Lastly, it was hypothesized that the α11 integrin would be important for collagen deposition in the heart. Indeed, mice null of the α11 integrin had reduced interstitial myocardial collagen and in reverse, α11 integrin expression increased in correlation with fibrosis development in both humans and mice. The results in the α11 deficient mice are quite novel, as they highlight that possibility that less, not more, collagen can also be an underlying cause of cardiac dysfunction, a relatively poorly understood phenomenon. Further, increases in α11 integrin expression in human HF patients and in mice subjected to TAC in correlation with collagen remodeling also suggests a potential dual function of the α11 integrin in both health and disease. It appears that a finite balance of α11 integrin expression and collagen deposition is needed in the heart to maintain normal cardiomyocyte function, with too little or too much leading to dysfunction.

Together the study findings presented in this thesis demonstrate that the α11 integrin is a cardiac fibroblast specific integrin that is important for postnatal cardiomyocyte growth, alignment and function, likely due to an important role in mediating interstitial myocardial collagen deposition (Figure 25). In all, results highlight the importance of the α11 integrin for mammalian heart development.
Figure 22. Proposed mechanism: loss of the fibroblast specific α11 integrin disrupts fibroblast-cardiomyocyte communication and organization due to impaired collagen remodeling. (Left) Following collagen type I ligand binding or mechanical activation the α11 integrin becomes activated leading to fibroblast-induced changes along the cardiomyocyte. Furthermore, collagen acts as a necessary structural framework for normal cardiomyocyte function. (Right) Without the α11 integrin, collagen deposition is impaired, possibly leading to impaired fibroblast-cardiomyocyte communication and ultimately resulting in abnormal cardiomyocyte development. Furthermore, lack of interstitial collagen may lead to disrupted mechanical support or stiffness of cardiomyocytes, resulting in impaired cardiomyocyte function.

6.1 Cardiomyocyte Development – Tool For Regenerative Therapies

Ieda et al. (2009) were instrumental in demonstrating that cardiomyocyte development is also dependent on paracrine signaling via fibroblasts and that this was mediated by the β1 integrin complex (18). Here, it is shown that the α11 integrin is one of the
important β1 integrins expressed by fibroblasts that mediates cardiomyocyte development and overall cardiac function. This work adds to our understanding regarding the critical mediators of cardiomyocyte development and continues to highlight the complexity of this relationship. It appears that integrins expressed by other cells in the heart, and not just cardiomyocyte specific integrins, are critical for cardiomyocyte development. This suggests that furthering our understanding of the vast cellular milieu that cardiomyocytes are exposed to is necessary in order to appreciate and grasp the complexity of in vivo cardiac development. In particular, this study highlights that exploring the ways in which the ECM, integrins and other cells in the heart mediate cardiomyocyte development may be instrumental for generating a complete picture of in vivo regulators. The idea that cardiomyocyte development is dependent on a vast network of matrix proteins, integrins and other cells in the heart may be an important paradigm to consider for furthering the development of cell-based therapies for the treatment of heart disease.

The adult mammalian heart, unlike some organs, has little to no capacity for self-regeneration; following insult, injury, hypertension or coronary artery disease, the ability of the heart to generate new cardiac muscle cells is limited (173,174). Currently, clinical interventions work solely to improve cardiac function transiently, none able to replace the damaged or lost cardiomyocytes or improve the surrounding cellular milieu (175). As such, new therapeutic strategies have been aimed at discovering novel ways to generate cardiomyocytes in vitro as a potential cell-based, regenerative therapy to replace lost cells in vivo. Since this idea came to fruition, multiple studies have shown that the generation of cardiomyocytes is possible, with studies utilizing human
embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs) as well as direct reprogramming of other cell types, including fibroblasts (176-179). However, the ability to generate mature, functional cardiomyocytes that are similar to native tissue and can be used for the treatment of human disease has been a challenge. Many of the above studies highlighted above relied on differentiating hESCs and iPSCS or reprogramming fibroblasts into cardiomyocytes via the use of a growth factor ‘cocktail’. For example, Song et al. (2012) demonstrated that forced expression of four transcription factors, GATA4, Hand2, Mef2c and Tbx5, could cooperatively reprogram mouse cardiac fibroblasts into beating cardiomyocyte-like-cells in vitro (177). The main issue with these approaches, as evident in this study and others, is that cardiomyocyte development is dependent on a complex set of critical interactions, ones that cannot be simply replicated by adding a set of transcription factors.

Indeed, with a vast number of studies demonstrating a critical role of the ECM in mediating cardiac development, novel new approaches have focused on incorporating ECM scaffolds to promote the differentiation of cells into cardiomyocytes. Multiple studies have shown that the addition of matrices into cell culture systems promotes the development of mature cardiomyocytes. Fetal cardiac ECM was shown to promote the adhesion and proliferation of neonatal cardiomyocytes (180). Moreover, adding an ECM monolayer in combination with application of growth factors induced differentiation of human iPSCs into cardiomyocytes with an exceedingly high purity (98%) and yield (11 cardiomyocytes per iPSC) and generated significantly more cardiomyocytes compared with growth factors alone (181). Further, growth with an ECM monolayer resulted in more mature cardiomyocytes at an earlier timeframe than adding growth factors alone.
This study and others highlight the importance of growing cells on ECM substrates rather than stiff conventional culture surfaces (182). Other work has also shown that the addition of ECM as well as integrins improves the effectiveness of cell-based therapies and cardiac regeneration (183-185). Collectively, these studies highlight the importance of the ECM in mediating cardiomyocyte maturation and development and emphasize the importance of replicating in vivo systems in vitro to enhance the efficiency and effectiveness of cell-based therapies.

### 6.2 Clinical Significance

In response to pathological stimuli, cardiac fibroblasts begin to differentiate into pro-fibrotic, highly contractile, myofibroblasts characterized by the abundance of α-SMA. Overtime, the accumulation of myofibroblasts in the myocardium results in increased mechanical stiffness of the ECM, impairing contractility of cardiomyocytes and ultimately resulting in reduced cardiac function (85,186). As such, increased production of myofibroblasts has been shown to increase the progression of cardiac disease, evident by the abundance of myofibroblasts in failing human hearts (187). The α11 integrin has been shown to regulate myofibroblast differentiation through TGF-β signaling. α11 integrin expression increases following myofibroblast differentiation with inhibition preventing this transformation (23,119). Furthermore, treatment with TGF-β isoforms has been shown to induce α11 integrin expression, with later work revealing the α11 integrin promoter contains binding sites for Smad proteins (23,138,140). These results, in line with current work, suggest that the α11 integrin may be an important mediator of fibrosis progression in failing human hearts.
Overall, it appears that the α11 integrin serves a critical dual function: (1) during cardiac development the α11 integrin is necessary for normal cardiomyocyte development and function, however, (2) in response to pathological stimuli, TGF-β signaling induces α11 integrin expression, which promotes myofibroblast differentiation, collagen deposition and ultimately increases the progression of heart failure. As such, the second potential function of the α11 integrin suggests that therapeutic strategies aimed at reducing α11 integrin expression or associated signaling molecules in the heart may be important for reducing fibrosis development. Taken a step further, improving our understanding of how the α11 integrin coordinates collagen remodeling in the heart may be vital for reducing ECM stiffness, potentially enhancing reverse remodeling by preventing the availability of adverse signaling molecules. This idea is supported by work showing that mechanically strained (stiff) ECM enhances TGF-β bioavailability in comparison to relaxed ECM, suggesting modifying ECM strain may be an important therapeutic consideration (188). Still, given the described role of the α11 integrin in development, therapeutic strategies must be carefully planned to avoid adverse outcomes.

Current findings also suggest that disruptions in particular integrins or associated signaling pathways may be an underlying manifestation of cardiac abnormalities. Intriguingly, other studies have demonstrated this finding. Mutations within the laminin, integrin and integrin-linked kinase were found in patients with severe cardiomyopathy and not in any unaffected individuals (189). Additional functional data in zebrafish demonstrated that these mutations lead to defects in cardiomyocytes and endothelial cells (189). Moreover, while mutations in some cases of hypertrophic cardiomyopathies are largely thought to be due to sarcomeric proteins, dilated cardiomyopathies have
been suggested to be caused by defects in several cell systems in the heart (189). This suggests that mutations in integrins, such as the α11 integrin, that are not expressed on cardiomyocytes but are on other cells in the heart may also contribute to particular forms of heart disease. Further investigation in human heart disease is certainly warranted regarding whether or not other integrin mutations contribute to disease manifestation.

### 6.3 Future Directions

To overcome limitations of off-target effects utilizing a germline mutation of the α11 integrin, fibroblast- and cardiac-specific conditional knockout mice are in the process of being generated and bred for experimental purposes. The α11 integrin floxed mice will be crossed with mice expressing Cre-recombinase under the control of the type I collagen promoter (obtained from Barbara Kream, University of Connecticut). In this mouse, cre expression is restricted to cardiac fibroblasts, hence the mice generated will be cardiac fibroblast restricted α11 integrin KO−/−. Obtaining these mice will allow for more specific investigation into the contribution of the α11 integrin during cardiac development.

Furthermore, a new technique has been described to co-culture cardiomyocytes with cardiac fibroblasts, potentially without many of the technical issues that had arisen previously. Utilizing a neonatal cardiomyocyte isolation protocol, towards the end of isolation cardiac fibroblasts remain co-cultured with cardiomyocytes, limiting the use of two separate systems. Therefore, instead of purifying cardiomyocytes completely, the final step of fibroblast removal will not be performed, leaving both cell populations in the
tissue culture. Having a co-culture system of fibroblasts and cardiomyocytes will then allow for more finite mechanistic studies. In particular, α11 integrin expression can be manipulated in fibroblasts by transfecting cells with siRNA to the α11 integrin. Changes in cardiomyocyte and fibroblast development can then be examined *in vitro* following loss of the α11 integrin. Furthermore, signaling molecules involved in these processes can also be examined, furthering our knowledge of the contribution of the α11 integrin to fibroblast-cardiomyocyte interactions.

Finally, to assess the potential clinical significance of the α11 integrin, cardiac fibroblast restricted α11 integrin KO−/− mice should be examined following TAC. Given that the α11 integrin has been shown to be involved in myofibroblast differentiation through TGF-β signaling and collagen remodeling, this study may demonstrate that deletion of the α11 integrin results in reduced ECM deposition. However, given the α11 integrin has been shown to have an important developmental role in the heart, ideally, using an inducible gene silencing system, such as tamoxifen-induced gene knockdown, at a period of time post TAC would be the best methodological approach to assess clinical and therapeutic significance.

Ultimately, what is really interesting about this work is it suggests that cardiomyocyte development is more complex than we had anticipated. It appears that not only are transcription factors and proteins surrounding and on sarcomeres important for cardiomyocyte development but also other cells and proteins may interact to mediate critical processes. As such, important questions arise from this work concerning our understanding of cardiomyocyte biology. In particular, do other cells, other than fibroblasts, communicate information to cardiomyocytes and if so, how? In addition, our
understanding of the importance of cardiac fibroblasts is vastly ill defined, with this work and others suggesting the role of the cardiac fibroblast in the heart should not go unnoticed. This work also raises important considerations for the development of effective and efficient cell-based therapies for the treatment of human heart disease. The idea that we should be differentiating cardiomyocytes in a dish devoid of extracellular matrix proteins, integrins and other cells, such as cardiac fibroblasts, appears contrary to the necessary in vivo interactions that ultimately contribute to cardiomyocyte development. Furthermore, by manipulating these interactions it may be possible to also enhance current strategies aimed at differentiating mature and functional cardiomyocytes. Thus, future work is necessary to uncover fundamental cardiac developmental processes and attempt to utilize this knowledge for the development of effective regenerative therapies.

6.4 Conclusion

This study shows for the first time that loss of the α11 integrin, a fibroblast specific integrin, disrupts cardiomyocyte growth and alignment as well as impairs normal cardiac relaxation in vivo. This study also highlights the importance of the α11 integrin for interstitial myocardial collagen deposition in the heart. Together, study findings suggest that the α11 integrin is a critical component of the cardiac fibroblast that is required for normal communication with cardiomyocytes. Without the α11 integrin, cardiomyocytes appear to develop abnormally and are smaller in size. Over time the hearts of the α11 KO−/− mice demonstrated a reduced ability to relax in comparison to the α11 WT+/* mice, which may be due to impairments in collagen. Collectively, these findings suggest a
dynamic and novel relationship between the ECM-integrin-fibroblast-cardiomyocyte that is critical for regulating the normal development of the cardiomyocyte.
Appendices

The TAC model

Methods

Echocardiography

All echocardiographic assessments were done using the Vevo® 2100 system and a MS-400 Red probe (VisualSonics, Toronto, Ontario). Baseline echocardiography was performed prior to surgery to ensure normal heart function. Echocardiography was then performed 3 days post TAC to confirm successful aortic banding. Mice were then echoed at 4 weeks to confirm left ventricular remodeling and at 6 weeks at end study.

Cardiac Catherization

Cardiac catherization was performed to examine pressure and volume changes at 6 weeks (end study), as previously published (147). In brief, animals were placed on a heating pad to maintain a body temperature of 37 ± 1 °C, intubated and placed on a respirator. Mice were secured in a recumbent position (sagittally) and the right jugular vein was cannulated. Pressure was calibrated after warming the catheter (Model SPR-838; Millar Instruments, Houston, Texas, USA) in 0.9% NaCl at 37 °C for 30 minutes. The right internal carotid artery was then identified and ligated cranially. A 2F miniaturized combined conductance catheter-micro-manometer was inserted into the carotid artery to obtain aortic blood pressure, and then advanced into the LV until stable PVx loops were obtained. The abdomen was opened and the inferior vena cava and portal vein identified. A cotton-tip applicator was pressed on these vessels to allow rapid reduction in cardiac preload. All loops were obtained with the respirator turned off for 5–10 s and the animal apneic. Data were then acquired under steady state conditions and during preload reduction. Calibration from relative volume units (RVUs) conductance signal to absolute volumes (in µl) was undertaken using a previously validated method of comparison to known volumes in Perspex wells (148). Using the pressure conductance data, a range of functional parameters was then calculated (Millar analysis software PVAN 3.4).
Histology

For overall morphology, midsections of the LV were isolated, immediately fixed in 10% formalin for 4-8 hours and embedded in paraffin. Paraffin-embedded longitudinal (4 µm thick) were prepared and stained with hematoxylin and eosin. Ten, 40 x images at the level of the subendocardium of the LV were imaged and the cross-sectional area of >50 randomly chosen circular-shaped nucleated cardiomyocytes were measured (146) using AxioVision software (Version 4.8.2).

RNA isolation and quantification

Total RNA was isolated from homogenized cardiac tissue, which had been stored at -80°C using TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA (2 µg) was converted to cDNA (for a 20 µL reaction) using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Life Technologies, Grand Island, NY) and stored at -20°C until further analysis.

Measurement of gene expression was expressed relative to RPL13a, using MicroAmp® Optical 384-Well Reaction Plates with a ViiA™ 7 Real-Time PCR System (Life Technologies Inc., Burlington, Ontario). Experiments were performed in triplicate and data analysis was performed using Applied Biosystems Comparative C_\text{T} method.

Statistics

An unpaired t-test was used to assess differences between the sham and TAC mice. Statistical significance was accepted with a value of P less than 0.05. All statistical tests were performed using the software program GraphPad Prism (version 5, GraphPad Software, Inc.). Data are presented as mean ± SD, unless specified otherwise.
Results

Animal Characteristics

At 6 weeks post-surgical intervention, TAC animals had undergone significant heart remodeling with cardiac hypertrophy evident (Table 1). Heart weight was significantly increased in comparison to sham animals \((p<0.05, \text{ Table 1})\). LV weight was also significantly elevated in comparison to sham animals \((p<0.05, \text{ Table 1})\), indicating LV remodeling. When indexed to tibial length, heart weight and LV weight were significantly increased \((p<0.05, \text{ Table 1})\). No differences in body weight or tibial length were observed, confirming similar baseline characteristics.

Table 1. Characteristics of sham and TAC mice 6 weeks post operation.

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<th>Sham</th>
<th>TAC</th>
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<tr>
<td>N</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>29 ± 3</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>123 ± 10</td>
<td>181 ± 37‡</td>
</tr>
<tr>
<td>LV Weight (mg)</td>
<td>87 ± 7</td>
<td>136 ± 25§</td>
</tr>
<tr>
<td>Lung Weight (mg)</td>
<td>128 ± 14</td>
<td>158 ± 42*</td>
</tr>
<tr>
<td>Tibial Length (mm)</td>
<td>15 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>HW: TL (mg: mm)</td>
<td>8.23 ± 1.35</td>
<td>11.27 ± 3.03†</td>
</tr>
<tr>
<td>LV: TL (mg: mm)</td>
<td>5.83 ± 0.97</td>
<td>8.53 ± 2.10‡</td>
</tr>
<tr>
<td>LW: TL (mg: mm)</td>
<td>8.57 ± 1.30</td>
<td>10.02 ± 3.45</td>
</tr>
</tbody>
</table>

\(N\), number per group; HW, heart weight; LW, lung weight; LV, left ventricle, TL, tibial length; HW:TL and LW:TL indicates the ratio of wet heart and lung weight to tibial length, respectively. Values are mean ± SD. \(^*P < 0.05\) when compared to sham, \(^†P < 0.01\) when compared to sham, \(^‡P < 0.001\) when compared to sham.

Pressure-Volume Loop Analysis

At 6 weeks, TAC induced various hemodynamic changes (Table 2). Heart rate was elevated in TAC animals compared with sham \((p<0.05, \text{ Table 2})\) TAC also induced pressure fluctuations, with greater end-systolic \((p<0.05, \text{ Table 2})\) and end-diastolic blood pressure \((p<0.05, \text{ Table 2})\) evident. Pressure changes suggest the aortic constriction adequately induced pressure-overload resulting in a compensatory rise in heart rate.
Table 2. PV loop analysis in sham and TAC mice 6 weeks post operation.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (BPM)</td>
<td>393 ± 78</td>
<td>508 ± 79†</td>
</tr>
<tr>
<td>ESP (mm Hg)</td>
<td>85 ± 9</td>
<td>135 ± 15§</td>
</tr>
<tr>
<td>EDP (mm Hg)</td>
<td>6 ± 2</td>
<td>10 ± 7*</td>
</tr>
</tbody>
</table>

N, number per group; HR, heart rate; ESP and EDP, end-systolic and end-diastolic pressure, respectively. Values are mean ± SD. *P < 0.05 when compared to sham, †P < 0.01 when compared to sham, §P < 0.0001 when compared to sham.

Echocardiography

Functional assessment obtained by echocardiography revealed marked LV hypertrophy accompanied with moderate dilation in TAC mice, ultimately resulting in reduced cardiac function (Figure 1; Table 3). LV mass, LV anterior and poster wall diameter (LVAWd, LVPWd, respectively) were all significantly increased 6-weeks post TAC (p < 0.05, Table 3), demonstrating LV hypertrophy. TAC mice also demonstrated LV dilation, as evident by increased LV internal diameter during systole (LVIDs; p < 0.05, Table 3), as well as elevated end-diastolic and end-systolic volume (EDV, ESV, respectively; p < 0.05, Table 3). LV internal diameter during diastole (LVIDd) was moderately elevated, albeit non-significant (Table 3). In conjunction with structural changes, TAC mice also demonstrated a marked reduction in ejection fraction (EF), fractional shortening (FS) and fractional area change (p < 0.05, Table 3) compared to sham mice, demonstrating impaired LV function. No changes were evident in end-diastolic and end-systolic endothelial area (EDA, ESA, respectively) or cardiac output (CO; Table 2).
Table 3. Echocardiographic characteristics of sham and TAC mice 6 weeks post operation.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVM (mg)</td>
<td>106 ± 11</td>
<td>187 ± 47§</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.876 ± 0.124</td>
<td>1.18 ± 0.15§</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.838 ± 0.148</td>
<td>1.14 ± 0.16§</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>4.10 ± 0.35</td>
<td>4.44 ± 0.49</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.61 ± 0.54</td>
<td>3.28 ± 0.66*</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>74 ± 15</td>
<td>91 ± 25*</td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>27 ± 14</td>
<td>46 ± 24*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>65 ± 13†</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36 ± 10†</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>FAC (%)</td>
<td>54 ± 11†</td>
<td>41 ± 13</td>
</tr>
</tbody>
</table>

N, number per group; LVM, left ventricular mass; LVAWd, left ventricular anterior wall thickness in diastole; LVPWd, left ventricular posterior wall thickness in diastole; LVIDd and LVIDs, left ventricular internal diameter in diastole and systole; EDA and ESA, end-diastolic and end-systolic endothelial area, CO, cardiac output; EDV and ESV, end-diastolic and end-systolic volume; EF, ejection fraction; FS, fractional shortening; FAC, fractional area change. Values are mean ± SD. *P < 0.05 when compared to sham, †P < 0.05 when compared to TAC, §P < 0.0001 when compared to sham.

Histology

Along with functional changes, TAC induced cellular adaptation 6 weeks post operation (Figure 1) Myocyte cross-sectional area was significantly elevated in TAC animals (p < 0.05, Figure 2A-C), demonstrating cellular hypertrophy.

Figure 1. TAC animals demonstrate increased cardiomyocyte hypertrophy. H & E stained heart sections in sham (A) and TAC animals (B), demonstrating cardiomyocyte size. Quantification of myocyte cross-sectional area (µm²) in (C). Values are mean ± SEM. §P < 0.0001 when compared to sham.
Activation of the ‘Fetal Gene Program’

Pressure-overload (PO) induced pathological hypertrophy of the heart via TAC has been shown to re-initiate fetal activation of various signaling cascades (190-192). To confirm our model induced similar changes, we examined the major fetal genes in mice 6 weeks post operation (Figure 2). Consistent with other models, TAC induced mRNA expression of atrial natriuretic factor (ANP), α-skeletal muscle actin (α-SKA) and β myosin heavy chain (MHC) ($p < 0.05$, Figure 2), whilst repressing sarcoplasmic reticulum Ca$^{2+}$ adenosine triphosphatase–2 (SERCA2a) and α-MHC expression ($p < 0.05$, Figure 2).

![Figure 2. Evidence for activation of the fetal gene program demonstrating cardiomyocyte hypertrophy in TAC mice 6 weeks post operation.](image)

TAC induced mRNA expression of ANP, α-SKA and β-MHC, whilst repressing SERCA2a and α-MHC expression. $C_T$ values were RPL13a-corrected (AU) and analyzed by a t-test to determine statistical difference between animals. Data are the mean ± SEM. $^*P < 0.05$ when compared to sham, $^{†}P < 0.01$ when compared to TAC.
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Figure 2 – Approved

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Figure 4 – Approved

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