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TITLE: Epicatechin as a Therapeutic Strategy to Mitigate the Development of Cardiac Remodeling and Fibrosis

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Heart failure with preserved ejection fraction (HFpEF) continues to increase and little is known about its pathophysiology. About 2/3 of patients are women and risk factors include aging, hypertension and metabolic syndrome. A feature of the disease is cardiac fibrosis. Currently, no drugs target HFpEF and the development of animal models can assist in therapy evaluation. We developed a female rat model of aging, estrogen depletion and metabolic syndrome to evaluate the role of these factors in altering cardiac structure/function. Aged female Fischer F344 rats were allocated into an aging group, aging + ovariectomy and aging + ovariectomy + 10% fructose in drinking water. At 22 months of age, animals were anesthetized and left ventricular (LV) function was evaluated. Histological measures were also obtained. Intraventricular pressure-volume loop analysis evidenced significant decreases in stroke work cardiac output and increases in myocardial stiffness with ovariectomy. Histological analysis indicated increasing levels of inflammatory infiltration, perivascular and interstitial fibrosis with ovariectomy and with fructose supplementation. In conclusion, with aging, estrogen deprivation, markedly deteriorates myocardial microstructure which may facilitate the loss of diastolic and systolic function. This model may serve to understand the role that aging and menopause may have in the development of HFpEF.

15. SUBJECT TERMS
fibrosis, myocardium, heart failure, stiffness, estrogen, aging, metabolic syndrome

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INTRODUCTION

The project proposes that treatment with the flavanol (-)-epicatechin (Epi) will ameliorate adverse tissue remodeling and cardiac fibrosis in female animal models developing diastolic dysfunction as seen in women with heart failure with preserved ejection fraction (HFpEF). The project's 3 specific aims are (1) to determine if early use of Epi in female animal models of fibrotic hearts will reduce collagen deposition and preserve function, (2) to determine if late use of Epi in female animal models of fibrotic hearts will reduce collagen deposition and recover function, and (3) to investigate if the beneficial effects of Epi are due to its action on the cardiac fibroblast which are the cells mainly responsible for the production of fibrillar collagens. The mechanism(s) and functional outcomes of oral Epi preventive and therapeutic treatments will be defined in a relevant female animal model of diastolic dysfunction and can potentially lead to the design and implementation of clinical trials for the treatment for myocardial fibrosis leading to improved function.

KEYWORDS

Fibrosis, myocardium, heart failure, aging, estrogen, metabolic syndrome, stiffness, collagen, compliance, remodeling, epicatechin, flavanols, left ventricle.

ACCOMPLISHMENTS

I. Major goals of the project

<u>Major Goals (Aims)</u>: The following are the **major tasks** identified in the Statement of Work associated with each aim.
Aim 1 related: Early preventive treatment with (-)-epicatechin (Epi) prevents myocardial fibrosis
1. Characterize effects of aging on myocardial fibrosis in untreated animals (completed)
2. Characterize effects of early Epi treatment on myocardial fibrosis in a model of estrogen depletion and aging (90% completed)
3. Characterize effects of early Epi treatment on myocardial fibrosis in a model of estrogen depletion, aging and fructose supplementation (90% completed)
Aim 2 related: Late treatment with Epi reverses myocardial fibrosis
4. Characterize long-term baseline effects of aging on myocardial fibrosis (completed)
5. Characterize the reversal of myocardial fibrosis by late Epi treatment in a model of estrogen depletion and aging (completed)
6. Characterize effects of estrogen depletion, aging and fructose supplementation on myocardial fibrosis and its reversal by late Epi treatment (completed)
Aim 3 related: The anti-fibrotic effects of (-)-epicatechin are mediated by TGF-β1 inhibition
7. Cardiac fibroblast phenotype characterization (40% completed)
8. Effects of profibrotic phenotype stimulation/inhibition (40% completed)
9. Gene expression modulation (ongoing)

II. Accomplishments

Aim 1 related

A. *Characterizing the effects of aging, estrogen depletion and excess weight on left ventricular (LV) remodeling*

During year 2, a large part of the project's effort related to completing the implementation and assessment of the female model of aging driven cardiac remodeling and fibrosis that was to be compounded by ovariectomy (estrogen depletion) and fructose supplementation (yielding excess weight gain). We completed the characterization of changes in cardiac structure/function that develop as a function of aging (alone), aging + ovariectomy and aging + ovariectomy + fructose supplementation. A highly detailed and extensive characterization of the models has been published (see bibliography list). We are actively using data gathered from these in vivo studies to implement computer models that will allow us to simulate the impact that structural changes have on diastolic function (mechanical properties).

As listed in the proposed project plans, we completed the following tasks/subtasks:
- Assessed serial changes in cardiac structure/function using echocardiography
- Implemented terminal studies and recorded detailed carotid and intraventricular hemodynamics
- Performed ex vivo pressure-volume curves to examine changes in global left ventricular (LV) compliance
- Performed ex vivo pressure-strain curves to examine changes in free wall LV epicardial strains
- Fixed hearts, measured and recorded detailed histomorphometric parameters
- Compiled and summarized all data and performed a rigorous statistical evaluation, wrote reports and shared data
- Analyze by histological methods the role that inflammation may play in modulating the process of aging/ovariectomy/weight gain associated LV remodeling
- Analyze by multiplex immunoassays the role that select circulating proinflammatory cytokines may play in the remodeling process

Methods utilized include echocardiography to measure in vivo changes in heart morphology, diastolic and systolic function. In vivo carotid and LV hemodynamics utilized a Millar pressure conductance catheter. At the time of the terminal study, an ex vivo assessment of LV pressure volume and strain was implemented using an inflatable balloon as well as video recording of the inflating hearts to monitor the displacement of epicardial markers and calculate two-dimensional strains. Finally, hearts were fixed and sectioned for detailed histological analysis using hematoxilin and eosin and Sirius Red staining. Once stained, sections were visualized using standard microscopy and images analyzed for LV morphometry and collagen density by using a high resolution digital system (HALO system). We have also utilized immunohistochemistry, tunnel assays, multiplex immunodetection of pro-inflammatory cytokines in plasma samples. All data was summarized in databases and subjected to rigorous statistical analysis using GraphPad software.

Aim 1 results have led to the publication of an extensive research article in the *Journal of Physiology*. <u>The following is the abstract of the submitted manuscript</u>:

Unmasking of Estrogen Dependent Left Ventricular Dysfunction in Aged Female Rats: A Potential Model for pre-HFpEF
Two-thirds of patients with heart failure with preserved ejection fraction (HFpEF) are older women and risk factors include hypertension and excess weight/obesity. Pathophysiological factors that drive early disease development (before heart failure ensues) remain obscure and female animal models are lacking. The study evaluated the intersecting roles of aging, estrogen depletion and excess weight on altering cardiac structure/function. Female, 18 month old, Fischer F344 rats were divided into aging group, aging + ovariectomy (OVX) and aging + ovariectomy plus 10% fructose (OVF) in drinking water (n=8-16/group) to induce weight gain. Left ventricular (LV) structure/function was monitored by echocardiography. At 22 months of age, animals were anesthetized and catheter-based hemodynamics evaluated, followed by histological measures of chamber morphometry and collagen density. All aged animals developed hypertension. OVF animals increased body weight. Echocardiography only detected mild chamber remodeling with aging while intraventricular pressure-volume loop analysis showed significant ($p<0.05$) decreases vs. aging in stroke volume (13% OVX and 15% for OVF), stroke work (34% and 52%), cardiac output (29% and 27%), and increases in relaxation time (10% OVX) with preserved ejection fraction. Histology indicated papillary and interstitial fibrosis with aging, which was higher in the endocardium of OVX and OVF groups. With aging, ovariectomy leads to the loss of diastolic and global LV function while preserving ejection fraction. This model recapitulates many cardiovascular features present in HFpEF patients and may help understand the roles that aging and estrogen depletion play in early (pre-HFpEF) disease development. [**end of abstract**].
   Details pertaining to each of the findings can be found in the attached (appendix) manuscript.

B. *Characterizing the effects of early Epi treatment on aging, estrogen depletion and excess weight associated LV remodeling/fibrosis*

During year 2 and 3 of the project, we successfully implemented all of the required in vivo studies to assess the effects of early Epi treatment on aging associated cardiac remodeling in the absence or presence of estrogens and excess weight as per fructose supplementation. Altogether, we completed 90% of the project and we are only waiting to receive the results of the histological analysis of tissue samples for determinations of fibrosis. No major issues arose during the studies and we expect to be 100% completed with the next 2 months. In the interest of report length, and since we have completed aim 2 studies (reported in a summarized version below) we will refrain from presenting these results until the final report is provided next year. However, a preliminary analysis of structural and functional data is encouraging.

Aim 2 related

Characterizing the effects of late Epi treatment on aging, estrogen depletion and excess weight associated LV remodeling/fibrosis

During the last year, we completed all of the in vivo studies assessing the effects of late Epi treatment on aging associated cardiac remodeling in the absence or presence of estrogens and/or excess weight. A summary of results is presented below for studies performed in aged female rats subjected to ovariectomy + fructose supplementation (OVF) vs. those treated with epicatechin (OVFE) for a period of 1 month prior to their terminal study.

Echocardiography

As shown in figure 1, results reveal comparable modest changes in OVF -/+ epicatechin groups. Anterior wall thickness in diastole (AWThD) decreased modestly as a function of time (A). For posterior wall diastolic wall,thickness (PWThD) there was also a minor decrease in thickness over time without differences between groups (B). There was a significant time dependent increase in LV diastolic and systolic chamber diameters (C, D) in all groups. Heart rate (HR), ejection fraction (EF) and fractional shortening (FS) were also not different between OVF groups (data not shown).

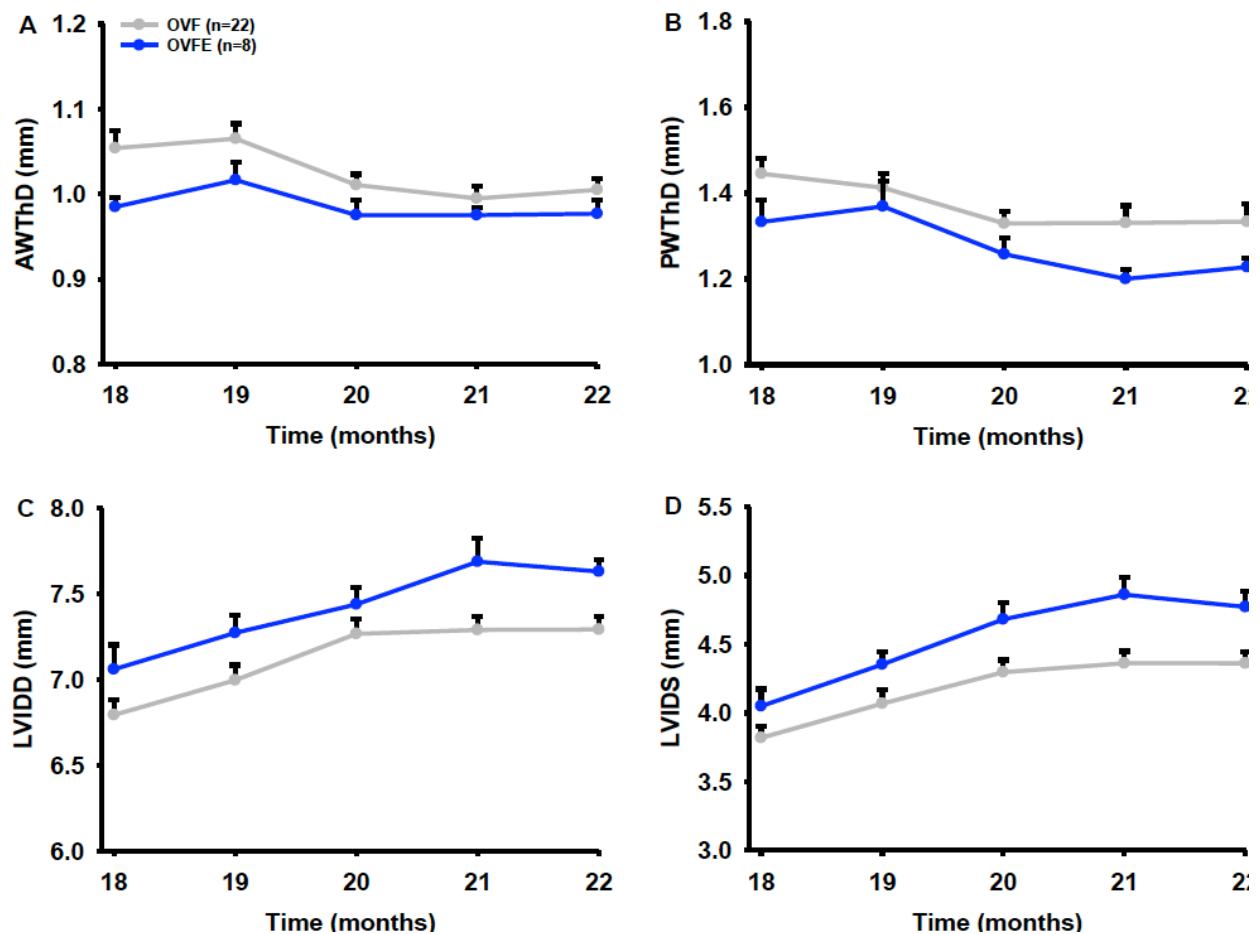**Figure 1.** Left ventricular (LV) remodeling as serially tracked by echocardiography. (A) Anterior wall thickness in diastole (AWThD). (B) Posterior wall thickness in diastole (PWThD). (C) LV internal diameter diastole (LVIDD). (D) LV internal diameter in systole (LVIDS). For panels C and D, $p < 0.001$ for time dependent changes in all groups. OVF: ovariectomized + fructose. OVFE: supplemented with epicatechin. Values are mean ± SEM.

Hemodynamic measurements

As shown in figure 2, for systolic aortic pressure (Pao) there were not differences between OVF and epicatechin treated animals. Cardiac index (output normalized to body weight), stroke volume index and ejection fraction were also comparable. Figure 3 depicts isovolumic relaxation time constant (IVRT) also known as "Tau" and arterial elastance (Ea) respectively. No differences were noted between OVF and epicatechin groups (OVFE).
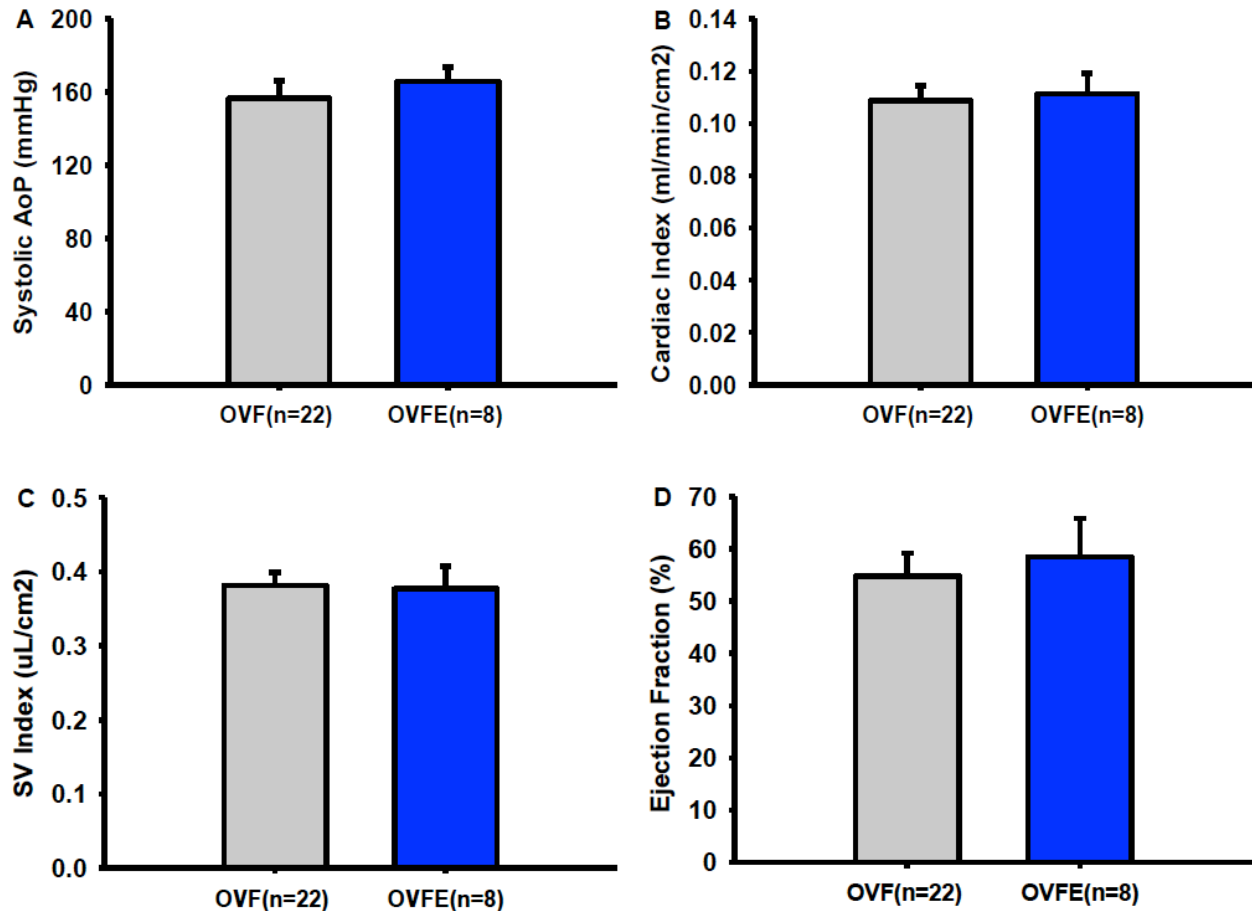
Figure 2. Hemodynamic values derived from arterial and left ventricular (LV) conductance catheter measurements during the terminal study. (A) Systolic aortic pressure. (B) Cardiac index, (C) Stroke volume (SV) index and, (D) Ejection fraction. OVF: ovariectomized + fructose, OVFE: supplemented with epicatechin. Values are mean ± SEM.
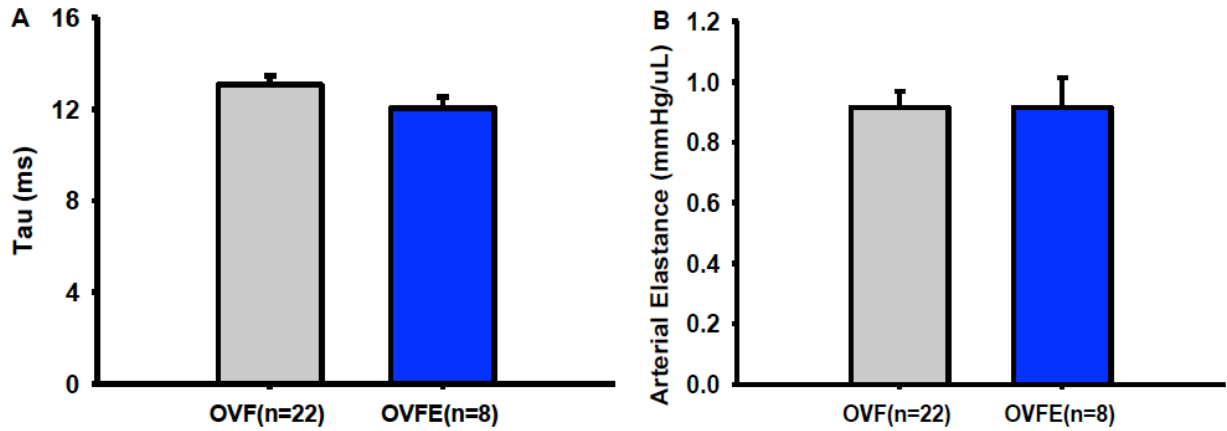
Figure 3. Hemodynamic values derived from arterial and left ventricular (LV) conductance catheter measurements during the terminal study. (A) LV isovolumic relaxation time constant (Tau), (B) Arterial elastance. OVF: ovariectomized + fructose, OVFE: supplemented with epicatechin. Values are mean ± SEM.

Ex-vivo LV mechanics

As shown in figure 4, analysis of LV PV curves (A) demonstrate a significant right shift in epicatechin vs. OVF while chamber stiffness (B) remained similar at the pressures examined. Epicardial circumferential strain analysis revealed no differences in E_{11} between OVF and OVFE groups or in longitudinal strains (E_{22}).

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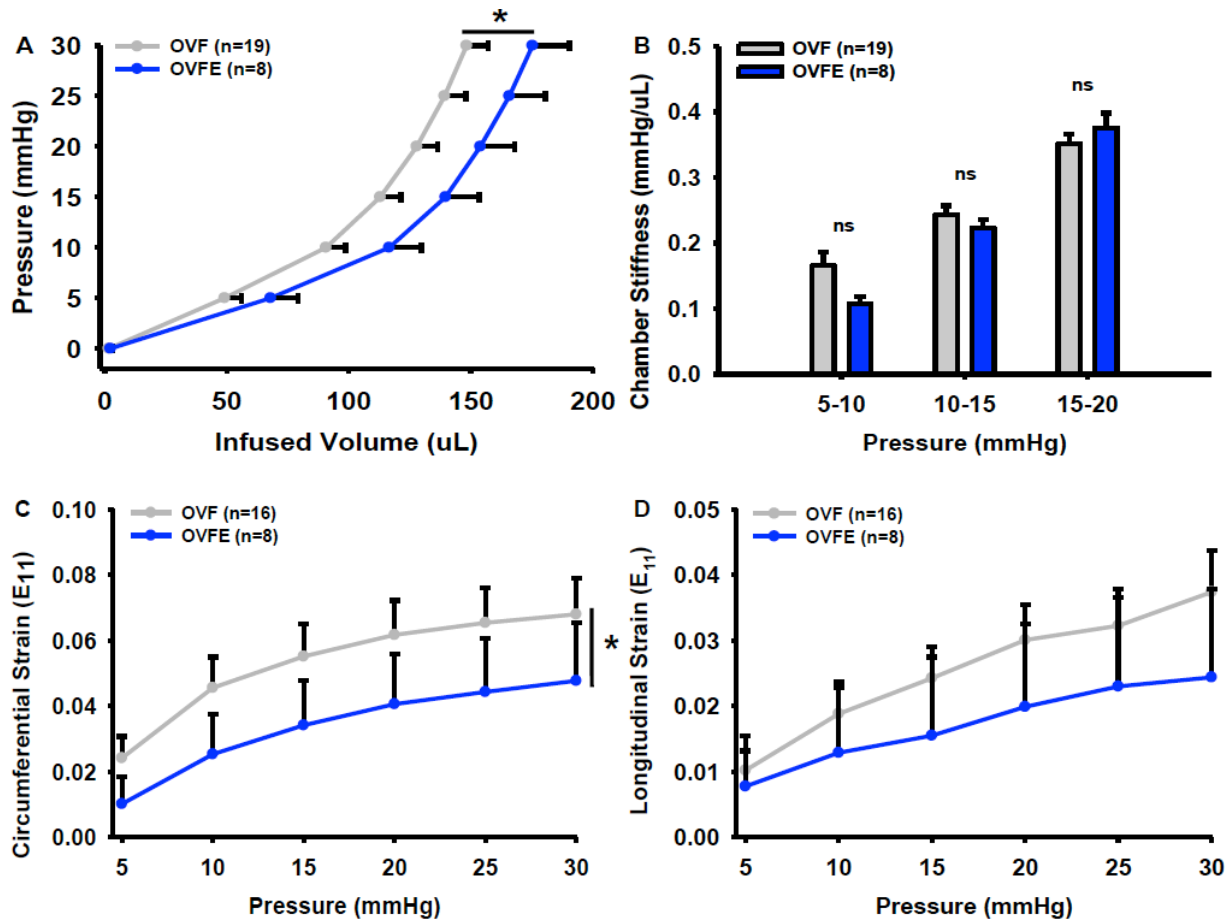
Figure 4. Ex-vivo analysis of left ventricular (LV) mechanics. (A) Passive LV pressure-volume (PV) curves for all groups at 21 months of age (p<0.05 aged, OVF vs. OVFE). (B) analysis of chamber stiffness at different pressures. Two-dimensional circumferential (E_{11}) and longitudinal (E_{22}) LV epicardial strains at incremental LV pressures (C, D). OVF: ovariectomized + fructose. OVFE: supplementec with epicatechin. Values are mean ± SEM.

Histological analysis and collagen quantification

Representative Sirius Red stained cross-sections of hearts from select animals of the different groups at low and high magnifications are shown in figure 5. Images similar to those shown in the panel were used to quantify collagen abundance in the LV by segments (12 free wall and 6 septal). Results from morphometry and histology are summarized in figure 6. Animals treated with epicatechin exhibited significantly decreased LV collagen area, % free wall (p=0.06), septal and total LV collagen vs. OVF. Thus, late epicatechin treatment appears to exert an antifibrotic effect.

Figure 5. Representative images 2 and 20 X magnification from Sirius Red staining of hearts from all groups. Histomorphometric analysis of LV tissue sections.

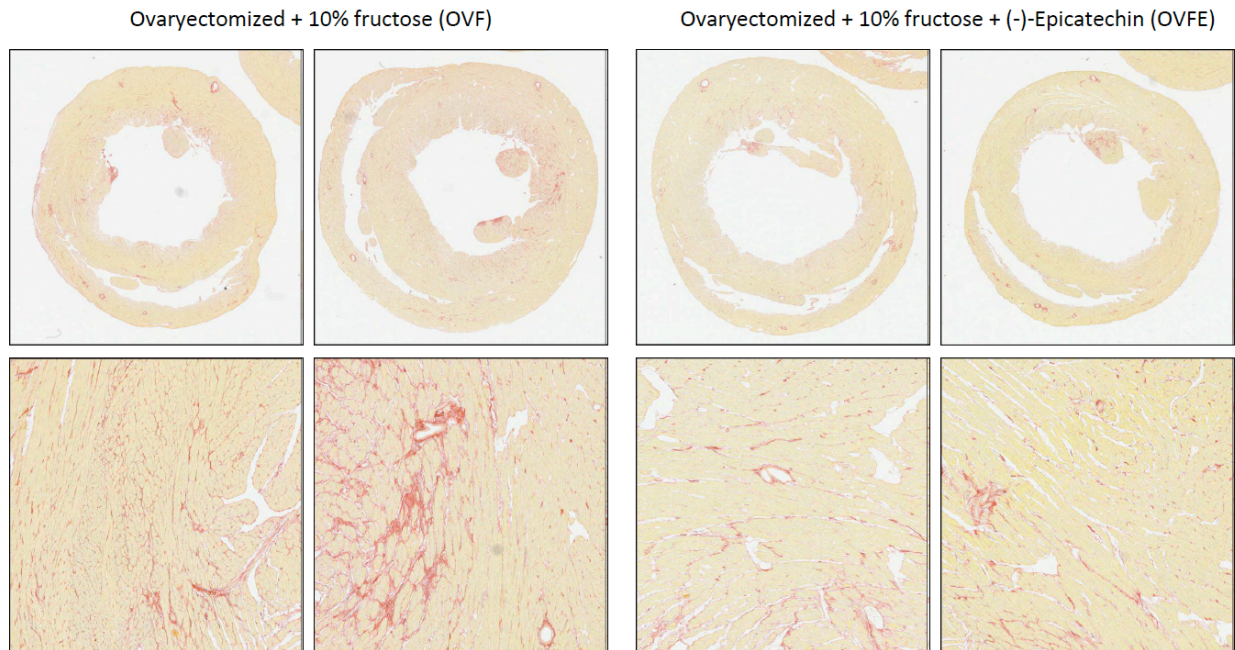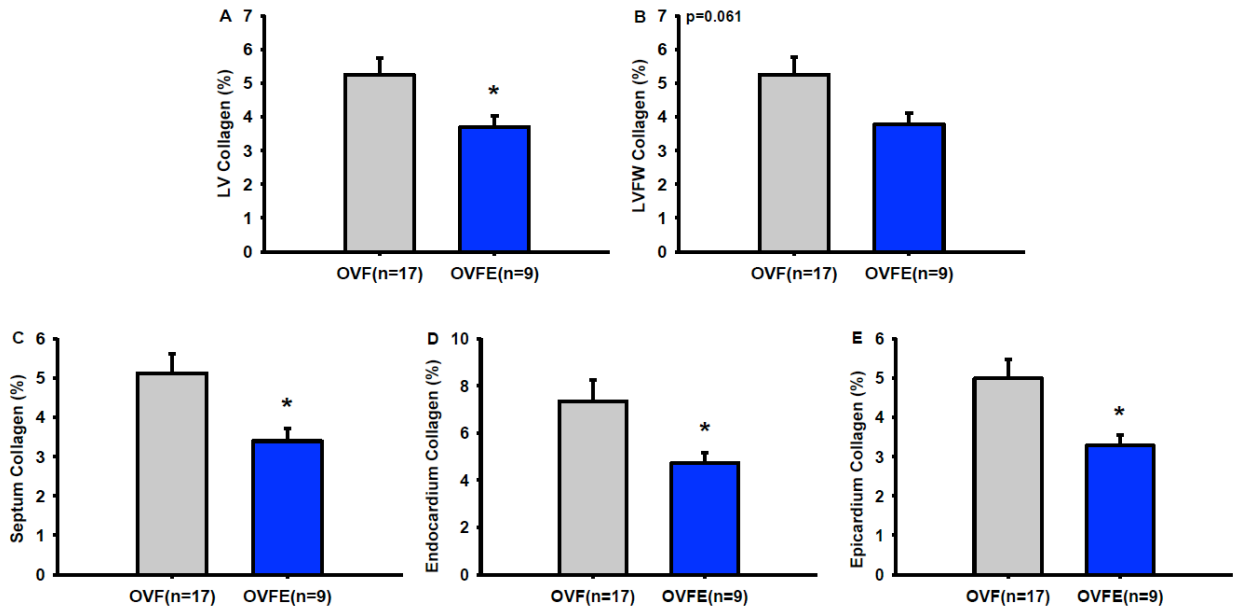**Figure 6**. Histomorphometric analysis of LV tissue sections. (A) LV collagen area, (B) LV collagen area free wall % collagen, (C) septum % collagen, (D) endocardium, (E) epicardium collagen ratio. Values are mean ± SEM. *$p<0.05$ vs. OVF by unpaired t-test.

Major findings from this study can be summarized as follows:
- As per echo results, all groups developed slowly over time and in a similar magnitude, modest eccentric LV remodeling (i.e. chamber dilation)
- With late epicatechin treatment, half-time for diastolic relaxation and increases in arterial elastance remained unchanged vs. OVF
- All groups demonstrate unaltered systolic hypertension
- Systolic and diastolic function measures remained unaltered with epicatechin treatment
- Ex vivo LV pressure-volume curves indicate a right shift in animals treated with epicatechin suggesting greater tissue compliance which did not reach statistical significance
- An notable presence of LV fibrosis occurs in OVF rats that is significantly reduced with epicatechin treatment

Conclusions and Clinical Perspectives

The results presented here are comparable to those previously published by us in aged rats subjected to ovariactomy and fructose supplementation. Late (1 month) treatment with epicatechin was effective in altering the ex vivo passive mechanical properties of the LV while also suppressing fibrosis throughout the myocardial walls. While these results are encouraging as per the potential of epicatechin to reduced tissue fibrosis, it had a minor impact on LV structure and function suggesting that earlier and longer treatment is likely required. In this regards, studies to be reported from aim 1 which are 90% completed should be able to shed light into this possibility. Once the analysis is complete we will be able to gauge the potential to act as an effective reducer of fibrosis that positively impact LV function which can then be explored for its potential clinical use in patients with HFpEF.

Aim 1 and 2 related: Mathematical modeling studies

Fibrillar collagen plays a major role in the passive material properties of the myocardium. Hearts with severe fibrosis have significantly higher tissue stiffness, which can affect overall filling mechanics of the ventricular chamber. It has been shown that cardiac fibrosis is not a homogenous process, and we are employing modeling approaches of diastolic ventricular function to examine the significance of these regional differences in fibrosis. Many studies have modeled infarct zones using finite-element methods, generally by drastically increasing the stiffness of the infarct scar and border zone. To model the pathophysiology of cardiac fibrosis, a similar approach was used in our preliminary studies to model rat LV during passive inflation. Utilizing realistic ventricular geometry, muscle fiber structure and collagen distribution, the goals of the model include quantifying the local material properties, deformations and stresses, and overall passive filling function. Passive material properties are important to diastolic function, and can be used to quantify the efficacy of treatments for heart failure in animal models. A transverse isotropic constitutive law with respect to the local fiber axis was used for this initial model. Experimental results from aim 1 studies showed increased collagen area fraction in the subendocardium vs. epicardium. Simulations using a bulk measurement of collagen versus a transmural variation were compared. These preliminary results show only modest differences between models with a single material vs. those with transmurally varying properties. We are currently awaiting the completion of aim 1 studies to determine if greater differences in tissue fibrosis become evident and what their impact may be in LV material properties.

In collaboration with senior expert from UCSD's department of Bioengineering we are currently also working on developing a testable hypothesis for the potential cause of HFpEF in female patients. As this hypothesis is to be put forward we will be able to propose a series of modeling studies using data derived from these experiments and others. We anticipate to be able to generate a manuscript on this concept within the next 3 months for submission in late 2019.

Aim 3 related

The anti-fibrotic effects of (-)-epicatechin are mediated by TGF-β1 inhibition

Over the course of the last year and a half, we implemented the culturing of cardiac fibroblasts isolated from young and aged female hearts. However, after many "logical" adjustments to the isolation protocol, we consistently detected a lower yield of cells vs. male hearts. Furthermore, in attempting to challenge the cells to develop a pro-fibrotic phenotype using high glucose media or angiotensin II, we noted a limited responsiveness to the stimulation vs. our historical strong response from male derived cells. On these basis, we proceeded to utilize male rat derived cells from our frozen allotments of cells. In figure 7 below, we summarize ELISA results for the determination of TGFβ-1 levels in cell lysates and cell culture media for male cardiac fibroblasts obtained from young hearts stimulated with high glucose for 48 h while figure 8 reports on changes in total collagen levels as per hydroxyproline assays.
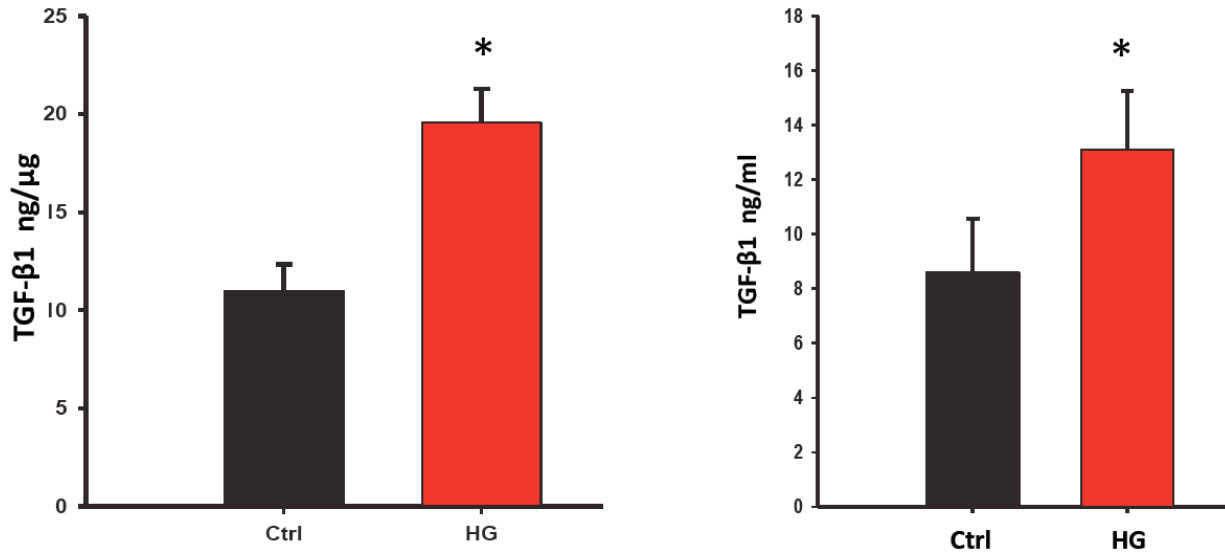
Figure 7. High glucose (HG) media effects on rat cardiac fibroblasts TGF-β1 protein levels as per ELISA. Results from n=5 experiments each, in triplicate.
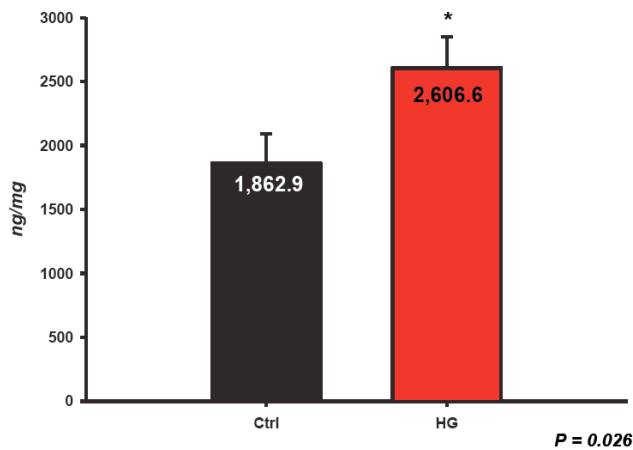
Figure 8. High glucose (HG) media effects on rat cardiac fibroblast collagen levels in cell lysate as per hydroxyproline assays. Results from n=5 experiments each, in triplicate.

III. Opportunities for training

As stated in our 2018 report, the pre-doctoral fellows (Moises Bustamante and Elva Garate) continue to be trained in techniques related to in vivo physiology and pharmacology. Both students are 100% committed to the project and they are now extensively trained on in vitro (cell culture), in vivo and ex vivo methods to assess for changes in cardiac fibroblast or heart structure and function and the impact that Epi treatment yields on relevant endpoints. Training activities also include one-on-one mentoring with senior staff so as to achieve technical proficiency in the methods they are being exposed to. Moises Bustamante is the 1st author listed on the published manuscript and Elva Garate as a middle author. Both students are also listed as authors in the listed abstracts and had the opportunity to present the study results at the 2018 and 2019 Experimental Biology meetings in San Diego, California and Orlando, Florida. Both students are

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currently working on two additional manuscripts to be submitted in 2019 for publication purposes.

IV. Dissemination of results

As noted above, we have disseminated our initial results during the 2018 and 2019 Experimental Biology meetings. We also reported to the international heart failure community, the initial results of the effects of epicatechin in mitigating adverse changes driven by aging/estrogen and/or fructose induced metabolic syndrome at the European Society of Cardiology, Heart Failure meeting in May of 2018 in Vienna, Austria. We reported on further advances to the local academic community (presentation held October 12th, 2018). We will submit the most recent results of our studies to National and International meetings.

V. Plans

The project continues to progress well within the anticipated general plan as essentially all three large in vivo studies have been completed generating a highly detailed database of results. Over the course of the next 2-3 months, as the pending histology results emerge will be able to draw solid conclusions as to the impact that each of the examined factors have on cardiac structure/function and on the capacity of Epi to limit/reverse these. As noted above, all of the relevant data generated are being incorporated into computer based simulations to examine the effect that changing specific variables has on diastolic function. In vitro studies have quicken their pace significantly and are beginning to generate an ample body of evidence for the antifibrotic effects of epicatechin and associated mechanisms of action.

IMPACT

The work being generated by this project has begun to unmask an important role for estrogens in mitigating the risk of developing adverse changes in cardiac structure/function that are slowly compounded with aging and that can remain undetected in female subjects for years. This area of cardiology continues to be underappreciated, as the number of relevant publications that address these factors systematically is very limited. This study also lays the ground for the systematic analysis of differences in sex as it relates to the development of various types of cardiometabolic diseases. As our data begins to validate the potential of epicatechin to exert antifibrotic effects we may then design additional preclinical studies to support this concept and eventually evolve to the planning of clinical studies.

CHANGES/PROBLEMS

We repeatedly modified cell culture conditions and media to optimize the growth of female cells and have encountered a reticence of the cells to growth in a significant manner at passage 2. Thus, limiting greatly a capacity to have enough material to perform the planned experiments. We have thus, opted to use male cardiac fiberoblasts from our frozen lots to perform the planned experiments. Cells are derived from young 3 month old healthy male rats. As documented above, we have implemented an initial round of cell culture experiments with encouraging results and will eventually culture cells from aged male rats to also examine the effects that aging has on

extracellular matrix production and explore the inhibitory effects of epicatechin as well as underlying mechanisms.

PRODUCTS

Journal Publications

1. Bustamante M, Garate-Carrillo A, Ito B, Garcia R, Carson N, Ceballos G, Ramirez-Sanchez I, Omens J, Villarreal F: Unmasking of Estrogen Dependent Left Ventricular Dysfunction in Aged Female Rats: A Potential Model of Early Stage HFpEF. J Phys, 597.7:1805-1817, 2019

2. Saucerman J, Tan P, Buchholz K, McCulloch A, Omens J. Mechanical regulation of gene expression in myocardium. Nat Rev Cardiol, 16:361-378, 2019
[Abstract: The intact heart undergoes complex and multiscale remodelling processes in response to altered mechanical cues. Remodelling of the myocardium is regulated by a combination of myocyte and non-myocyte responses to mechanosensitive pathways, which can alter gene expression and therefore function in these cells. Cellular mechanotransduction and its downstream effects on gene expression are initially compensatory mechanisms during adaptations to the altered mechanical environment, but under prolonged and abnormal loading conditions, they can become maladaptive, leading to impaired function and cardiac pathologies. In this Review, we summarize mechanoregulated pathways in cardiac myocytes and fibroblasts that lead to altered gene expression and cell remodelling under physiological and pathophysiological conditions. Developments in systems modelling of the networks that regulate gene expression in response to mechanical stimuli should improve integrative understanding of their roles in vivo and help to discover new combinations of drugs and device therapies targeting mechanosignalling in heart disease].

3. Carruth E, The I, Schneider J, McCulloch A, Omens J, Frank L. Regional Variations in Diffusion Tensor Anisotropy are Associated with Myocyte Remodeling in Left Ventricular Pressure Overload. Resubmitted, Am J Phys, 2019
[Abstract: In this study we hypothesized that regional (especially transmural) gradients in structural properties of myocardium would become more spatially uniform and would be reduced in pressure overload such as seen with hypertension to maintain uniformity of fiber strain. To test our hypothesis, we performed high-resolution, high-fidelity Diffusion Tensor-MRI (DTI) on rat hearts isolated/fixed from transverse aortic constricted and sham control animals and investigated the regional variations in DTI-derived parameters of orientation, diffusivity, and anisotropy in the LV. We found that there are indeed regional variations in myocyte geometry and structural organization, which become more uniform with pressure overload. Additionally, several structural features correlated significantly with DTI-derived parameters, regardless of phenotype. These results will be valuable in understanding the mechanisms by which cardiac myocytes in different regions of the LV respond to hypertension and may provide a tool for diagnosing the early stages of hypertrophy or other remodeling clinically and non-invasively, allowing for earlier lifestyle changes or interventions to reverse early stage remodeling before the progression to heart failure occurs].

Conference Papers

1. Bustamante M, Garate-Carrillo A, Loredo M, Garcia R, Carson N, Ito B, Ceballos G, Omens J, Ramirez-Sanchez I, Villarreal F. Detrimental Effects of Aging, Ovariectomy and Weight Gain on Left Ventricular Structure and Function: A Potential Preclinical Model of Early Stage HFpEF. FASEB J, 32, S1, 2018

2. Bustamante M, Garate-Carrillo A, Ito B, Ceballos G, Omens J, Ramirez-Sanchez I, Villarreal F. Development of an aging female rat model of HFpEF and evaluation of the antifibrotic potential of (-)-epicatechin. European Journal of Heart Failure, 20, S1, 2018

3. Bustamante M, Garate-Carrillo A, Ito B, Garcia, R, Carson N, Ceballos G, Ramirez-Sanchez I, Omens J, Villarreal F. Antifibrotic Effect of (-)-Epicatechin in a Rodent Model of Early Stage HFpEF. FASEB J, 33, S1, 2019

Presentations

1. Presented Conference Paper listed as #1 above at the Experimental Biology meeting in April, 2018 in San Diego, CA

2. Presented Conference Paper listed as #2 above at the European Society of Cardiology, Heart Failure meeting in May, 2018 in Vienna, Austria

3. Presented in October 2018 at the UCSD Cardiology seminar series. Title: A female rodent model of early HFpEF: A potential critical role for estrogen depletion in the aged heart.

4. Presented Conference Paper listed as #3 above at the Experimental Biology meeting in April, 2019 in Orlando, FL

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

I. Individuals involved in the project

Francisco Villarreal (Principal Investigator)   no change
Jeffrey Omens (Co-Investigator)   no change
Israel Ramirez-Sanchez (Project Scientist)   no change
Diane Huang (SRA 2)   no change

Name	Moises Bustamante
Project role:	Graduate student
ID	114959
Cal months	12
Contribution to project	In vivo physiology and pharmacology
Funding support	CONACyT and DoD (this award)

Name	Elva Garate
Project role:	Graduate student
ID	000921750
Cal months	12
Contribution to project	In vitro biology and in vivo pharmacology
Funding support	CONACyT and DoD (this award)

II. Changes in other support

Nothing to report.

III. What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Not applicable

APPENDICES (Manuscripts)

1. Bustamante M, Garate-Carrillo A, Ito B, Garcia R, Carson N, Ceballos G, Ramirez-Sanchez I, Omens J, Villarreal F: Unmasking of Estrogen Dependent Left Ventricular Dysfunction in Aged Female Rats: A Potential Model of Early Stage HFpEF. J Phys, 597.7:1805-1817, 2019

2. Saucerman J, Tan P, Buchholz K, McCulloch A, Omens J. Mechanical regulation of gene expression in myocardium. Nat Rev Cardiol, 16:361-378, 2019

Unmasking of oestrogen-dependent changes in left ventricular structure and function in aged female rats: a potential model for pre-heart failure with preserved ejection fraction

Moises Bustamante[1,2], Alejandra Garate-Carrillo[1,2], Bruce R. Ito[1], Ricardo Garcia[1,3], Nancy Carson[3] (iD), Guillermo Ceballos[2] (iD), Israel Ramirez-Sanchez[1,2], Jeffrey Omens[1] (iD) and Francisco Villarreal[1,4] (iD)

[1]*Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, CA, USA*
[2]*Seccion de Estudios de Posgrado e Investigacion, Escuela Superior de Medicina, Instituto Politecnico Nacional, Mexico, DF*
[3]*Bristol-Myers Squibb, New York, NY, USA*
[4]*VA San Diego Health Care, San Diego, CA, USA*

Edited by: Don Bers & Beth Habecker

The Journal of Physiology

Key points

- Heart failure with preserved ejection fraction (HFpEF) is seen more frequently in older women; risk factors include age, hypertension and excess weight.
- No female animal models of early stage remodelling (pre-HFpEF) have examined the effects that the convergence of such factors have on cardiac structure and function.
- In this study, we demonstrate that ageing can lead to the development of mild chamber remodelling, diffuse fibrosis and loss of diastolic function.
- The loss of oestrogens further aggravates such changes by leading to a notable drop in cardiac output (while preserving normal ejection fraction) in the presence of diffuse fibrosis that is more predominant in endocardium and is accompanied by papillary fibrosis.
- Excess weight did not markedly aggravate such findings.
- This animal model recapitulates many of the features recognized in older, female HFpEF patients and thus, may serve to examine the effects of candidate therapeutic agents.

Abstract Two-thirds of patients with heart failure with preserved ejection fraction (HFpEF) are older women, and risk factors include hypertension and excess weight/obesity. Pathophysiological factors that drive early disease development (before heart failure ensues) remain obscure and female animal models are lacking. The study evaluated the intersecting roles of ageing, oestrogen depletion and excess weight on altering cardiac structure/function. Female, 18-month-old, Fischer F344 rats were divided into an aged group, aged + ovariectomy (OVX) and aged + ovariectomy + 10% fructose (OVF) in drinking water ($n = 8$–16/group) to induce weight gain.

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Left ventricular (LV) structure/function was monitored by echocardiography. At 22 months of age, animals were anaesthetized and catheter-based haemodynamics evaluated, followed by histological measures of chamber morphometry and collagen density. All aged animals developed hypertension. OVF animals increased body weight. Echocardiography only detected mild chamber remodelling with ageing while intraventricular pressure–volume loop analysis showed significant ($P < 0.05$) decreases *vs.* ageing in stroke volume (13% OVX and 15% for OVF), stroke work (34% and 52%) and cardiac output (29% and 27%), and increases in relaxation time (10% OVX) with preserved ejection fraction. Histology indicated papillary and interstitial fibrosis with ageing, which was higher in the endocardium of OVX and OVF groups. With ageing, ovariectomy leads to the loss of diastolic and global LV function while preserving ejection fraction. This model recapitulates many cardiovascular features present in HFpEF patients and may help understand the roles that ageing and oestrogen depletion play in early (pre-HFpEF) disease development.

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Introduction

Heart failure (HF) is the most common cause for hospitalization in older patients and represents the greatest cost for Medicare (Owan *et al.* 2006; Lam *et al.* 2011). Currently, up to 50% of HF patients are now recognized to have what is termed HF with preserved ejection fraction (HFpEF). This is a poorly understood disease and there are no therapies identified as clearly effective in mitigating its pathology (Omar *et al.* 2016; Barandiarán Aizpurua *et al.* 2019). Certain features such as the preservation of left ventricular (LV) geometry and ejection fraction (while at rest) in the setting of diastolic dysfunction are recognized as 'most common' in HFpEF patients (Omar *et al.* 2016). To better understand its pathophysiological underpinnings and the effects of 'preventive' therapies, animal models of pre-HFpEF would be desirable. HFpEF is more predominant in elderly, post-menopausal female patients (2:1) *vs.* men (Pacher *et al.* 2008; Lam *et al.* 2011) and the causes for this unequal distribution remain unclear. In women, the disease is also closely associated with the presence of hypertension as well as excess weight/obesity (Eaton *et al.* 2016). The development of pre-HFpEF animal models would thus require the convergence of 'risk' factors known to be associated with the disease while recapitulating features commonly seen with the pathology (Borlaug, 2016; Omar *et al.* 2016).

Systemic processes suspected to play a prominent role in the development of diastolic dysfunction with HFpEF include endothelial and mitochondrial dysfunction as well as oxidative stress (Borlaug, 2014), all of which have been associated with the presence of menopause in women, as well as with ageing (Takahashi & Johnson, 2015). However, very few female animal models have examined the impact that the loss of oestrogens has in the evolution of LV function, in particular, as low oestrogen levels interact with ageing, hypertension and/or excess weight before the onset of HF (Omar *et al.* 2016).

The Fischer F344 rat developed by NIH investigators has been extensively used as a model to study processes associated with ageing (Boluyt, 2004; Pacher *et al.* 2004). However, the great majority of published studies using rodent models of heart disease have used young, male animals with only an extremely limited number of studies focused on the female sex and the role of oestrogens (Conceição *et al.* 2016; Valero-Muñoz *et al.* 2017). While aged female rodents do not truly develop menopause, ovariectomy has been widely used and validated as a research tool to examine the role that oestrogen deprivation has on the control of multiple physiological systems (Sohrabji, 2005; Knowlton & Lee, 2012).

The main objective of this study was to develop and characterize a female rat model where ageing, oestrogen deprivation, hypertension and excess weight converge, with the intent to produce structural and functional changes that while not leading to HF, parallel those found in female HFpEF patients. To achieve this goal, older, female F344 rats undergoing ovariectomy and fructose supplementation were assessed for changes in LV structure and function, highlighting the changes associated with the presence of HFpEF in older, female patients.

Methods

Study design

Young (3-month-old) and aged (18-month-old) female Fischer F344 rats were used. Aged animals were obtained from the NIH/NIA ageing colony. Young animals were used as a reference control for all measurements. Animals were housed in pairs and maintained with a 12-h light–dark cycle with *ad libitum* intake of standard rat chow and sterilized tap water. Animal care and use

followed National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* guidelines, and the Institutional Animal Care and Use Committee of the University of California San Diego School of Medicine approved the protocol for this study. Figure 1 summarizes the study design and protocol. Aged rats were acclimated to the environment for 1 week and then randomly assigned to three groups: aged ($n = 8$), ovariectomized (OVX, $n = 16$) and ovariectomy + 10% fructose in drinking water (OVF, $n = 16$). OVX animals underwent bilateral oophorectomy performed under isoflurane anaesthesia via a dorsal incision as previously described (Stout Steele & Bennett, 2011). OVF animals also had this surgery and were started on 10% fructose (w/v) in their drinking water 1 week after ovariectomy. Fructose intake was used to induce weight gain and replicate metabolic syndrome-like features known to be commonly present in female HFpEF patients. All aged animals were maintained under the same conditions for 3 months (to 21 months of age) with weekly measurement of body weight. At 21 months, all rats were subjected to a terminal study under iso-flurane anaesthesia to measure *in vivo* LV haemodynamics, and *ex vivo* passive LV pressure–volume and epicardial strain–pressure curves. Young rats underwent the same terminal study after a 1 week period of acclimatization. Blood and select tissues including tibias were collected for further analysis.

Echocardiography

Closed-chest echocardiography was performed monthly for 3 months (between 18 and 21 months of age) in isoflurane-sedated animals using a GE Vivid 7 machine and an i12L probe (GE Healthcare, Milwaukee, WI, USA). Young rats were evaluated only once after the 1 week period of acclimatization. Measured parameters included anterior wall thickness in diastole and systole (AWThD/AWThS), posterior wall thickness in diastole and systole (PWThD/PWThS), LV internal diameter in

diastole and systole (LVIDD/LVIDS), heart rate (HR), ejection fraction (EF) and fractional shortening (FS).

In vivo haemodynamics

At the terminal time point, animals were anaesthetized using 2.5% isoflurane, intubated and mechanically ventilated. To measure haemodynamics, a 2 French pressure transducer/conductance catheter (SPR-838 Millar Instruments; Houston, TX, USA) was introduced via the carotid artery into the aorta and LV. LV pressure and volume data were acquired at baseline and during temporary occlusion of the inferior vena cava at the level of the diaphragm to change LV filling. At the end of each experiment, parallel conductance was determined using an intravenous saline injection (Pacher *et al.* 2008). Measurements were recorded at a sampling rate of 1000 Hz using ADInstruments Powerlab (Colorado Springs, CO, USA) hardware and LabChart software. Parameters recorded included stroke work (SW), cardiac output (CO, ml min^{-1}), stroke volume (SV, μl), end-diastolic and end-systolic volumes (μl) and indexes, ejection fraction (EF, %), LV first derivative of pressure (dP/dt maximum and minimum), isovolumic relaxation time constant (Tau, ms) and systolic and diastolic aortic pressure (P_{ao}).

Ex vivo passive LV mechanics

After *in vivo* cardiac measurements were obtained, the heart was arrested with a slow infusion of 2 ml of a cardio-plegia solution containing 0.03 M 2,3-butanedione mono-xime and high potassium solution. The heart was rapidly excised and rinsed in sterile ice-cold saline, and remaining connective tissue was trimmed. The aorta was cannulated using a modified Langendorff system where it was perfused with the cold cardioplegia solution at 10–15 mmHg. A balloon was inserted into the LV from the left atrium and connected to a pressure transducer and an infusion pump as done previously (Omens *et al.* 1995).

To generate passive LV pressure–volume (PV) curves, the LV balloon was inflated at a constant rate of 200 μl min^{-1}. Pressure and infused volume data were acquired using DATAQ Software and Data Acquisition Systems (Akron, OH, USA) at a sampling rate of 10 Hz. Just prior to initiation of the infusion–deflation cycles, discrete epicardial markers were placed on the anterior LV epicardial surface. During a PV inflation–deflation cycle, synchronized video images were acquired using a ×250 digital USB microscope (Plugable Technologies, Redmond, WA, USA). The displacements of the epicardial markers were obtained during each infusion cycle to calculate two-dimensional circumferential (E_{11}) and longitudinal (E_{22}) strains relative to the long axis of the heart. Two to three preconditioning inflation runs
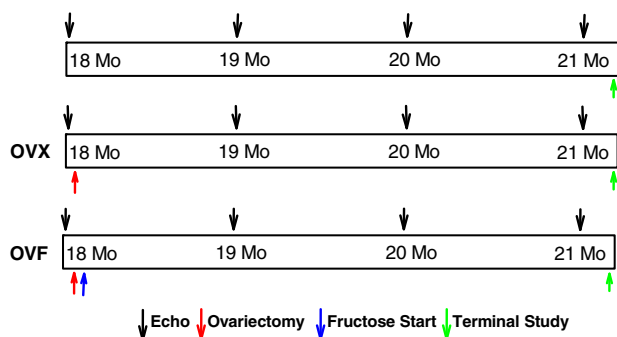

Figure 1. Scheme depicting the timeline of the study, measures and interventions used over the course of 3 months

were followed by two to three data acquisition runs with a maximal LV pressure of ~30 mmHg. Epicardial strains were calculated using customized strain software. Validation of these types of passive functional studies has been done previously (Omens *et al.* 1993).

Following these mechanical measures, hearts were perfusion fixed for 10 min using 10% formalin (EMD Millipore Corp., Billerica, MA, USA) introduced via the aortic cannula and with the LV balloon maintained at 10–15 mmHg. Fixed tissues were stored in conical tubes at a temperature of 4°C for 2 weeks until histological evaluation.

Histological analysis and collagen quantification

As shown in Fig. 2, formalin-fixed hearts were weighed and trimmed before sectioning at the midventricular region for subsequent embedding in paraffin. LV base and apical rings were then sectioned and mounted onto slides for Sirius Red staining and microscopic imaging. Using HALO® Digital Pathology Software (Indica Labs, Corrales, NM, USA) the analysis of the LV area and collagen positive area was performed by subdividing the LV ring into 18 discrete epicardial to endocardial wall sections. Of these regions, 12 were identified as free wall and six as septal. The ring sections of the LV were also digitally divided into inner half (subendocardium) and outer half (subepicardium) to allow comparison of endocardial *vs.* epicardial collagen distribution.

Statistical analysis

All data shown is presented as the mean ± standard error of the mean (SEM). Statistical analyses used are one-way or two-way ANOVA and Holm–Sidak's *post hoc* test and Student's unpaired *t* test as appropriate using SigmaPlot (Systat Software, 2008, San Jose, CA, USA). Results were considered statistically significant at a value of $P < 0.05$.

Results

General parameters

Total body weight and percentage body weight gain over time of aged, OVX and OVF rats are shown in Fig. 3*A* and *B*. Aged and OVX animals showed a 6% and 10% increase in weight respectively while the OVF group gained 26% ($P < 0.05$, OVF *vs.* OVX and aged). As shown in Table 1, there were no differences in heart weights between aged groups, but there was a difference between OVF *vs.* young ($P < 0.05$ by unpaired *t* test). Calculated body surface area was higher in OVF and OVX *vs.* aged ($P < 0.001$).

Echocardiography

Echocardiographic results are summarized in Table 1. As shown in Fig. 4*A–D*, results reveal a time-dependent effect on cardiac morphometry in aged, OVX and OVF groups. AWThD significantly decreased as a function of time with no differences between aged, OVX and OVF groups (Fig. 4*A*). In AWThS, there was a significant difference between aged *vs.* OVX and OVF ($P < 0.05$). For PWThD and PWThS there was also a decrease in thickness over time without difference between aged, OVX and OVF groups (Fig. 4*B*). While LVIDD and LVIDS demonstrated no differences between aged, OVX and OVF groups, there was a significant time dependent increase in chamber diameters (Fig. 4*C* and *D*). HR, EF and FS were also not different between aged, OVX and OVF groups. BW, AWThD and AWThS of aged, OVX and OVF were different *vs.* young ($P < 0.001$). LVIDD, LVIDS and PWThD of aged, OVX and OVF were significantly different *vs.* young ($P = 0.003$, $P = 0.014$ and $P = 0.002$, respectively).

Haemodynamic measurements

Results from *in vivo* haemodynamic measurements are summarized in Table 2 and selected data are shown in Fig. 5*A–C*. For systolic P_{ao}, there was a significant increase in aged groups *vs.* young ($P = 0.009$). Cardiac index was decreased in aged, OVX and OVF *vs.* young animals ($P < 0.05$) while OVX and OVF were different *vs.* aged ($P < 0.001$). Stroke volume index was reduced in aged, OVX and OVF *vs.* young ($P < 0.05$), and differences between OVX and OVF were also present *vs.* aged ($P < 0.001$) indicating an effect of ovariectomy and fructose on cardiac function. EF was stable and not different between groups, being preserved at the normal range of ≥50% (Fig. 5*D*). Figure 6*A* and *B* depicts iso-volumic relaxation time constant (IVRT), also known as 'Tau', and arterial elastance (E_a), respectively, in the different groups. An increase in Tau was noted in aged, OVX and OVF groups *vs.* young ($P < 0.05$). Arterial elastance was significantly elevated in OVX and OVF *vs.* young ($P < 0.05$) with a trend from an increase in the aged group. The end-diastolic pressure–volume relationship (EDPVR) slope demonstrated differences between aged and OVX ($P < 0.05$).

Ex vivo LV mechanics

As shown in Fig. 7*A*, the analysis of LV PV curves did not demonstrate differences among the aged, OVX and OVF groups. However, all these groups were different *vs.* young ($P < 0.001$) demonstrating a global right-shift in aged, OVX and OVF animals. Epicardial circumferential strain analysis (Fig. 7*B*) revealed no differences in E_{11} between aged, OVX and OVF groups or when compared to young.

In longitudinal strain E_{22}, the maximal strain observed at 30 mmHg was ~0.04 (4%) and no differences were detected amongst all groups. Overall, E_{11} strains were higher than E_{22} for all groups, where the range for E_{11} was ~0.06–0.09 (i.e. 6–9%).

Histological analysis and collagen quantification

Representative Sirius Red-stained cross-sections of hearts from select animals of the different groups at low and high magnifications are shown in Fig. 8. Images similar to those shown in the panel were used to quantify collagen abundance in the LV by segments as outlined in Methods. Interestingly, a visual inspection of large areas of abnormal (patch like) fibrosis present in papillary muscles indicated that aged hearts showed such lesions in 1/4 aged animals (25%), 4/6 OVX (67%) and 5/7 OVF (71%) *vs.* none in young animals. Results from morphometry and histology are summarized in Fig. 9*A–F*. Aged rats demonstrated a higher LV tissue area *vs.* young ($P < 0.05$). Aged, OVX and OVF groups exhibited increased LV collagen area, percentage free wall, septal and total LV collagen *vs.* young ($P < 0.05$). Whereas the endocardial/epicardial ratio did not demonstrate significant overall differences by ANOVA among the groups, there were differences between OVX and OVF *vs.* young ($P < 0.05$, by unpaired *t* test).

Discussion

Findings from this study indicate that with ageing in female rats, a modest degree of LV remodelling is observed which is accompanied by the prolongation of relaxation time. With ovariectomy, these changes are compounded by significant reductions in stroke volume and CO in the setting of preserved EF. Ageing in this animal model also showed the development of diffuse myocardial fibrosis, which becomes greater in the endocardium
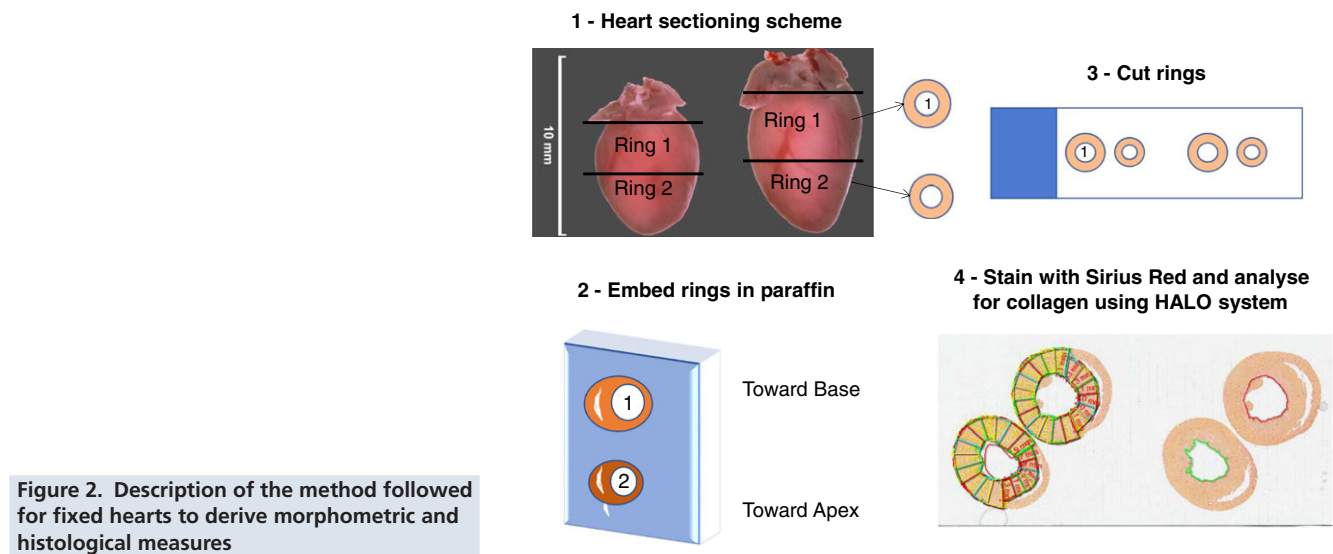
Figure 2. Description of the method followed for fixed hearts to derive morphometric and histological measures
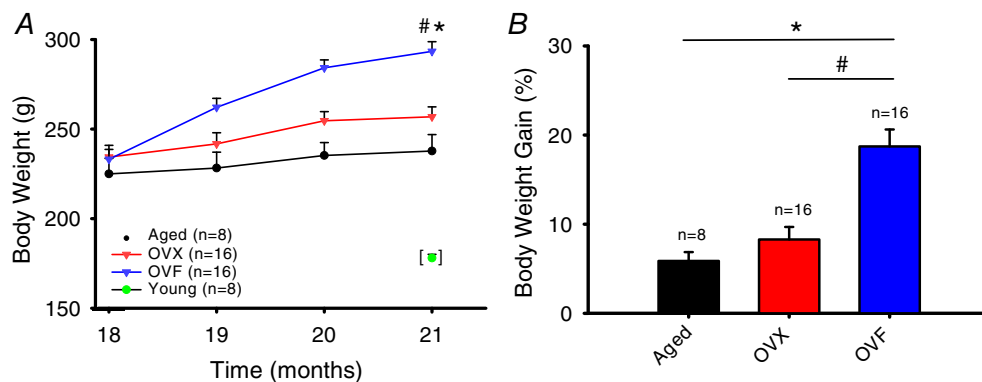
Figure 3. Changes in body weight observed in the different animal groups over 3 months
A, body weight gain in grams ($P < 0.001$, OVF *vs.* OVX and aged). *B*, body weight gain as a percentage of the initial weight ($P < 0.001$, OVF *vs.* OVX and aged). Young group single time-point data included as reference. Values are means ± SEM. OVF, ovariectomized + fructose; OVX, ovariectomized.

Table 1. Body weight, heart weight and echocardiographic assessment of systolic and diastolic function of young and aged, ovariectomized (OVX) and ovariectomized + fructose (OVF) Fischer 344 female rats

| | | | | | | | P | | | |
| | | | | | | | Two-way ANOVA (aged, OVX and OVF) | | | One-way ANOVA (sacrifice) |
Parameter	Group	3 months	18 months	19 months	20 months	21 months	Group	Interval	Interaction	All group
BW (g)	Young	178 ± 2.07								<0.001
	Aged		225.00 ± 8.58	228.25 ± 8.84	235.25 ± 7.23	237.75 ± 9.15	<0.001	<0.001	<0.001	
	OVX		234.38 ± 6.54	241.75 ± 6.21	254.53 ± 5.09	256.88 ± 5.53				
	OVF		233.13 ± 5.53	262.13 ± 5.03	284.19 ± 4.43	293.38 ± 5.42				
HW (g)	Young	0.730 ± 0.032								0.300
	Aged					0.846 ± 0.054				
	OVX					0.896 ± 0.054				
	OVF					0.895 ± 0.037				
AWThD (mm)	Young	0.91 ± 0.006								<0.001
	Aged		1.09 ± 0.022	1.05 ± 0.023	1.03 ± 0.014	1.03 ± 0.020	0.809	<0.001	0.407	
	OVX		1.07 ± 0.013	1.07 ± 0.016	1.02 ± 0.009	1.01 ± 0.013				
	OVF		1.10 ± 0.017	1.08 ± 0.022	1.03 ± 0.014	1.01 ± 0.018				
AWThS (mm)	Young	1.20 ± 0.027								<0.001
	Aged		1.70 ± 0.074	1.55 ± 0.072	1.53 ± 0.030	1.51 ± 0.034	0.017	<0.001	0.044	
	OVX		1.51 ± 0.032	1.52 ± 0.043	1.43 ± 0.030	1.33 ± 0.021				
	OVF		1.47 ± 0.033	1.56 ± 0.047	1.44 ± 0.025	1.40 ± 0.031				
LVIDD (mm)	Young	6.74 ± 0.088								0.003
	Aged		6.65 ± 0.089	6.76 ± 0.133	6.80 ± 0.124	6.99 ± 0.110	0.205	<0.001	0.142	
	OVX		6.81 ± 0.110	6.87 ± 0.112	7.09 ± 0.084	7.32 ± 0.097				
	OVF		6.59 ± 0.091	6.85 ± 0.097	7.19 ± 0.109	7.20 ± 0.095				
LVIDS (mm)	Young	3.82 ± 0.065								0.014
	Aged		3.64 ± 0.079	4.00 ± 0.113	4.04 ± 0.128	4.06 ± 0.141	0.388	<0.001	0.275	
	OVX		3.74 ± 0.064	4.05 ± 0.123	4.25 ± 0.099	4.44 ± 0.154				
	OVF		3.61 ± 0.061	3.91 ± 0.114	4.25 ± 0.118	4.30 ± 0.108				
PWThD (mm)	Young	1.10 ± 0.028								0.002
	Aged		1.49 ± 0.030	1.44 ± 0.045	1.38 ± 0.027	1.44 ± 0.065	0.729	<0.001	0.483	
	OVX		1.46 ± 0.036	1.45 ± 0.038	1.35 ± 0.026	1.34 ± 0.047				
	OVF		1.52 ± 0.025	1.44 ± 0.042	1.35 ± 0.036	1.36 ± 0.051				
PWThS (mm)	Young	1.97 ± 0.280								0.216
	Aged		2.44 ± 0.035	2.33 ± 0.056	2.35 ± 0.041	2.41 ± 0.084	0.298	0.003	0.951	
	OVX		2.52 ± 0.044	2.37 ± 0.055	2.33 ± 0.038	2.36 ± 0.074				
	OVF		2.58 ± 0.041	2.43 ± 0.071	2.39 ± 0.057	2.45 ± 0.065				
HR (bpm)	Young	302.15 ± 10.972								0.199
	Aged		292.75 ± 9.100	307.88 ± 11.038	291.13 ± 14.111	311.50 ± 8.113	0.225	0.006	0.275	
	OVX		297.69 ± 5.702	335.38 ± 7.061	309.81 ± 8.930	302.38 ± 9.707				
	OVF		305.00 ± 7.484	323.13 ± 5.989	301.50 ± 11.431	326.56 ± 8.703				
EF (%)	Young	79.87 ± 0.429								0.109
	Aged		81.75 ± 0.701	77.00 ± 1.239	77.00 ± 1.180	78.25 ± 1.449	0.597	<0.001	0.295	
	OVX		81.56 ± 0.223	77.25 ± 1.039	76.44 ± 0.962	75.00 ± 1.497				
	OVF		81.75 ± 0.520	79.25 ± 1.135	77.00 ± 1.057	76.38 ± 1.197				
FS (%)	Young	43.33 ± 0.419								0.146
	Aged		45.38 ± 0.596	40.75 ± 1.146	40.75 ± 1.098	42.13 ± 1.302	0.548	<0.001	0.333	
	OVX		45.19 ± 0.277	41.00 ± 0.949	40.19 ± 0.823	39.38 ± 1.316				
	OVF		45.38 ± 0.523	42.94 ± 1.086	41.06 ± 0.994	40.50 ± 1.021				

Values are means ± SEM. AWThD, anterior wall thickness diastole; AWThS, anterior wall thickness systole; BW, body weight; EF, ejection fraction; FS, fractional shortening; HR, heart rate; HW, heart weight; LVIDD, left ventricle internal diameter diastole; LVIDS, left ventricle internal diameter systole; PWThD, posterior wall thickness diastole; PWThS, posterior wall thickness systole.

with ovariectomy, accompanied by a marked development of papillary fibrosis. The presence of modest chamber geometric remodelling, preservation of EF, prolongation of LV relaxation time, loss of CO and development of fibrosis recapitulates several of the features recognized in older, female HFpEF patients and suggests this model may be suitable to explore the role that risk factors play in early disease development and the effects of therapeutic interventions.

Multiple rodent models have been employed in an attempt to examine suspect pathophysiological events linked to the development of HFpEF (Conceição *et al.* 2016; Valero-Muñoz 2017). While models commonly incorporate suspect aetiological factors such as pressure overload, obesity and diabetes, there is much less emphasis on the use of female or aged animals, thus incompletely replicating the prototypical human female HFpEF patient (Omar *et al.* 2016). Common findings using young, male rodents with aortic banding/or hypertension include LV hypertrophy, inflammation, fibrosis, stiffer end-diastolic pressure–volume relationships (EDPVR), and prolongation of relaxation with minimal changes in EF (Conceição *et al.* 2016; Valero-Muñoz *et al.* 2017). Diabetic models also demonstrate hypertrophy and interstitial fibrosis, while obese animals show decreases in the E/A ratio (i.e. impaired LV relaxation) (Valero-Muñoz *et al.* 2017). There are certainly relevant applications for these animal models, but they do not replicate the convergence of 'typical' factors found in ageing female patients that subsequently develop HFpEF.

Ageing models have used various rat strains (mostly male) to characterize changes in cardiac structure/function (Boluyt, 2004; Pacher et al. 2004, 2008; Valero-Muñoz *et al.* 2017). When using ~2-year-old female Fischer 344 rats, ageing results in LV hypertrophy and diastolic dysfunction, while males from the same strain/age develop eccentric remodelling, mitral regurgitation, interstitial fibrosis and impaired systolic function (Forman *et al.* 1997). Hybrid Brown Norway–Fischer 344 rats (F344BN) have also been used to study the effects of ageing. Female rats of up to 30 months of age show development of LV hypertrophy, dilatation and diastolic dysfunction (Fannin *et al.* 2014), while decreases in collagen area fraction have been reported (Hacker, 2005). In our study, using echocardiography, we document the development of mild eccentric chamber remodelling from 18 to 21 months of age, with no apparent changes in diastolic or systolic function, including EF. However, direct catheter-based haemodynamics reveal with ageing hypertension, the prolongation of relaxation and a modest but significant reduction in CO while maintaining EF. Histology demonstrated an approximate doubling of collagen area fraction in the absence of chamber stiffening as revealed by *ex vivo* LV passive PV curves and epicardial strains. Therefore, the prolongation of relaxation is likely derived from impaired active LV relaxation, and not the direct effect of excess collagen and tissue stiffening at the levels measured here (~5%). Thus, in aged female rats, there can be modest LV chamber remodelling, prolonged relaxation and interstitial fibrosis while maintaining normal EF.

The high prevalence of HFpEF in older women suggests a strong link between low oestrogen levels and the disease (Zhao *et al.* 2014). Interestingly, published studies suggest the development of greater levels of myocardial fibrosis in post-menopausal women *vs.* men (Barasch *et al.* 2009; Liu & Liu, 2014) with normal ageing or with HF. In rats, unlike menopausal women, oestradiol levels during ageing can be near their younger counterpart even past 20 months of age (Fannin *et al.* 2014). Thus, ovariectomy is commonly used to examine the role that low oestrogen levels play in altering cardiac structure/function (Knowlton & Lee, 2012). Unfortunately, only an extremely limited number of studies have used this approach. Using young male and female rats undergoing aortic banding (Douglas *et al.* 1998), a lesser degree of LV chamber remodelling, loss of function and fibrosis were noted in female animals. However, the protective role of oestrogens in rodent models of pressure overload is greatly diminished with ovariectomy (Bhuiyan *et al.* 2007). Stice *et al.* (2011) reported that ovariectomy in 20-month-old Norway Brown rats decreases LV fractional shortening from
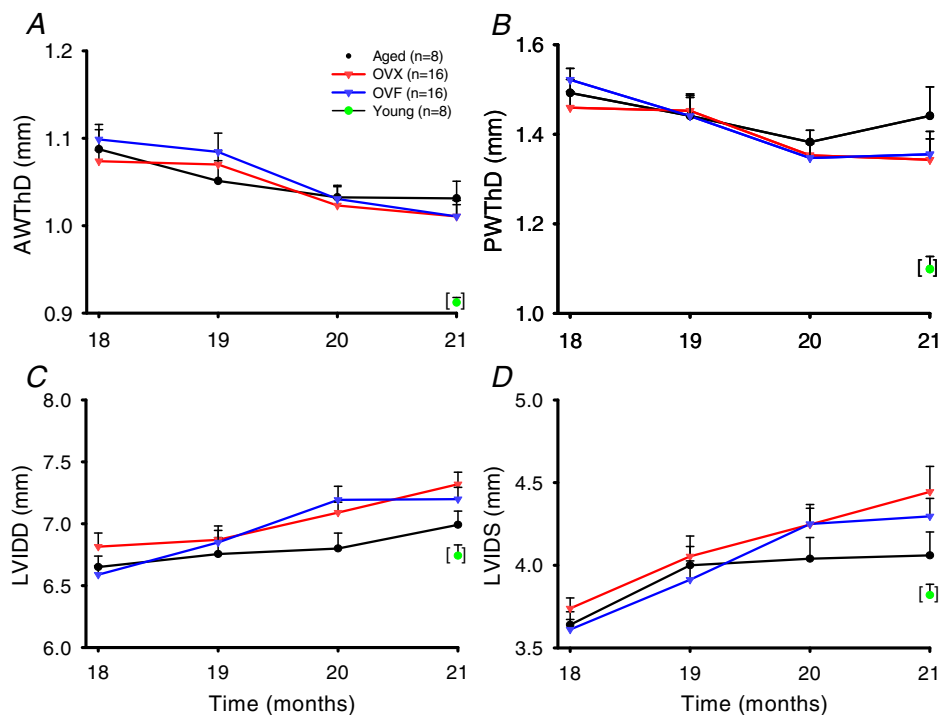
Figure 4. Left ventricular (LV) remodelling as serially tracked by echocardiography
Young group single time-point data included as reference. *A*, anterior wall thickness in diastole (AWThD). *B*, posterior wall thickness in diastole (PWThD). *C*, LV internal diameter diastole (LVIDD). *D*, LV internal diameter in systole (LVIDS). Values are mean ± SEM. For panels *A–D*, $P < 0.001$ for time-dependent changes in all groups. OVF, ovariectomized + fructose; OVX, ovariectomized.

Table 2. Arterial and left ventricular (LV) haemodynamics by conductance catheter of young (Y), aged (A), ovariectomized (OVX) and ovariectomized + fructose (OVF) Fischer 344 female rats

| | Group | | | | | Two-way ANOVA | | | | | |
Parameter	Y (n = 8)	A (n = 8)	OVX (n = 16)	OVF (n = 16)	P	A vs. Y	OVX vs. Y	OVF vs. Y	OVX vs. A	OVF vs. A	OVF vs. OVX
SAC BW (g)	178.0 ± 2.1	238.3 ± 8.6	253.4 ± 5.4	290.8 ± 4.9	<0.001	<0.001	<0.001	<0.001	0.083	<0.001	<0.001
BSA (cm^2)	311.0 ± 2.4	377.4 ± 8.9	393.4 ± 5.7	431.3 ± 4.9	<0.001	<0.001	<0.001	<0.001	0.077	<0.001	<0.001
SW (mmHg ml)	157.2 ± 12.0	214.1 ± 18.6	143.4 ± 8.7	146.3 ± 10.8	0.001	0.031	0.824	0.789	0.001	0.002	0.842
CO (ml/min)	63.5 ± 6.3	63.5 ± 6.3	44.8 ± 2.4	46.3 ± 2.8	< 0.001	0.899	<0.001	0.002	0.002	0.004	0.723
Cardiac index	0.212 ± 0.010	0.169 ± 0.017	0.114 ± 0.006	0.108 ± 0.007	< 0.001	0.015	< 0.001	<0.001	<0.001	<0.001	0.579
SV (μl)	194.8 ± 9.6	214.9 ± 29.3	167.2 ± 7.9	161.8 ± 8.8	0.031	0.605	0.404	0.323	0.051	0.049	0.730
SV index	0.627 ± 0.032	0.573 ± 0.080	0.427 ± 0.022	0.376 ± 0.022	<0.001	0.378	0.002	<0.001	0.024	0.002	0.426
V_{max} (μl)	386.6 ± 28.4	373.2 ± 29.1	339.0 ± 17.8	351.5 ± 31.9	0.667	NA	NA	NA	NA	NA	NA
V_{min} (μl)	191.8 ± 24.7	158.3 ± 27.8	171.8 ± 14.8	183.4 ± 30.3	0.870	NA	NA	NA	NA	NA	NA
V_{es} (μl)	208.1 ± 23.8	176.1 ± 27.4	198.5 ± 16.2	213.1 ± 32.5	0.830	NA	NA	NA	NA	NA	NA
V_{ed} (μl)	347.6 ± 31.9	350.6 ± 31.9	312.8 ± 17.1	318.9 ± 32.1	0.746	NA	NA	NA	NA	NA	NA
EDV index	1.121 ± 0.107	0.936 ± 0.093	0.800 ± 0.047	0.745 ± 0.078	0.013	0.434	0.040	0.013	0.432	0.361	0.563
P_{max}	114.1 ± 5.2	148.2 ± 11.6	138.0 ± 6.6	142.9 ± 6.1	0.042	0.050	0.133	0.049	0.740	0.636	0.831
P_{dev}	112.8 ± 5.3	146.3 ± 10.8	135.0 ± 6.0	140.8 ± 5.9	0.031	0.044	0.140	0.046	0.631	0.603	0.744
P_{es}	111.5 ± 5.0	144.1 ± 11.0	134.9 ± 6.2	139.6 ± 6.0	0.038	0.047	0.946	0.049	0.813	0.617	0.946
EF%	55.1 ± 4.1	61.4 ± 7.0	53.2 ± 2.6	56.3 ± 5.7	0.747	NA	NA	NA	NA	NA	NA
E_a	0.58 ± 0.03	0.759 ± 0.098	0.867 ± 0.094	0.913 ± 0.065	0.006	ns	<0.05	<0.05	ns	ns	ns
dP/dt_{max}	6645 ± 453	7902 ± 880	6459 ± 367	7001 ± 454	0.282	NA	NA	NA	NA	NA	NA
dP/dt_{min}	−9018 ± 621	−9377 ± 1158	−7746 ± 456	−8112 ± 638	0.192	NA	NA	NA	NA	NA	NA
dV/dt_{max}	8587 ± 516	7607 ± 734	6776 ± 734	6719 ± 438	0.077	NA	NA	NA	NA	NA	NA
dV/dt_{min}	−12937 ± 2451	−13087 ± 1762	−9511 ± 1525	−9189 ± 1192	0.228	NA	NA	NA	NA	NA	NA
Tau	10.0 ± 0.3	12.6 ± 0.9	13.8 ± 0.4	13.4 ± 0.5	<0.001	0.044	<0.001	<0.001	0.441	0.692	0.958
$P_{ao,sys}$	122.1 ± 6.9	180.5 ± 9.2	163.6 ± 7.4	166.7 ± 11.6	0.009	0.010	0.034	0.024	0.604	0.594	0.800
$P_{ao,dia}$	99.9 ± 3.3	115.3 ± 5.6	112.2 ± 3.8	111.6 ± 3.8	0.318	NA	NA	NA	NA	NA	NA
$P_{ao,mean}$	110.4 ± 4.1	142.7 ± 7.4	134.1 ± 5.0	134.1 ± 5.0	0.051	NA	NA	NA	NA	NA	NA
HR	339.0 ± 7.1	302.2 ± 11.3	271.4 ± 11.3	287.9 ± 9.9	0.002	0.220	0.001	0.017	0.193	0.391	0.404
PRSW	64.1 ± 4.5	73.8 ± 7.3	75.2 ± 4.5	74.3 ± 6.8	0.654	NA	NA	NA	NA	NA	NA
ESPVR slope	0.397 ± 0.03	0.617 ± 0.21	0.496 ± 0.101	0.675 ± 0.10	0.179	NA	NA	NA	NA	NA	NA
EDPVR slope	0.042 ± 0.006	0.0311 ± 0.005	0.0573 ± 0.007	0.0410 ± 0.004	0.022	0.484	0.285	0.896	0.030	0.610	0.111
EDVPR inter	−9.749 ± 3.55	−3.87 ± 2.01	−10.01 ± 2.22	−6.602 ± 1.87	0.329	NA	NA	NA	NA	NA	NA

Values are means ± SEM. NA, subgroup analysis not applicable. BSA, body surface area; CO, cardiac output; dP/dt_{max}, maximal rate of LV pressure rise over time; dP/dt_{min}, minimum rate of LV pressure decrease over time; E_a, arterial elastance; EDPVR, end-diastolic pressure–volume relationship slope; EDPVR inter, end-diastolic pressure volume relationship intercept; EDV, end-diastolic volume; EF, ejection fraction; ESPVR, end-systolic pressure–volume relationship slope; HR, heart rate; $P_{ao,dia}$, aortic diastolic pressure; $P_{ao,mean}$, mean aortic pressure; $P_{ao,sys}$, aortic systolic pressure; P_{dev}, LV developed pressure; P_{es}, end-systolic pressure; P_{max}, maximal LV pressure; PRSW, preload recruitable stroke work; SAC BW, terminal body weight; SV, stroke volume; SW, stroke work; Tau, isovolumic relaxation time constant; V_{max}, LV maximal volume; V_{ed}, end-diastolic volume; V_{es}, end-systolic volume; V_{min}, LV minimal volume.

50% (observed in control 4-month-old animals) to 40% and was restored by oestradiol supplementation. These changes were associated with the activation of regulators of inflammation in isolated myocytes, which were also suppressed by oestradiol. In a recent study by Alencar *et al.* (2017), the influence of ageing and oestrogen depletion on LV structure/function was examined in hybrid female F344BN rats. A subgroup of animals at 18 months of age underwent an ovariectomy for 2 months and relevant endpoints were examined. In their study, ovariectomy did not alter blood pressure, systolic function or lead to the development of hypertrophy or additional interstitial fibrosis beyond that noted for ageing. Significant alterations were only noted in LV relaxation and increases in filling pressure. Our echocardiography remodelling and systolic function data essentially replicate their results pertaining to the apparent lack of 'added'

effects derived from ovariectomy. However, our use of a high-fidelity conductance catheter allowed us to provide evidence for a rather large (~30%) significant loss in stroke volume index, cardiac output index and increased arterial elastance in the presence of preserved EF in OVX rats that was not detected by echocardiography. *Ex vivo* LV passive PV curves did not demonstrate global changes in passive properties. However, OVX group EDPVR slopes were modestly higher *vs.* ageing. Conductance catheter results also replicated the increased in Tau noted in intact aged rats. Ovariectomy did not lead to further increases in collagen area fraction but did appear to uniquely influence the distribution of myocardial fibrosis towards the endocardium (*vs.* epicardium). Interestingly, papillary fibrosis was notably increased in OVX animals *vs.* ageing alone and may have important implications for disease development in the presence of mitral valve dysfunction.

In an attempt to incorporate excess body weight into our animal model, we utilized 10% fructose in water in OVX rats. As expected, ageing over 3 months led to a modest increase in body weight of ∼6% while OVX yielded ∼8% and OVF ∼19%. However, none of the *in vivo*, *ex vivo* or histological endpoints showed greater differences between the OVF *vs.* the OVX groups. Thus, using this model, excess weight did not lead to greater, adverse changes in LV structure/function. A limitation of the approach used is that a blood-based metabolic profiling of these animals groups was not pursued.

As noted above, 'typical' female patients with HFpEF are older and suffer from hypertension and excess weight. Their hearts typically show minimal degrees of LV remodelling with fibrosis, and increases in active relaxation times while preserving EF. Reductions in CO typically become apparent upon physical exertion. The aged, female animal model implemented in this study recapitulates many of these features, and highlights an

important role for ovarian hormones in the maintenance of CO and possibly in the redistribution of excess interstitial cardiac collagens across the LV wall, and development of papillary fibrosis. These changes would likely be accentuated if animals were allowed to survive for a longer period of time and could lead to the development of a detectable HF phenotype. Furthermore, changes in the quality of collagen fibres would also need to be determined as, for example, the crosslinking of fibres can alter their mechanical properties. Surprisingly, the development of interstitial fibrosis as a function of ageing (about double *vs.* young) did not alter the mechanical properties of the LV in a uniform manner. However, an increase in Tau suggests impaired 'active' relaxation, likely related to alterations in calcium handling, which has been reported in other studies (Yang *et al.* 2017). The loss in stroke volume (and consequently in CO) in OVX does indicate that oestrogen depletion can contribute to impaired pump function. These data suggest that the use of invasive methods may
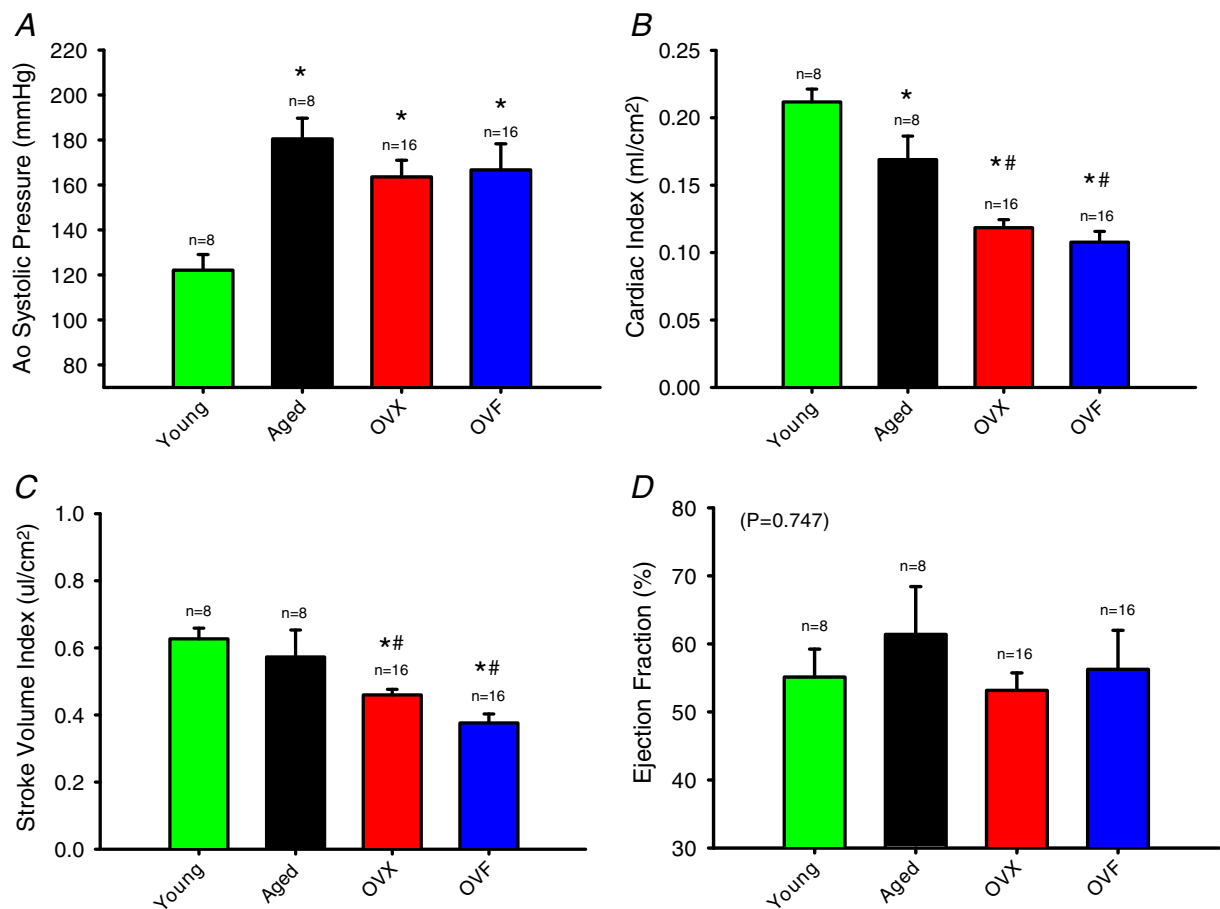

Figure 5. Haemodynamic values derived from arterial and left ventricular (LV) conductance catheter measurements during the terminal study
A, systolic aortic pressure ($P < 0.05$ aged, OVX and OVF *vs.* young). *B*, cardiac index (*$P < 0.05$, aged, OVX and OVF *vs.* young; and #$P < 0.05$, OVX and OVF *vs.* aged). *C*, stroke volume index ($P < 0.05$ OVX and OVF *vs.* young, and #$P < 0.05$, OVX and OVF *vs.* aged). *D*, ejection fraction. Values are means ± SEM. OVF, ovariectomized + fructose; OVX, ovariectomized.

be required to accurately detect HFpEF in animal models. It is worth noting that all active functional measurements in the current studies were done with the animal in a 'resting' state. The true unmasking of the effect that specific interventions have in aged models may require the use of 'stress' interventions such as a dobutamine infusion during the acquisition of LV haemodynamics. This modality of testing would be equivalent to that done in HFpEF patients when stressed by exercise or heart rate stimulation to truly unmask the degree of LV dysfunction.
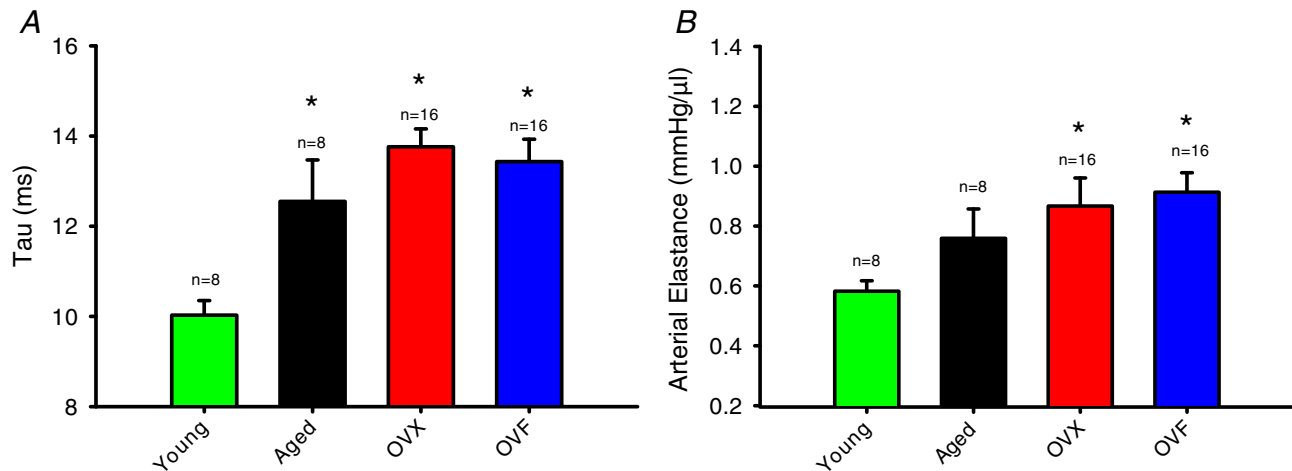

Figure 6. Hemodynamic values derived from arterial and left ventricular (LV) conductance catheter measurements during the terminal study
A, LV isovolumic relaxation time constant (Tau) (*P* < 0.05 aged, OVX and OVF *vs.* young). *B*, arterial elastance (*P* < 0.05 OVX and OVF *vs.* young). Values are means ± SEM. OVF, ovariectomized + fructose; OVX, ovariectomized.

Figure 7. *Ex vivo* analysis of left ventricular (LV) mechanics
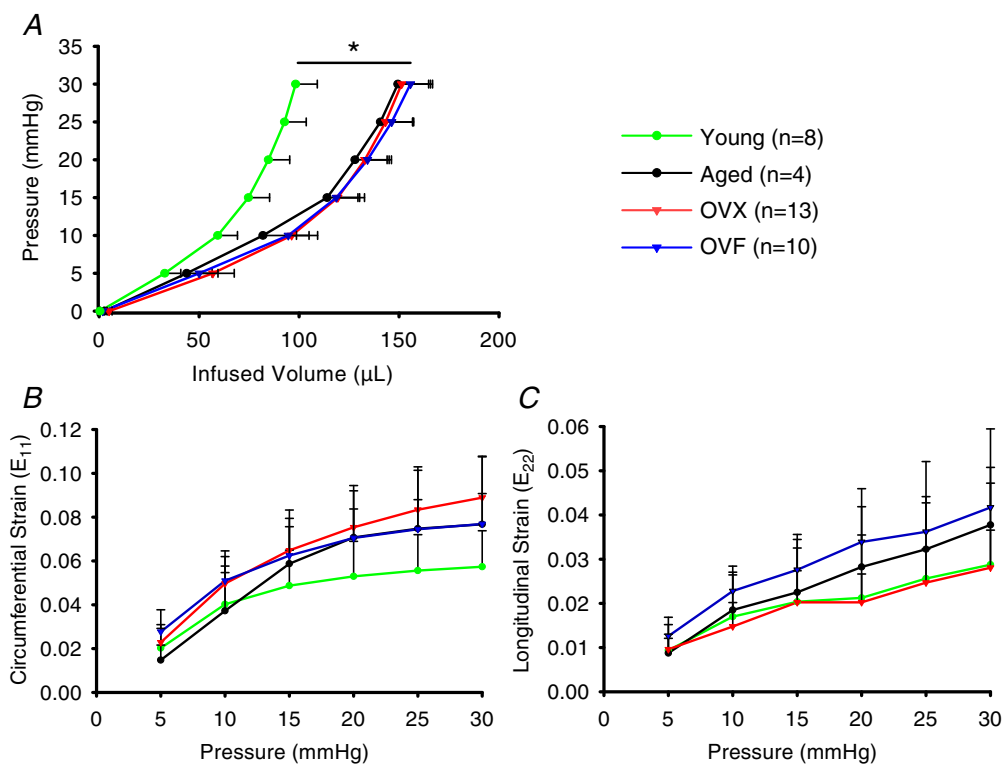*A*, passive LV pressure–volume (PV) curves for all groups at 21 months of age (*P* < 0.001 aged, OVX and OVF *vs.* young). *B* and *C*, two-dimensional circumferential (E_{11}) and longitudinal (E_{22}) LV epicardial strains at incremental LV pressures. **P* < 0.001, OVX *vs.* young. Values are means ± SEM. OVF, ovariectomized + fructose; OVX, ovariectomized.

As the pathophysiology of HFpEF remains unclear, the identification and modelling of early stages of the disease is critical to its understanding. It is difficult to envision such measurements in a patient population at risk since the disease is likely to progress slowly over time. Thus, the need to develop and implement animal models that incorporate recognized risk factors and 'mimic' early stages of the pathology allow for the evaluation of therapeutic strategies targeting those elements identified as critical to its evolution. Thus, the use of aged female rats undergoing ovariectomy may allow investigators to further examine the role that oestrogen depletion plays in
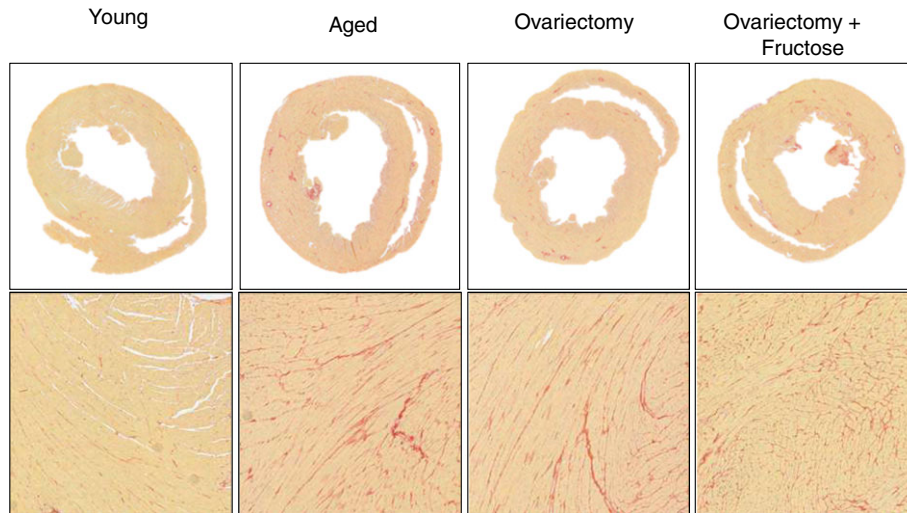
Figure 8. Composite of representative panels for left ventricular collagen as derived from Sirius Red staining
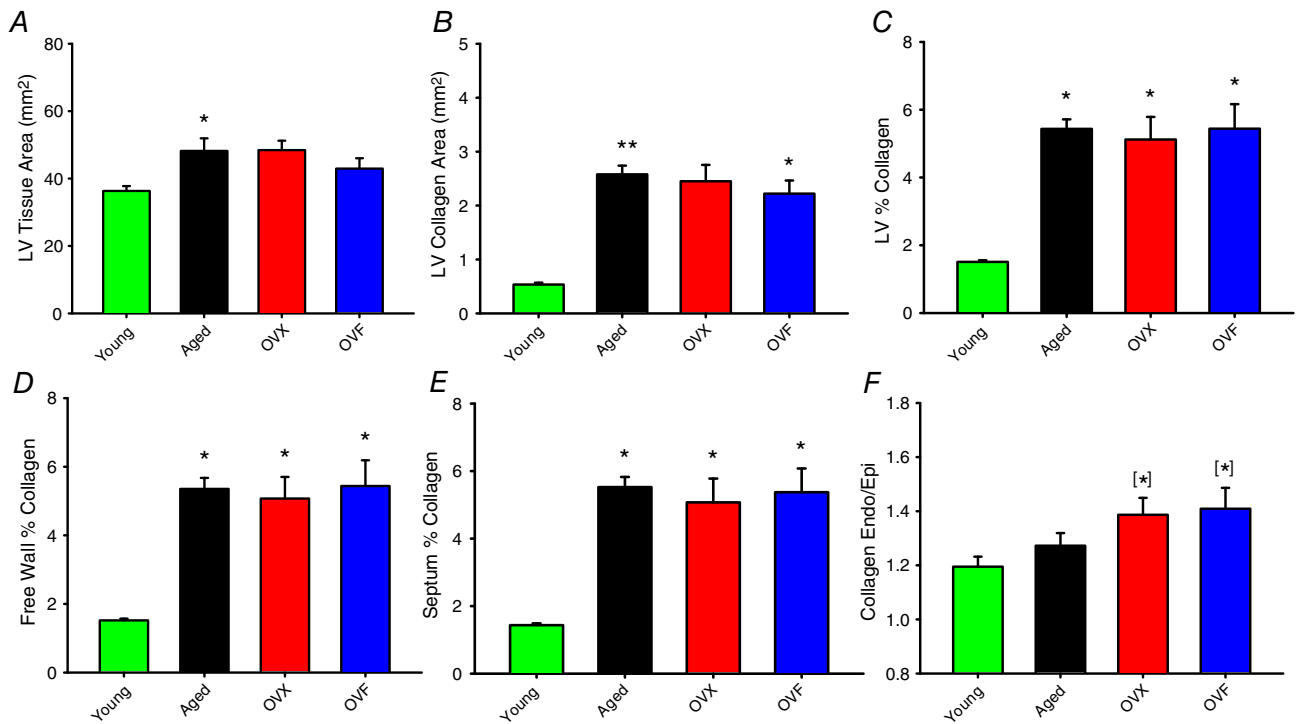
Figure 9. Morphometric and histological analysis of left ventricular (LV) tissue sections
A, LV tissue area; *B*, LV collagen area; *C*, LV % collagen (collagen/LV area); *D*, free wall percentage collagen; *E*, septum percentage collagen; *F*, endocardium/epicardium collagen ratio. Values are means ± SEM. *$P < 0.05$ *vs.* young, ANOVA. [*]$P < 0.05$ *vs.* young, unpaired *t* test. Endo: endocardium; Epi: epicardium; OVF, ovariectomized + fructose; OVX, ovariectomized.

the early stages of HFpEF development, as well as to test for pharmacological interventions that may modify this process.

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Additional information

Competing interests

Author contributions

MB, BI, RG, GC, JO and FV conceptualized and designed the work. All authors contributed to acquisition, analysis, interpretation, and drafting. Experiments were performed at UCSD. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Mechanical regulation of gene expression in cardiac myocytes and fibroblasts

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Abstract | The intact heart undergoes complex and multiscale remodelling processes in response to altered mechanical cues. Remodelling of the myocardium is regulated by a combination of myocyte and non-myocyte responses to mechanosensitive pathways, which can alter gene expression and therefore function in these cells. Cellular mechanotransduction and its downstream effects on gene expression are initially compensatory mechanisms during adaptations to the altered mechanical environment, but under prolonged and abnormal loading conditions, they can become maladaptive, leading to impaired function and cardiac pathologies. In this Review, we summarize mechanoregulated pathways in cardiac myocytes and fibroblasts that lead to altered gene expression and cell remodelling under physiological and pathophysiological conditions. Developments in systems modelling of the networks that regulate gene expression in response to mechanical stimuli should improve integrative understanding of their roles in vivo and help to discover new combinations of drugs and device therapies targeting mechanosignalling in heart disease.

Physiological and pathological cardiac structural remodelling are commonly associated with chronic alterations in haemodynamics, chamber shape and myocardial mechanics that can initially compensate for, but ultimately exacerbate, the physical triggers of cardiac remodelling[1,2]. Cell-mediated mechanotransduction responses are important regulators of adaptive and maladaptive myocyte and matrix remodelling[3]. Mechanical loading also induces the release of factors such as angiotensin II, endothelin 1 and transforming growth factor-β (TGFβ), which are potent activators of myocyte hypertrophy and matrix remodelling[4–6].

At the organ and tissue scales, concentric hypertrophy during pressure overload and exercise-induced physiological hypertrophy can be homeostatic or compensatory by normalizing wall stress or increasing cardiac output. These hypertrophic responses can also be accompanied by adaptive remodelling of the extracellular matrix (ECM) and coronary vasculature. In vitro studies suggest that these responses can be derived from fundamental regulatory mechanisms that drive normal sarcomerogenesis and match ventricular structure to mechanical workload demands[7]. However, under pathological conditions, myocardial mechanoregulated remodelling responses frequently become maladaptive, leading to decompensation and failure associated with elevated wall stresses, insufficient or inappropriate cardiomyocyte hypertrophy, apoptosis, pathological fibrosis or energetic mismatches between supply and demand[8–10]. Haemodynamic loading itself is an important therapeutic target, and mechanical unloading with left ventricular assist devices (LVADs) or other device strategies can, under some conditions, reverse changes in gene expression and structural remodelling and partially restore ventricular function[11–13].

Cardiac tissue remodelling is regulated by multiple cell types, including cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells and haematopoietic-derived cells. Endothelial cells constitute the majority of non-cardiomyocytes in the heart[14] and are involved in multiple regulatory and disease responses in the myocardium. Because of the central roles of cardiomyocytes and fibroblasts in cardiac structural remodelling, in this Review, we focus on these cell types and their responses to multiple biomechanical signals[15–17], although other cells types are clearly also mechanoregulated. The cellular mechanosensors, signalling pathways, timescales and functional responses share similarities between these two cell types. Fibroblast–cardiomyocyte crosstalk during mechanical stimulation is clearly present and important, although still not fully understood[18,19]. All whole-tissue and many in vitro studies reflect the

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REVIEWS

Key points

- The complex remodelling processes in the myocardium are regulated by mechanical signals that are sensed and transduced into transcriptional responses by cardiac myocytes and fibroblasts.
- Mechanosensitive pathways regulate expression of genes that encode proteins mediating cardiac myocyte hypertrophy, myofibroblast differentiation and remodelling of the extracellular matrix.
- Mathematical systems models are beginning to address outstanding challenges regarding how cardiac cells integrate complex mechanical and biochemical signals to coordinate gene expression and cell remodelling.
- Integrative experimental and computational mechanotransduction studies should provide further insights into mechanisms and potential therapies for mechano-based diseases, including chronic tissue and chamber pathological remodelling.

combined gene-expression response in myocytes and non-myocytes.

Cardiac myocytes and fibroblasts have been shown to respond to a variety of mechanical stimuli, including static and dynamic, isotropic and anisotropic, compressive and tensile stresses and strains, as well as fluid flow shear stresses and alterations in substrate stiffness. The features of the mechanical stimulus can induce distinct signalling mechanisms and gene-expression profiles. A variety of molecular mediators and pathways have been identified. Regardless of the specific experimental system or stimulus, there seem to be common mechano-regulated gene programmes, such as the re-expression of fetal gene programmes[20] and the induction of genes encoding ECM[21] and cytoskeletal proteins[22], suggesting that these genes might be critical in the remodelling responses of the heart to altered mechanical conditions. We review the major pathways shown to be mechano-regulated and the major downstream transcriptional responses in cardiac myocytes and fibroblasts (FIG. 1).

Nevertheless, how these numerous mechanical signals and pathways are integrated in vivo, and how they determine hypertrophic versus fibrotic responses, eccentric versus concentric hypertrophy, hypertrophic versus dilated cardiomyopathy[23,24], compensated versus decompensated adaptation, or heart failure with reduced ejection fraction versus heart failure with preserved ejection fraction remains poorly understood[25,26]. Therefore, we also emphasize the need for more integrative analyses of mechanoregulatory mechanisms in cardiac cells and discuss new, integrative approaches to analysing cardiac cell mechanosignalling that promise to advance this synthesis. Given that these approaches need to account for the wide variety of mechanical stimuli, mechanosensors, signalling pathways, and transcriptional and phenotypic responses, we conclude by surveying new systems biology approaches, such as multiscale computational models that promise to advance our understanding of the relationships between altered cardiac wall mechanics, changes in gene expression, and chronic tissue and chamber remodelling.

Cardiac mechanosensitive pathways

The myocardium in adult mammals is a structurally complex tissue composed of many cell types, including myocytes, fibroblasts, endothelial cells and perivascular smooth muscle cells. Myocytes make up >70%

of the myocardial volume, but only 25–40% of the cells by number[14,27]. In this section, we review cardiac cell structural and functional pathways that mediate mechanosensitive responses in two predominant and well-characterized cell types in the heart: myocytes and fibroblasts. These pathways are especially active during cardiac growth and development and are downregulated under normal homeostatic conditions in adults. Many pathways are also altered or reactivated by abnormal or pathological conditions in the mature heart. Mechanosensing and the subsequent signalling processes and gene expression are associated with structures and protein complexes on the surface of, inside and between these cells (FIG. 1).

Cell membrane and downstream pathways

The integrin complex in cardiac myocytes. Transmission of forces between the interior and exterior of cardiac myocytes is facilitated by transmembrane proteins such as integrins and their associated intercellular complexes. Forces generated by the contractile filaments are transmitted outside the cell through this complex and, likewise, cells sense external mechanical cues via these connections. Integrins are receptors that form dimers with α and β subunits, which can bind to ECM proteins, including fibronectin, laminin and collagen[28]. In response to pressure-overload-induced hypertrophy, the expression of α1, α5, α7 and β1D integrin subunits increases[29]. Hearts deficient in β1 integrin have a blunted hypertrophic response to transverse aortic constriction (TAC)[30]. Integrins are connected to intracellular signalling proteins, such as integrin-linked protein kinase (ILK), an important regulator of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and phospholamban, which both regulate cardiomyocyte contractility[31].

The costamere is an organized membrane complex localized at the Z-disc in muscle cells that incorporates integrins and other proteins such as vinculin and talin, mechanically linking cytoskeletal structures and the sarcomeres to the sarcolemma and ECM[32]. Forces at the costamere not only cause cell deformations[33] but also induce activation of vinculin, focal adhesion kinase (FAK), proto-oncogene tyrosine-protein kinase SRC and the small GTPase RhoA[34,35]. Vinculin also localizes to the intercalated disc, which has made it harder to isolate the specific function of vinculin in mechanotransduction[36]. Vinculin heterozygous-null mice have decreased cardiac function after 6 weeks of TAC[37]. Mice deficient in talin 1 have a blunted hypertrophic response to TAC[38]. Talin can recruit FAK to the costamere[39]. FAK is an important regulator of cytoskeletal organization and hypertrophic gene expression[40], has been shown to mediate integrin signalling leading to hypertrophy and is activated by α_1-adrenergic stimulation[41]. In addition to its function at the costameres, FAK and its carboxy-terminal-binding partners p130 CRK-associated substrate (p130Cas) and paxillin also translocate to the Z-disc with hypertrophy. Therefore, the assembly of signalling complexes that include the costameric proteins as well as p130Cas, FAK and paxillin at Z-discs might regulate, either directly or indirectly, both cytoskeletal
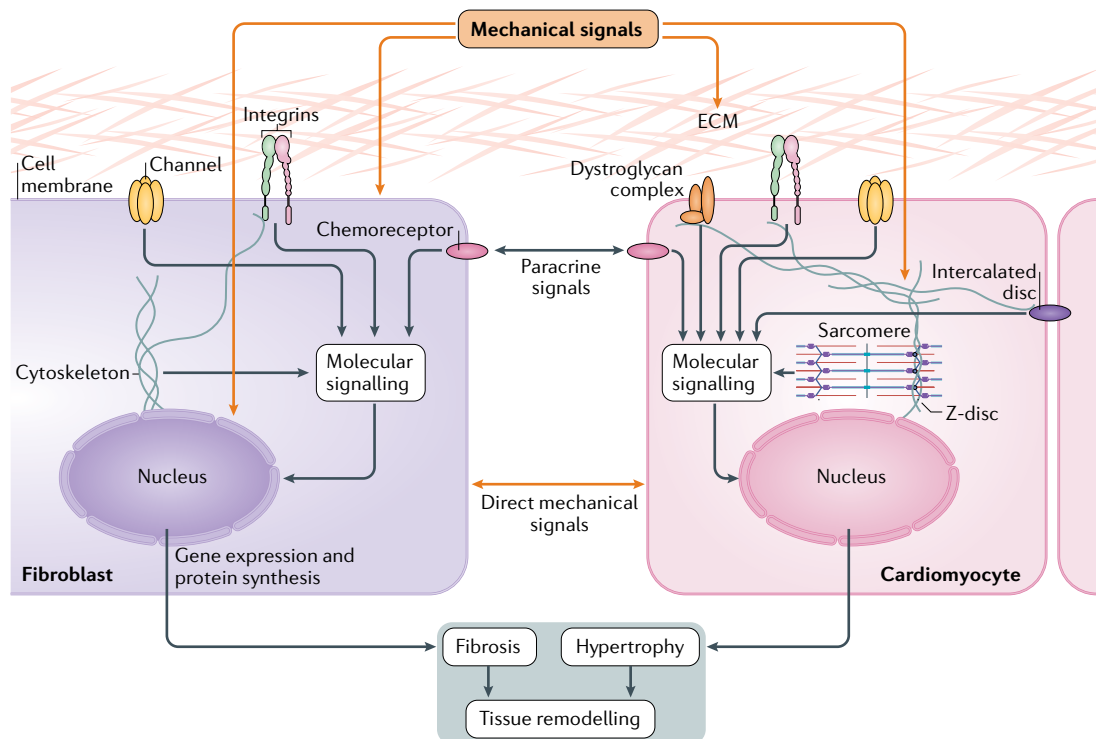
Fig. 1 | **Major mechanosensitive mechanisms and pathways in cardiac fibroblasts and myocytes.** Mechanical signals that act on the extracellular matrix (ECM) and cell membranes and internally on the cytoskeleton and nucleus initiate complex molecular signalling cascades, leading to changes in gene expression and protein synthesis in both cell types. Direct and indirect interactions between cells also mediate these responses. The net result of the changes in the mechanical cues is tissue remodelling and, in many cases, pathophysiological outcomes.

organization and gene expression associated with the cardiac myocyte hypertrophic stretch response[40].

The integrin complex in fibroblasts. Cardiac fibrosis and the accumulation of ECM is found in almost all forms of cardiac disease[42], and the transformation of cardiac fibroblasts to myofibroblasts and their regulation of tissue fibrosis and ECM is mediated by a variety of chemical and physical stimuli[43]. The ECM is an important mediator in the regulation of cell surface growth factor receptors and adhesion molecules in fibroblasts, such as integrins[43]. When cardiac fibroblasts bind to ECM ligands, these cells can sense and respond to mechanical signals via membrane receptors, including integrins, which cluster to form focal adhesions. In fibroblasts, the focal adhesion–integrin complex is a primary mechanosensing organelle[44]. Integrins interact with >150 known partners, making the focal adhesion complex the initiator of many downstream signalling pathways[45]. These pathways include crosstalk with other membrane receptors, including G-protein-coupled receptors and tyrosine kinase receptors[46,47].

Activation of fibrotic signalling depends on specific integrins[48] and ECM ligands[43,49]. Multiple components of the focal adhesion complex and their interactions result in fibrosis-related signalling cascades in response to mechanical stimuli. Integrins also directly activate non-receptor tyrosine-protein kinases, such as FAK, SRC and FYN in response to mechanical cues[49,50], and pathways such as mitogen-activated protein kinases (MAPKs) and

p38 (REF.[51]), leading to ECM production and fibrosis[52,53]. Therefore, strong feedback exists between integrin-mediated responses to the stretched ECM and the fibroblast-mediated remodelling of ECM synthesis, degradation and crosslinking[49], as discussed further below.

The dystroglycan complex in cardiac myocytes. The dystroglycan complex is another mechanical link between the cardiomyocyte cytoskeleton and the ECM[54]. Proteins associated with this transmembrane complex include dystrophin, sarcoglycans, dystroglycan, dystrobrevins, syntrophins, sarcospan, caveolin 3 and neuronal nitric oxide synthase[54]. Dystroglycan attaches to the actin cytoskeleton via dystrophin[55] and connects externally to the ECM protein laminin[56]. Dystrophin mutations lead to Duchene muscular dystrophy[57,58], and force production and power output are substantially reduced in the skeletal muscle of dystrophin-deficient mice[59]. Disruption of the dystrophin–dystroglycan complex impairs the capacity of cardiomyocytes to produce nitric oxide and might increase cell slippage and chamber dilatation in response to stretch[60,61].

Changes in gene expression in models with dystroglycan complex defects are consistent with an inflammatory response[62] and involve genes encoding both cellular (actins, cardiac ankyrin repeat protein (CARP; also known as ANKRD1) and cathepsins) and ECM (procollagens, biglycan, matrix metalloproteinases and tenascin C) proteins[54]. Genes in metabolic and energetic pathways tend to be downregulated[63].

Mechanosensitive channels in cardiac myocytes. Many mechanoregulated responses, such as mechanoelectric feedback, in cardiac myocytes have been attributed to stretch-activated ion currents. Mechanosensitive channels are important mediators of sarcolemmal stretch sensing and have been implicated in arrhythmias induced by acute and chronic changes in cardiac mechanical loading[64]. Mechanosensitive ion channels have been reported in the sarcolemma and transverse-tubule system in cardiac myocytes and regulate transmembrane fluxes of sodium, potassium, calcium and chloride ions[64].

Intracellular calcium release was reported in some of the earliest studies of myocyte responses to acute stretch[65–67], but it has taken many years to identify the origins of this calcium. Among the calcium channels claimed to be stretch-sensitive, the L-type calcium channel (LTCC)[68–70] and members of the transient receptor potential (TRP) channel family[71–73] have the most evidence. As is the case with angiotensin II receptor activation, calcium release triggers the upregulation of a wide range of hypertrophic factors[74]. Many of these calcium-dependent effects are mediated by the calcineurin–nuclear factor of activated T cells (NFAT) pathway[75,76]. Importantly, the mechanosensing capacity of cardiac calcium and other ion channels has relevance for electrophysiology as well as cellular hypertrophy[77]. As further studies clarify the role of LTCCs and TRP channels in the heart, their pharmacological relevance will continue to increase[78].

Mechanosensitive channels in fibroblasts. Although well documented in cardiac myocytes, mechanosensitive channels in cardiac fibroblasts and their role in regulating cell function are not as clear. Cardiac fibroblasts express several ion channels that are not necessarily mechanically sensitive ($Na_v1.5$, K_{ATP} and BK_{Ca})[79], but fibroblasts might have stretch-activated ion currents through channels such as TRPs[64] and other nonselective cation conductance channels[80] that might affect mechanoelectric feedback[81]. Other functions in cardiac fibroblasts, such as myofibroblast differentiation in response to TGFβ and matrix stiffness, are regulated by mechanosensitive ion channels, such as TRPs generally[82] and TRPV4 specifically[83], and involve downstream signalling of AKT, SMAD and myocardin-related transcription factor A (MRTF-A) pathways. Studies in mouse pressure-overloaded hearts have shown that inhibition of TRPC3 activity reduces fibrosis, suggesting a role for these types of stretch-sensitive ion channels in fibrosis signalling[82]. Studies have also suggested that mechanosensitive TRPV4 channels are involved in the integration of mechanical and TGFβ signals into myofibroblast differentiation[84] and thereby the cardiac fibrosis response.

Calcium-related signalling in cardiac myocytes. In addition to its well-described function in excitation–contraction coupling, calcium is an important second messenger in the regulation of metabolism, apoptosis and transcription[85]. A variety of external and internal mechanical loads affect intracellular calcium signalling[86]. Stretch induces transient increases in intracellular calcium[87,88] and sarcoplasmic reticulum calcium spark rate[89] via multiple mechanisms, including the influx of calcium

via membrane channels (as described above), triggering sarcoplasmic reticulum calcium release via ryanodine receptors[87]. Studies suggest that stretch induces an increase in reactive oxygen species (ROS) in a process dependent on membrane-bound NADPH oxidase 2 (NOX2) and microtubules (termed X-ROS signalling), which increases the calcium sensitivity of ryanodine receptors and the frequency of calcium sparks[90]. This stretch-induced X-ROS signalling has been associated with arrhythmia and diseases such as muscular dystrophy[91]. Myofilaments can also supply the increase in intracellular calcium via load-dependent changes in myofilament calcium sensitivity[92] or by crossbridge detachment leading to calcium dissociation from troponin C[93]. Elevated intracellular calcium can increase protein kinase C (PKC), calcineurin and calcium–calmodulin-dependent protein kinase II (CaMKII) signalling and thereby lead to downstream gene-expression changes[85]. Calcium dynamics in atrial myocytes are also sensitive to mechanical stimuli, possibly mediated by a surface-membrane-associated compartment[94].

Several calcium-related pathways are associated with regulation of cellular hypertrophy and might be one mechanism by which cardiac myocytes sense external loads and respond with long-term gene regulation and cell growth. The calcium–calmodulin pathway regulates hypertrophic signalling and myocyte growth[95]. Related to calcium signalling and cardiac hypertrophy are the CaMKII–histone deacetylase (HDAC) and calcineurin–NFAT pathways. HDACs can be regulated by hypertrophic stress signals[96] and have been associated with transcription factors including activator protein 1 (AP-1), activating transcription factor 1 (ATF1), serum response factor (SRF), cAMP-response element binding protein (CREB) and myocyte enhancer factor 2 (MEF2)[97]. NFAT signalling is downstream of calcineurin and low-frequency calcium changes[98]. Several hypertrophic pathways are related to NFAT signals via transcription factors such as GATA4 (REF.[99]), and calcium-dependent calcineurin–NFAT pathway activation by stretch in myocytes is implicated in cellular hypertrophy[100].

Calcium-related signalling in fibroblasts. Stretch-activated ion channels in cardiac fibroblasts create an early signal within the cell that is responsive to stretch of the membrane[101]. Deformation of the fibroblast membrane is associated with fluxes of calcium and other ions, and the calcium influx is seen in various cell-stretch studies and is associated with fibrosis in the tissue[21]. Increases in calcium signals activate multiple signalling pathways in fibroblasts including MAPK signalling[102] and CaMKII pathways, which activate transcription factors, such as CREB[103]. The TRPC6 calcium channel in fibroblasts has been shown to be critical for myofibroblast differentiation via angiotensin-II-stimulated and TGFβ-stimulated calcineurin pathways[104].

Angiotensin II signalling in cardiac myocytes. Angiotensin II has multiple effects in the myocardium, and specific signalling pathways have been discovered in cardiac myocytes. Angiotensin II, via specific angiotensin II membrane receptors, mediates cardiac

contractility, coupling between myocytes and electrical propagation, and long-term regulation of growth and remodelling[105]. Angiotensin II is likely to be produced and released by cardiac myocytes, in particular under pathological conditions[106]. The angiotensin II type 1 receptors (AT1Rs) start the signalling cascades associated with angiotensin II in the heart and are associated with short-term blood-pressure control and long-term cardiomyocyte growth. AT1R was one of the first molecules implicated in cardiac mechanosignalling[107,108]. In cardiac myocytes, angiotensin II directly leads to cell growth, but this effect is mostly seen in neonatal cells rather than in adult myocytes[109]. In response to stretch, AT1R signalling increases MAPK phosphorylation[110,111], JAK–STAT signalling[112,113] and expression of several hypertrophic markers[107,114]. Studies have also shown that matrix metalloproteinase (MMP) expression by cardiac myocytes is angiotensin-II-dependent[115]. AT1R has also proved to be directly stretch-sensitive, independent of the binding of ligands such as angiotensin II[116]. AT1R signalling via G-protein-coupled pathways is biased to β-arrestin-mediated pathways during mechanoactivation, suggesting that myocyte stretch can mediate AT1R signalling with or without ligand binding[14,117–119]. Interestingly, β-arrestin activity in coordination with AT1R was shown to mediate the Frank–Starling mechanism of cardiac contractility[120]. Although cardiomyocytes do release angiotensin II in response to stretch[107,108,110,121,122], both the mechanism underlying angiotensin II release and its specific effect on cardiomyocyte remodelling remain unclear[123]. Nonetheless, the importance of angiotensin II and AT1R in cardiomyocyte mechanosensing is firmly established, especially given the prevalence and efficacy of AT1R blockers for treating cardiovascular disease[124].

Angiotensin II signalling in fibroblasts. Angiotensin II has well-documented profibrotic effects in cardiac fibroblasts[125]. Angiotensin II can regulate cardiac ECM via an increase in collagen expression through activation of AT1Rs[126,127]. This type of fibrotic response is likely to be mediated through TGFβ synthesis and related pathways[128,129]. Evidence exists for both direct downstream activation of TGFβ by angiotensin II and via paracrine mechanisms[130,131]. Angiotensin II also exerts its profibrotic effects by decreasing MMP activity[132] and increasing tissue inhibitor of metalloproteinases (TIMP) activity[133]. Pathways that have been associated with angiotensin II signalling in the heart include syndecan[17], IL-6 and ERK–p38 MAPK–JNK[134]. Stretch and angiotensin II can mediate cytokine release from fibroblasts[135] that might further regulate fibrosis. In addition to the profibrotic effects of angiotensin II on cardiac fibroblasts, effects have also been documented on myofibroblast differentiation[136] and possibly proliferation of adult fibroblasts, probably via autocrine or paracrine mechanisms[137]; these pathways involved growth factors such as vascular endothelial growth factor[138] and endothelin 1 (REF.[139]).

TGFβ signalling in cardiac myocytes. TGFβ is well described as a contributor to fibrosis in many different tissues, including the myocardium[109], although TGFβ signalling in cardiac myocytes is not as well defined as in fibroblasts. The expression of TGFβ in cardiomyocytes increases in both dilated and hypertrophic cardiomyopathies[140,141]. In cultured cardiomyocytes, TGFβ mRNA and protein are upregulated by angiotensin II[142], and TGFβ itself promotes expression of the fetal gene programme associated with cell hypertrophy[143,144]. TGFβ expression in cardiomyocytes is regulated by several molecular signals including PKC, p38 MAPK and the AP-1 complex[142]. TGFβ signalling in myocytes might also be involved in maladaptive hypertrophy and cardiac dysfunction[145]. Stretch might directly regulate TGFβ–SMAD signalling in neonatal cardiomyocytes, modulating gene expression and inhibiting cardiomyocyte proliferation during development[146].

TGFβ signalling in fibroblasts. In cardiac fibroblasts, TGFβ is centrally involved in many aspects of fibrosis, including myofibroblast differentiation, inflammation, gene expression and ECM synthesis[147–149]. TGFβ receptors signal via SMAD proteins, which translocate to the nucleus and regulate gene transcription[150]. SMADs interact with a large number of DNA-binding transcription factors and therefore trigger a diverse set of gene transcription responses[151]. TGFβ also initiates transcription through noncanonical pathways including p38 MAPK, ERK, JNK, TAK1 and RhoA GTPase[147].

In addition to the many molecular signals that activate fibroblasts during injury response[152], mechanical stress stimulates TGFβ signalling in cardiac fibroblasts via regulation of ECM organization and TGFβ bioavailability. Stretched fibroblasts increase expression of TGFβ mRNA and protein[153]. TGFβ is secreted in latent form and then activated through contraction-mediated conformational changes in the integrin-latent TGFβ–ECM[154] complex[155] and by numerous other mechanisms including MMP2 and/or MMP9 proteolytic cleavage[156]. This system can involve complex feedbacks, because mechanical stretch of cardiac fibroblasts can modulate gene expression of *MMP2* and *TIMP2* (REF.[157]) as well as MMP activity[158], probably through the PKC and tyrosine kinase pathways[157]. Furthermore, mechanical loading of some ECM components, such as collagen, causes conformational changes that can shield them from MMP-mediated proteolysis[159,160]. Crosslinking enzymes, such as lysyl oxidase-like 2 (LOXL2), are upregulated in the heart in response to mechanical stress and are associated with cardiac fibrosis and increased stiffness[161]. In addition to increasing collagen organization and therefore TGFβ bioavailability, LOXL2 was found to stimulate cardiac fibroblasts to produce TGFβ, mediating TGFβ signalling. Inhibition of LOXL2 reduced cardiac fibrosis in response to left ventricular pressure overload and improved overall cardiac function[161].

Cytoskeletal complexes
The cytoskeleton has a complex structural arrangement in most cell types, and specific components have been associated with transduction of mechanical signals in cardiac myocytes and fibroblasts. Most cytoskeletal mechanotransduction processes in myocytes are associated with the sarcomere and Z-disc, whereas in fibroblasts, the actin cytoskeleton is central to these pathways (FIG. 1).

MLP, titin and associated complexes in cardiac myocytes.
Myofilament perturbations can affect mechanotransduction by altering either sarcomere contraction or calcium buffering[162,163]. The Z-disc of the sarcomere is directly connected to the cytoskeleton, but in addition to mediating force transmission[164], Z-disc-associated proteins are now well recognized to have important functions in mechanosensing and mechanotransduction.

Titin is a giant protein that connects the Z-disc to the M-line and is responsible for the passive stiffness of cardiac muscle[165]. Titin has been proposed to be an important mechanosensor and interacts with many proteins that have been implicated in mechanosensitive signalling pathways[166]. These cytoskeletal proteins that can bind to titin include muscle LIM protein (MLP), telethonin (also known as titin cap protein; TCAP), four-and-a-half LIM domains protein 1 (FHL1) and muscle ankyrin-repeat protein (MARP) family members[167].

MLP has been widely investigated as a prime mechanosensing element in the Z-disc, with direct binding to α-actinin[168,169]. In MLP-deficient mice, a lack of upregulation of fetal gene markers, such as *Nppa* (encoding atrial natriuretic peptide) and *Nppb* (encoding B-type natriuretic peptide), in response to mechanical stress suggests that MLP has a specific role in mechanotransduction and cardiomyocyte hypertrophy[170]. Mice deficient in MLP eventually develop heart failure and die prematurely[168], possibly owing to abnormal mechanotransduction at the Z-disc. The loss of MLP and its interactions with TCAP have also been suggested to alter the elastic properties of titin, leading to the inability of cardiac muscle cells to sense mechanical stress properly[170]. As shown in smooth muscle cells, MLP mediates gene expression via binding to GATA and SRF transcription factors[171] and possibly modulates gene expression through similar pathways in cardiac myocytes. Additionally, MLP has also been implicated in anchoring calcineurin at the Z-disc[169], which might be part of the calcineurin–NFAT hypertrophic pathway. PKC-interacting cousin of thioredoxin (PICOT; also known as glutaredoxin 3) is a protein found at the Z-disc and has also been implicated in hypertrophic signalling via the calcineurin–NFAT pathway[75]. PICOT interacts with MLP, which in turn mediates the binding of calcineurin to MLP, leading to its displacement from the Z-disc.

The Z-disc giant protein titin has been shown to be an important contributor to passive stiffness of the myocardium[172]. FHL1 binds to titin at its elastic region[173] and is upregulated in animal models of hypertrophy[174,175], implicating FHL1 in the biomechanical stress responses in cardiac hypertrophy. This stress response is likely to be related to the signalosome that encompasses components of the MAPK signalling pathway RAF1–MEK2–ERK2 at the stretch sensor domain of titin[173]. Further studies have suggested a direct link between FHL1-mediated mechanotransduction and G_q pathways, with FHL1 deficiency preventing ERK2 phosphorylation caused by constitutively active G_q overexpression in a mouse model[173]. FHL1 might also act as a scaffold for MAPK-mediated hypertrophic signalling[176].

Similar to FHL1, all three MARP members, including CARP, ankyrin repeat domain-containing protein 2 (ANKRD2) and diabetes-related ankyrin repeat protein (DARP; also known as ANKRD23), bind to titin. However, MARP members bind to the N2A region instead of the N2B region. In response to stretch of neonatal rat cardiac myocytes, CARP and DARP translocate to the nucleus[177]. Additionally, eccentric contractions of skeletal muscle cause upregulation of *CARP* and *ANKRD2* gene expression[178]. MARP members have been linked to protein kinase A and PKC[179].

Actin and associated complexes in fibroblasts. As in other cell types, the cytoskeleton in cardiac fibroblasts is a structural network responsible for maintaining cell shape and stability and is the mechanical structure that can transmit mechanosignals both outside-in and inside-out. The ECM is a regulator of cytoskeletal stress[180], and fibroblasts have been shown to use mechanotransduction signalling pathways from surface molecules (such as integrins) to control and maintain their actin cytoskeleton. RhoA, a member of the family of small Rho GTPases[181], activates Rho kinase (ROCK), which phosphorylates downstream targets associated with actin stress fibre regulation, including LIM kinases and myosin light chain[17]. RhoA-dependent signalling affects nuclear translocation of transcription factors in part through these changes in the actin cytoskeleton[17].

An important example of cytoskeleton-mediated control of transcription is via MRTF-A and MRTF-B. When fibroblasts undergo changes in their external mechanical environment, G-actin assembles into F-actin polymers, liberating MRTF-A and allowing it to enter the nucleus[182]. Deficiency of MRTF-A reduces fibrosis after myocardial infarction[183], implicating MRTF-A as an important component of the fibrosis pathway in response to mechanical signals. Another pathway associated with cytoskeletal dynamics and sensing of ECM mechanical signals is the Hippo signalling pathway[184,185]. Inhibition of Rho and disruption of F-actin results in pathway inactivation[186] and can lead to alterations in translocation of the transcription co-activators yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) between the cytoplasm and the nucleus[187]. These transcriptional co-activators, possibly interacting with SMAD3 from the TGFβ signalling pathway, might regulate fibroblast myofibroblast differentiation[188].

Nuclear mechanosensing

Cardiac myocytes. Evidence suggests that myocyte stretch can have direct effects on the nucleus via force transmission through the sarcolemma and cytoskeleton and that mechanosensing might occur inside the nucleus of myocytes via modulation of protein activity in the nucleus[189]. The nucleus has been shown to be mechanically connected to the cytoskeleton inside the cytoplasm by linker of nucleoskeleton and cytoskeleton (LINC) complexes[190], probably mediated by nuclear lamins[191,192]. Lamins are specialized structural proteins that provide structural stability to the nucleus. Other integral nuclear membrane proteins include emerin, inner nuclear membrane protein Man1, LEM domain-containing protein 2, spindle-associated membrane protein 1, barrier-to-autointegration factor and certain transcription

factors[193,194]. The LINC complex has been shown to be a direct nuclear mechanotransducer[195] and can regulate transcription factors and chromatin structure in the nucleus and, therefore, gene transcription[189]. Mutations in the proteins of the nuclear envelope can disrupt this type of force transmission and directly influence mechanotransduction and transcriptional regulation[196]. Without the intact cytoskeleton, in particular actin filament connections to the nuclear membrane, deformation of the nucleus can occur with defective mechanotransduction[197]. Force distributions within nuclear lamina are altered with varying nuclear properties[192]. Laminopathies, caused by mutations in the *LMNA* gene, are associated with muscular dystrophies, lipodystrophies and premature ageing syndromes[198], with an inflammatory component linked to some of these diseases[199]. Evidence also exists that stresses transmitted to the nucleus can affect chromatin structure, possibly regulating transcription factors directly[200]. In mice overexpressing high mobility group nucleosome-binding domain-containing protein 5 (HMGN5)[201], the mutation of the protein alters the interaction between H1 histone and chromatin, reducing chromatin compaction[202], which suggests a link between forces transmitted through the nuclear membrane and chromatin structure.

Fibroblasts. Direct cytoskeletal force transmission to the nucleus might also mediate mechanically regulated gene expression in fibroblasts[190]. This mechanism is likely to be a ubiquitous and efficient way for cells to respond rapidly to external mechanical forces[17]. Studies in fibroblasts with defective lamins have shown alterations in proliferation, suggesting this nuclear structural protein has a role in fibroblast activation and probably differentiation into myofibroblasts[203]. The lamins and emerin can regulate actin function in mouse embryonic fibroblasts, regulating MRTF-A and SRF activity[204] and cardiac myofibroblast differentiation[183]. Mechanosensing in the nucleus is suggested by changes in nuclear shape, and these shape changes have been associated with alterations in gene expression. For example, fibroblast collagen synthesis has been reported to depend on the shape of the nucleus[205]. The LINC complex is involved in nuclear mechanosensing and gene transcriptional changes in response to the altered mechanical environment of the cell[206]. Nuclear shape is associated with fibroblast cell spreading and migration speed, and this mechanosensing is defective when the LINC complex is disrupted[207].

As the heart develops and matures, the nuclei of cardiac fibroblasts and myocytes are exposed to varying degrees of mechanical forces. Therefore, in cells with a defective or fragile nuclear membrane and associated interconnections to the cytoskeleton, the nucleus can show decompacted chromatin and nuclear blebbing, as seen in cell death. These observations indicate the mechanosensitivity of nuclei and that its structural integrity is important for cardiac cell adaptation to physical loads[17].

Cell–cell interactions

Intercalated discs in cardiac myocytes. The intercalated disc is the cell–cell junction that longitudinally connects neighbouring cardiac myocytes. The intercalated disc has multiple functions related to maintenance of mechanical and electrical coupling between cardiomyocytes[208,209]. In response to cyclic cardiac volume overload, the intercalated disc undergoes ultrastructural changes that might be associated with sarcomere restructuring, implicating the cell–cell junction as part of the mechanotransduction pathway associated with myocyte hypertrophic growth[210]. The three types of cell junctions that make up an intercalated disc are fascia adherens, desmosomes and gap junctions. Fascia adherens are anchoring sites for cytoskeletal actin and connect to the closest sarcomere. Intermediate filaments bind to desmosomes[209]. Ions can pass through gap junctions, which allows action potentials to propagate along muscle fibres.

The fascia adherens junctional complex is composed of proteins such as cadherins, catenins and catenin-binding proteins[211]. N-Cadherin (also known as cadherin 2) can form attachment sites with neighbouring cardiac myocytes and transmit forces between cells[212]. N-Cadherin has also been shown to be upregulated in response to stretch[213]. Hearts from N-cadherin-deficient mice have altered cell–cell mechanosensing that affects their sarcomere structure and causes dilated cardiomyopathy[214]. Cardiac myocytes from these mice do not have identifiable fascia adherens junctions, and their sarcomeres are shorter in length. β1 Integrin levels are increased in hearts from N-cadherin-knockout mice, which indicates a compensatory response in which other mechanotransductive proteins increase in expression owing to the deficit of N-cadherin[214]. Catenins are also part of the fascia adherens junctional complex and regulate cadherin-based and binding proteins, such as muscle-specific mouse mXinα, vinculin–metavinculin and α-actinin, which link the fascia adherens to the cytoskeleton and modulate catenin activity[211,212]. α-Catenin recruits vinculin to the fascia adherens junction through force-dependent changes in α-catenin[215]. α-Catenins have also been shown to modulate cytoskeletal mechanotransmission, which can regulate YAP-mediated cell proliferation during development[216]. As with deficiency of N-cadherin, α-catenin deficiency alters the structure of the intercalated disc and leads to dilated cardiomyopathy. Vinculin localization is lost at the fascia adherens junction; however, vinculin also localizes to the costamere and remains there after α-catenin inactivation[217]. Experimental evidence also exists for a role of the striated muscle-specific protein, nebulin-related-anchoring protein (N-RAP), in myocyte mechanotransduction between the fascia adherens junction and the sarcomere[218,219].

Desmosomes connect intermediate filament cytoskeletal networks between cells and contain cadherins, desmocollin 2 and desmoglein 2. Mutations in these proteins are associated with arrhythmogenic right ventricular cardiomyopathy (ARVC)[209]. Desmoplakin connects desmosomes to the intermediate desmin filaments[209] in conjunction with desmosomal proteins junction plakoglobin (JUP) and plakophilin 2 (PKP2)[211]. A potential direct link between JUP and PKP2 in the cardiac muscle shear stress response has been shown[220]. Desmoplakin has been shown to be a critical component of the desmosome and cell–cell junctional integrity[221];

knocking out *Dsp* (encoding desmoplakin) causes sarcomeric defects and loss of desmosomal but not N-cadherin-based proteins and has been associated with ARVC[222]. Studies have suggested a role for desmosomes in transducing mechanical forces into multiple cellular responses related to tissue mechanics. Mutations in the desmosomal cadherins, desmocollin 2 and desmoglein 2 are linked to human ARVC[223].

Gap junctions are cell–cell connections that allow ions to pass between neighbouring cells, thereby electrically and metabolically connecting adjacent cells. Connexin 43 (also known as gap junction-α1 protein) is a gap junctional protein that is upregulated in response to mechanical stretch[224]. Upregulation of connexin 43 is accompanied by an increased number and size of gap junctions as well as by an accelerated conduction velocity[225]. Gap junctions are sensitive to mechanical stress[208] and remodel in several cardiac diseases. Crosstalk between adherens junctions, desmosomes and gap junction proteins, including α-catenin, tight junction protein ZO1, connexin 43 and PKP2, at the intercalated disc might account for the mechanisms of gap junction remodelling in cardiac disease[226].

Fibroblast–myocyte interactions. Interactions between cardiac myocytes and fibroblasts can be via paracrine cell–cell signalling or via direct physical coupling between the different cell types, possibly mediated by the ECM. Fibroblasts can mediate myocardial electrophysiology via electrical coupling to myocytes and alterations in myocyte membrane potential and therefore electrical conduction[18,227–229]. Many fibroblast–myocyte interactions have been implicated in cardiac arrhythmogenesis[230–232]. Coupling between myocytes and fibroblasts can occur in vivo and represents electrotonic coupling between these different cell types[233,234]. Ongstad and colleagues suggest several ways in which cardiac myocytes and fibroblasts can couple to each other, including fibroblasts acting as insulators between myocytes or with small numbers of gap junctional channels that could serve as short-range or long-range conductions of electrical excitation[234]. Modifying heterotypic cell–cell interactions and coupling might be useful in reducing arrhythmias after myocardial infarction[235]. Secreted factors such as angiotensin II, cardiotrophin 1, fibroblast growth factor, IL-6, insulin-like growth factor I, TGFβ and tumour necrosis factor have been shown to mediate cell–cell communication via indirect autocrine or paracrine mechanisms[236–238]. These factors regulate a host of physiological functions in the cells and tissue of the heart, most of which are described in the preceding sections involving signalling pathways related to development, electrical activity, contractile function and pathological tissue remodelling.

Systems-level cardiac mechanosignalling

As described in the previous section, a wide range of pathways have been implicated in mechanosensing. Appropriately, these studies have focused largely on gaining a more detailed understanding of particular mechanosensing mechanisms. However, as indicated in FIG. 1, how these mechanisms work in concert to mediate cardiac mechanoresponses is less understood. Global measurement and modelling approaches are complementing more mechanistically focused studies to progress towards a systems-level understanding of cardiac mechanosignalling.

Mechanosensitive gene expression

Heart. Studies of gene expression downstream of individual mechanosensitive pathways have most often focused on the fetal gene programme or a small number of candidate genes in that particular pathway. By contrast, gene expression profiling using cDNA microarrays or RNA sequencing have allowed for a more comprehensive view of the transcriptome and discovery of mechanosensitive gene programmes. Although the most prevalent causes of heart failure, including myocardial infarction and hypertension, have highly complex biochemical and mechanical perturbations that evolve slowly over time, studies of direct perturbations to myocardial mechanics have helped to inform which aspects are more closely associated with mechanosensing. Animal models of pressure overload by TAC, which induces concentric hypertrophy and interstitial fibrosis, have consistently demonstrated increased expression of natriuretic peptide genes, ECM-related genes (notably connective tissue growth factor and periostin) and cytoskeleton-related genes[239,240]. Knockout of *Nppa* further exacerbates the hypertrophic and fibrotic gene expression profiles caused by pressure overload[240]. Cardiac transcriptomes from rats subjected to pressure or volume overload identified consistent expression of *Nppa*, *Nppb*, *Mt1* (encoding metallothionein 1) and genes encoding proteins involved in mitochondrial metabolism or the cytoskeleton. Volume overload induced more distinct upregulation of genes encoding actin-binding proteins (such as tropomyosin 4 and thymosin-β4) and some ECM proteins (such as osteopontin)[241]. Volume overload and eccentric remodelling caused by mitral valve regurgitation is associated with increased expression of metalloproteinases and decreases in the expression of genes encoding non-collagen ECM proteins[242].

Several studies have characterized gene expression profiles from human failing hearts before and after mechanical unloading with an LVAD. Initial reports demonstrated that LVAD-induced reverse remodelling coincided with a decrease in the expression of genes associated with natriuretic peptides, ECM, sarcomeres, cytoskeleton and metabolism[243,244]. Subsequent studies, including comparisons with non-failing heart samples, demonstrated that LVAD-induced reverse structural remodelling does not similarly induce widespread reversal of gene expression but instead induces a distinct state[245–247]. In terms of functional responses related to gene expression, LVAD therapy has been shown, for example, to correlate with changes in calcium dynamics in cardiomyocytes[248]. Even in experiments and treatments involving direct mechanical intervention, the parallel effects on vascular remodelling, neurohormonal feedback and multiple cell types hinder the identification of genes whose expression is regulated directly by the mechanosensitivity of cardiac myocytes or fibroblasts.

Cardiac myocytes. In the first transcriptomic measurements of stretched cardiac myocytes, Weinberg and colleagues found strongly mechanosensitive expression of *Il1rl1*, which encodes suppression of tumorigenicity 2 (ST2; also known as IL-1 receptor-like 1)[249]. After confirming mechanoregulated expression by traditional methods, they demonstrated increased levels of soluble ST2 protein after myocardial infarction in the serum of mice and humans[249]. Subsequently, ST2 has been developed substantially as a clinical biomarker and is approved by the FDA to predict outcomes from myocardial infarction and heart failure[250,251]. Frank and colleagues used microarrays to identify highly mechanoresponsive genes in rat stretched cultured cardiomyocytes, identifying *Gdf15* and *Hmox1* in addition to strong induction of the fetal gene programme and other previously reported stretch-responsive genes[252]. The investigators further demonstrated that stretch-responsive transcription of several genes could be blocked by angiotensin II receptor antagonists or mimicked by angiotensin II[252], thereby linking transcription to a known mechanotransduction pathway. McCain and colleagues used a novel stretching device with micropatterned membranes to control cardiac myocyte alignment, characterizing how the direction and duration of stretch affects the transcriptome[253]. Clustering of genes by expression pattern across conditions elucidated coordinated expression of genes associated with ECM and cytoskeletal remodelling as well as those previously associated with pathological hypertrophy or heart failure in vivo[253]. This study nicely illustrated how clustering of transcriptomic data from multiple treatment conditions and time points aids in the identification of coordinated gene expression programmes. A subsequent study in rat isolated ventricular myocytes subjected to cyclic stretch investigated gene expression at even more time points between 1 h and 48 h (REF.[254]). Detailed data on signalling and expression time courses remain limited; however, as more studies on the sequences of signalling events become available, the data will provide new insight into which mechanisms are acting in parallel, which are dependent on upstream processes and which components of the network are sensors, transducers or effectors.

Fibroblasts. As an early application of cDNA microarrays, in 2001 Kessler and colleagues profiled the response of fibroblasts cultured in 3D collagen gels that were either mechanically restrained or unrestrained from cell-induced contraction[255]. Mechanical stress induced expression of many genes encoding proteins known to be related to focal adhesions, cytoskeletal remodelling and ECM, including those encoding collagen type I, MMP1 and connective tissue growth factor[255]. Driesen and colleagues demonstrated that spontaneous differentiation of cardiac fibroblasts on a rigid substrate could be prevented or even reversed by inhibition of TGFβ receptor type 1 (TGFβR1)[256]. Transcriptome profiling showed that compared with stiffness-induced myofibroblasts, TGFβR1-inhibited cells had lower expression of genes encoding proteins associated with fibrosis, adhesion and TGFβ signalling. By contrast, TGFβ-treated myofibroblasts had reduced expression of genes

encoding proteins related to the cell cycle[256]. Alam and colleagues used RNA sequencing to identify how fibroblast mechanosensitive gene expression and microRNAs are regulated by the LINC complex[206]. Disruption of the LINC complex by expressing a dominant-negative SUN domain-containing protein 1 (SUN1) caused increased expression of genes encoding proteins associated with ion transport, adhesion, motility and ECM organization on stiff but not soft substrates[206].

Studies of gene expression from cultured papillary muscles and engineered heart tissues have helped to bridge cellular and in vivo studies because these tissues are well suited for precise mechanical perturbations and contain multiple cardiac cell types. To elucidate transcriptomic responses to particular mechanical signals, Haggart and colleagues measured transcriptomic responses of contracting papillary muscles under combinations of physiological versus reduced myocyte shortening (as occurs with pressure overload) and mean stretch (as occurs with an LVAD)[257]. Reduced muscle shortening strongly regulated genes encoding proteins associated with the ECM and cardiomyocyte hypertrophy, partially overlapping with the gene expression seen with pressure overload in vivo. Hirt and colleagues found that engineered heart tissues with increased afterload developed hypertrophy and fibrosis consistent with that seen in pressure overload in vivo[258]. Transcriptome measurements demonstrated that increased afterload was associated with strong induction of genes associated with the fetal gene programme, ECM and glycolytic metabolism in a manner consistent with that seen with biochemically induced hypertrophy and in partial overlap with that seen with pressure overload in vivo[258]. Of note, in both papillary muscles[257] and engineered heart tissues[258], although only 10–15% of genes overlapped with in vivo reports, the most highly responsive overlapping genes were associated with the fetal gene programme and the ECM.

TABLE 1 compares mechanoresponsive gene sets across animal models, human studies and cardiac cells. Individual genes that were noteworthy in each study are also listed. Nearly all studies showed significant expression of genes encoding protein related to the ECM, with the gene encoding connective tissue growth factor often being among the most mechanoresponsive. Cardiac tissue or cardiac myocyte studies typically show increased expression of natriuretic peptides, consistent with their use as clinical biomarkers. Genes encoding focal adhesion proteins are also consistently responsive to stretch in cardiac myocytes and fibroblasts, whereas fibroblasts show additional expression of proliferation-related genes. Gene expression has been profiled with varying measurement technologies and analyses, which contributes to variation between studies in the consistency of reported gene sets. Cardiac myocyte cultures also contain non-myocytes, which might contribute to measured responses.

Regulation by microRNAs. Gene expression is also substantially regulated at the post-transcriptional level by microRNAs, and some studies have begun to focus specifically on their role in response to mechanical

stretch. MicroRNAs are short (~22-nucleotide), non-coding RNAs that bind complementary mRNAs to regulate mRNA degradation or protein translation. A large number of microRNAs are differentially expressed and control cardiac remodelling in vivo, as reviewed previously[259]. Microarrays were used to discover differentially expressed microRNAs in mice subjected to TAC-induced pressure overload or calcineurin overexpression, leading to identification of miR-195 as a regulator of cardiac hypertrophy[260]. To find microRNAs specifically responsive to cardiac myocyte stretch, Frey and colleagues used microarrays to identify eight stretch-responsive microRNAs[261]. Follow-up experiments identified miR-20a as being responsive to both stretch and simulated ischaemia–reperfusion, that overexpression of miR-20a was sufficient to protect cardiomyocytes from apoptosis and that expression of miR-20a was inversely correlated with pro-apoptotic miR-20a targets Egl nine homologue 3 and E2F family transcription factors[261]. Motivated by studies showing miR-208a regulation of cardiac hypertrophy[262,263], studies of cultured cardiac myocytes and myoblasts showed stretch-responsive TGFβ signalling that controlled miR-208a expression and either β-myosin heavy chain (also known as myosin 7) or collagen in the respective cell types[264,265]. Dynamics of mRNA and microRNA expression of stretched cardiac myocytes has also been examined by Rysa and colleagues, who predicted involvement of nuclear factor-like 2 and interferon regulatory transcription factors and let-7 family microRNAs[254].

Long (>200-nucleotide) intergenic non-coding RNAs[266] have also been shown to regulate cardiac remodelling[267] and more strongly correlate with reverse remodelling of human hearts after LVAD support[247]. Long non-coding RNAs have been shown to regulate the stretch response of vascular smooth muscle cells[268], but corresponding studies in stretched cardiac myocytes have not yet been reported.

Challenges of complexity
Despite a wealth of characterized mechanosensitive proteins, pathways and global transcriptional profiles, considerable knowledge gaps must be overcome to obtain a molecular systems-level understanding of how mechanical signals regulate gene expression and cardiac remodelling (BOX 1). Addressing these challenges of mechanosignalling complexity requires quantitative comparisons between combinations of mechanobiochemical perturbations, measurements and experimental contexts. However, testing of all combinations is clearly not feasible and would overwhelm rather than provide new conceptual insights. When closely integrated with experimental studies, mathematical systems models can provide rigorous frameworks for data integration, hypothesis generation, prioritization of experiments and understanding of complex systems[269]. In the following section, we provide an overview of how systems models have been applied to address complexity challenges for cardiac signalling networks, focusing on the latest work extending into mechanosignalling of cardiac myocytes and fibroblasts.

Table 1 | Mechanoresponsive gene programmes in the heart and specific cardiac cells

Experimental system	Gene sets	Example genes	Refs
Cardiac pressure overload	NP, ECM and metabolism	*CTGF* and *FHL1*	239
	NP and ECM	*POSTN* and *SPP1*	240
	NP and ECM	*LOXL1*, *MT1* and *PDK1*	241
Cardiac volume overload	NP, ECM and cytoskeleton	*MT1* and *TAGLN*	241
	Decreased ECM	*MMP1*, *MMP9* and decreased *CTGF*	242
Patients after LVAD implantation	NP, ECM and metabolism	*HSPB6* and *MT1*	243
	Sarcomere, cytoskeleton, ECM and FA	*ITGB1* and *VCL*	244
	Limited	*PDK1* and *SOCS3*	245
	Limited	*CRYM*, *TNNT3* and multiple microRNAs	246
	Limited	Multiple lncRNAs	247
Engineered heart tissue	NP, ECM, sarcomere and metabolism	*ACTA1* and *COL1A1*	258
Papillary muscle	NP, ECM and sarcomere	*CTGF* and *HSP90AB1*	257
Cardiac myocytes	Not reported	*IL1RL1*	249
	NP, sarcomere and cell adhesion	*FHL1*, *GDF15* and *HMOX1*	252
	ECM, FA and sarcomere	*TRPC1* and *ITGA6*	253
	ECM, cytoskeleton and metabolism	–	322
	NP and proliferation	*SERPINB2* and let-7 family	254
Fibroblasts	ECM, FA, cytoskeleton and proliferation	*CTGF* and *SERPINB2*	255
	FA, proliferation and TGFβ signalling	–	256
	ECM, FA, cytoskeleton and proliferation	–	206

Gene expression measured by cDNA microarrays or RNA sequencing. ECM, extracellular matrix; FA, focal adhesions; lncRNA, long non-coding RNA; LVAD, left ventricular assist device; NP, natriuretic peptides; TGFβ, transforming growth factor-β.

Systems models of mechanosignalling

Computational models of signalling and gene regulation in cardiac myocytes. Mathematical models have long provided crucial insights into how complex molecular systems regulate cardiac myocyte physiology[270]. Classic mathematical models provided insights into the molecular mechanisms of actin–myosin cross-bridge cycling[271] and feedback between ionic currents that drive cardiac pacemaking[272]. Over several decades, mathematical models have provided a wide range of systems-level insights into the molecular regulation of excitation–contraction coupling[273], arrhythmia[274], metabolism[275,276] and signalling pathways[277,278] in normal and pathological[279] conditions.

In contrast to the larger number of models of short-term (milliseconds to minutes) cardiac myocyte physiology, fewer models have examined the molecular networks that control longer-term gene expression and remodelling (hours to days)[269,280]. Tavi and colleagues used a model of calcium–calmodulin–calcineurin kinetics to show that calcineurin might act as a frequency-sensitive integrator of cytosolic calcium signals[281,282], which correlates with experimental measurements of NFAT activity, mRNA expression[281] and myocyte hypertrophy[283]. Other models coupled these systems to more detailed systems of upstream subcellular calcium dynamics[284] or mechanisms of NFAT nuclear transport[285]. Cooling and colleagues developed models of receptor-stimulated inositol 1,4,5-trisphosphate (IP$_3$) transients that regulate calcium–calcineurin, showing that the distinct kinetics of transients could be explained by differences in the kinetics of endothelin and angiotensin II receptors[286]. Combining simulations and perturbation experiments, Shin and colleagues characterized crosstalk between calcineurin, PI3K and ERK pathways that produce a biphasic switching mechanism by which calcipressin 1 regulates calcineurin–NFAT activity[287]. Ryall and colleagues developed a systems model of the overall cardiac myocyte hypertrophy signalling network, predicting RAS as an integrating network hub for many biochemical stimuli[288]. Their model also included a simplified integrin pathway that was sufficient to accurately predict stretch-responsive activation of three transcription factors and six hypertrophic genes[288]. This and other systems models were developed using data from cultured cardiac myocytes, whose relevance to in vivo hypertrophy has been debated[289,290]. To test the generalizability of this network model[288], overexpression of 34 genes was simulated and compared with in vivo data from cardiac-specific transgenic mice[291]. The model accurately predicted 72% of 168 in vivo measurements, as well as the hypertrophic phenotypes of four double-transgenic mice, with mispredictions guiding subsequent model revision on the basis of in vivo data[291].

Models linking mechanosignalling to cytoskeletal remodelling and gene expression. A number of mathematical models have provided insights into the biophysical mechanisms that guide cytoskeletal remodelling, as reviewed previously[292]. Biophysical models have demonstrated how long stress fibres and heterogeneous distributions of focal adhesions in fibroblasts arise from positive feedback between adhesion and stress fibre contractility[293], how further incorporation of cytoskeletal mechanics can predict regional orientation and density of stress fibres[294] and how cells re-orient in response to the direction of stretch[295]. Coupled biochemical–mechanical models have also been used to understand cardiac myocyte myofibre formation, accounting for differences from fibroblast stress fibres. Grosberg and colleagues accurately predicted myofibrillar patterning of geometrically constrained cardiac myocytes[296], which was driven by both fibre length–force dependence[293] and mutual alignment of myofibres due to parallel coupling[297]. Simulations by Yuan and colleagues predicted that cell–cell adhesion forces reduce local focal adhesion formation[298], helping cardiac myocytes to form a syncytium with aligned myofibrils, as found experimentally[299].

Systems-level models are further needed to provide insight into how mechanical cues are transduced and integrated into downstream signalling pathways and gene expression[269,292,300]. For example, discriminating the regulation by specific mechanical stimuli of particular signalling pathways is confounded by crosstalk and overlap between these different stimuli and downstream pathways. Several studies have illustrated the promise of such models to address challenges in mechanosignalling network integration.

Although studies of mechanosignalling have focused primarily on linear pathways from sensor to response, mechanoresponsiveness can also emerge from the interplay of more subtle relationships. Dingal and colleagues used a series of simplified models to illustrate

how tension-inhibited protein degradation[301] (such as is seen with many cardiac sarcomeric, cytoskeletal and matrix proteins[152,302]) coupled with positive regulation from protein to transcription can lead to tension-enhanced gene expression. Proteins such as lamin A with long protein half-lives but short mRNA half-lives were predicted to show steady, tension-enhanced mRNA and protein abundance, whereas proteins such as collagen with short protein half-lives were predicted to generate transient increases with stretch as seen experimentally. Coupling of mRNA–protein modules illustrated how tension-inhibited degradation of one protein might indirectly drive mechanoresponsive expression of other genes in a cytoskeletal network (for example, the effects of myosin 2 on lamin A) or coordinated gene expression in fibroblasts and cardiac myocytes[301].

Myofibroblast differentiation is highly responsive to mechanical signals as well as multiple growth factors, but the mechanisms underlying crosstalk between these signals are not well characterized. Schroer and colleagues developed a dynamic computational model of how integrin, TGFβ and fibroblast growth factor signals converge on p38 and ERK to coordinately regulate α-smooth muscle actin (αSMA) expression, a key marker of myofibroblasts[303]. Rather than using a single model, uncertainty regarding mechanisms involving SRC, ERK and p38 was addressed by comparing eight candidate models, each representing alternative hypotheses of the signalling network. Candidate networks incorporating SRC-dependent αSMA expression and SRC feedback provided the most parsimonious explanations for their experimentally observed responses to matrix stiffness, growth factors and genetic perturbations[303].

Mechanoresponsiveness of the transcriptional effectors YAP–TAZ has been shown to rely on cytoskeletal tension, non-muscle myosin and RhoA activity downstream of integrins and cell adhesion[304]. Sun and colleagues developed a systems model integrating these mechanisms and crosstalk with cell–cell adhesion-sensitive Hippo pathway members, such as serine/threonine-protein kinase LATS[305]. A strength of this model is that the equations were largely based on biophysical mechanisms of cytoskeletal proteins and their biochemical regulation by signalling, effectively linking cytoskeletal regulation to signalling pathways. The model recapitulated a range of previously reported effects of perturbations on YAP–TAZ nuclear translocation[304], including inhibitors for myosin, RhoA, ROCK, F-actin and altered matrix stiffness[305]. The model was then used to make new predictions regarding increased mechanosensitivity of YAP–TAZ compared with SRF as well as synergistic regulation of YAP–TAZ by LATS[306] and RhoA pathways[305].

Large-scale systems models can integrate heterogeneous data from many sources to identify network control principles. Zeigler and colleagues developed a systems model of the cardiac fibroblast signalling network, which integrated 11 biochemical or mechanical cues to regulate myofibroblast genes and ECM components in agreement with a wealth of independent literature[307]. Computational screening of network perturbations in multiple cell contexts predicted mechanoresponsive

αSMA expression to require an autocrine loop with AP-1-dependent TGFβ expression and TGFβR1–SMAD3 signalling. Further simulations and validation experiments of fibroblasts in mechanically restrained collagen gels confirmed that TGFβR1 inhibition prevented αSMA expression[307]. These predictions were consistent with subsequent in vivo experiments by Khalil and colleagues showing that fibroblast-specific deletion of *Tgfbr1* and *Tgfbr2* or *Smad2* and *Smad3* suppressed cardiac fibrosis in response to pressure overload[308].

Despite the wide range of mechanosensitive pathways that have been identified, the systems models described above examined crosstalk between biochemical signals and only a single mechanosensor. To assess how cardiomyocytes integrate mechanical signals from multiple mechanosensitive proteins, Tan and colleagues reconstructed a cardiomyocyte mechanosignalling network model linking nine mechanoresponsive proteins (five shown to be directly responsive to stretch in reconstituted assays) to cardiomyocyte size, protein synthesis and eight mechanosensitive genes[309] (FIG. 2). Comparison of model predictions to independent published experiments yielded a validation rate of 78% (134 out of 172). Although the 11 transcription factors showed selective sensitivity to particular mechanosensors, functional cooperativity between transcription factors was predicted to make downstream genes more broadly sensitive. Computational perturbation screens predicted combinatorial 'mechanotherapies' that might suppress hypertrophic gene expression, including combined stimulation of cGMP and inhibition of AT1R as might occur with the drug Entresto (Novartis; previous known as LCZ696, combining valsartan and sacubitril). The direct effects of Entresto on cardiomyocyte mechanosignalling have not yet been tested experimentally; however, in rats subjected to aortic constriction, Entresto suppressed cardiac expression of *Myh7* and *Nppa* and cardiomyocyte hypertrophy and increased expression of *Myh6* (REF.[310]) — all consistent with the previous model's predictions[309]. Entresto has also been shown to attenuate cardiac hypertrophy after myocardial infarction[311], in chronic kidney disease[312] and in angiotensin-II-stimulated cardiomyocytes[311].

Multiscale models of mechanoregulated ventricular growth and remodelling. Ultimately, mathematical models will need to link predicted mechanotransduction mechanisms, signalling networks and gene expression to cardiac hypertrophy and myocardial tissue remodelling in response to haemodynamic changes and other alterations that affect myocardial mechanics. To date, progress has been made in organ-scale modelling of biomechanically stimulated growth and remodelling using empirical growth and remodelling laws[313]. Witzenburg and Holmes examined the capacity of several proposed hypertrophic growth laws to predict remodelling in response to pressure or volume overload[314]. Kerckhoffs and colleagues assumed that cardiomyocytes grow by adding sarcomeres in series when maximum fibre lengthening (that is, normally during filling) exceeds a homeostatic threshold and by adding sarcomeres in parallel when maximum cross-fibre strain (that is, typically during systole) exceeds a homeostatic threshold[315].
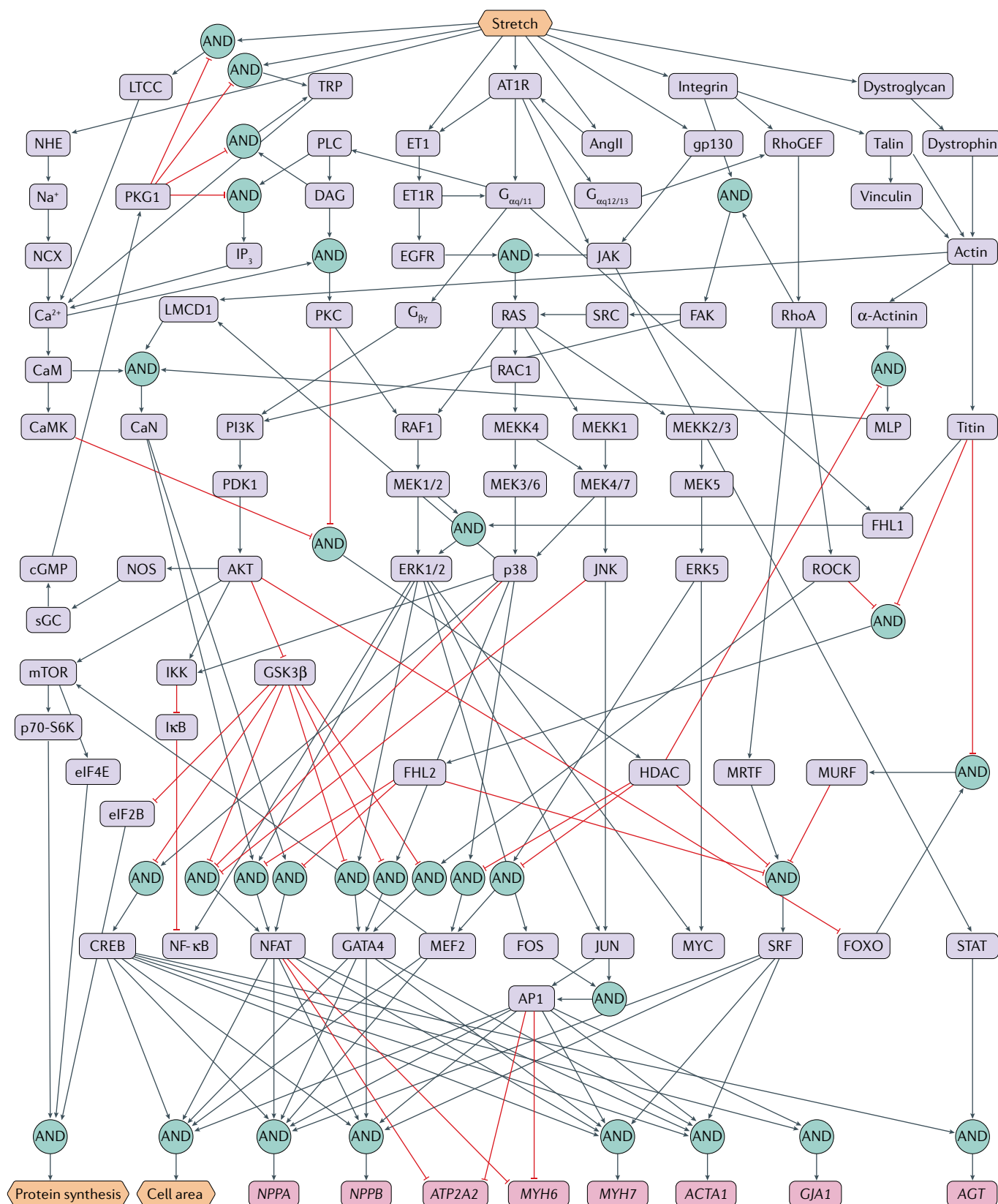
Fig. 2 | **Systems model of the cardiac myocyte signalling network.** The model predicts how mechanical stretch is sensed by nine proteins to regulate transcription factors, protein synthesis, cell size and gene expression of *NPPA* (encoding atrial natriuretic peptide), *NPPB* (encoding B-type natriuretic peptide), *ATP2A2* (encoding sarcoplasmic/endoplasmic reticulum calcium ATPase), *MYH6* (encoding α-myosin heavy chain), *MYH7* (encoding β-myosin heavy chain), *ACTA1* (encoding skeletal α-actin), *GJA1* (encoding connexin 43) and *AGT* (encoding angiotensinogen). AND logic gates indicate multiplicative activation or inhibition of the downstream node. Adapted from REF.[309], CC-BY-4.0 (https://creativecommons.org/licenses/by/4.0/), and details of the model logic can be found in this publication.

When the investigators implemented these rules in a 3D biomechanical model of the canine left and right ventricles coupled to haemodynamic models of the circulation, they found that the mechanical changes induced by simulated mitral valve regurgitation indeed predicted a pattern of eccentric ventricular hypertrophy consistent with experimental observations[316]. Similarly, the same growth rules under conditions of simulated aortic stenosis gave rise to a pattern of concentric hypertrophy similar to observations in canine experiments[317]. Multiscale models of extracellular remodelling by mechanically stimulated fibroblast activity have also been created. Rouillard and Holmes used a technique known as agent-based modelling to simulate collagen matrix synthesis and remodelling by fibroblasts in response to physico-chemical stimuli and incorporated the multicellular model into a 3D model of post-infarction scar formation in the ventricular wall[318]. Taken together, these different modelling approaches provide the foundations for future, more mechanistic and integrated multiscale models that represent the biophysical mechanisms of cellular mechanotransduction of specific mechanical stimuli, the cell signalling pathways that integrate these responses, their downstream regulation of gene expression and phenotypic responses, the multicellular and tissue-scale changes in tissue structure and mechanical properties, and the feedback effects of these changes on the driving mechanical stimuli. Multiscale models could be particularly valuable for assessing potential therapies that combine pharmacological and device-based interventions.

Conclusions

A wide range of signalling pathways have been identified that mediate mechanosensing by cardiac myocytes and fibroblasts. The molecular responses to mechanical stimuli have also been profiled with increasing scope. However, the complexity of molecular networks has challenged attempts to understand more fully how particular mechanical signals are processed and integrated to control gene expression and cardiac remodelling. Systems models of mechanosignalling have begun to provide insights into how mechanical signals are integrated from multiple mechanosensors and crosstalk with biochemical stimuli to regulate fibroblast and cardiac myocyte gene expression. These models will need to be closely integrated with extensive quantitative experiments that compare distinct mechanosignals and combinations of perturbations to mechanosensors and biochemical pathways. Such integrative systems analyses promise to integrate data across diverse experimental contexts, improve understanding of multiscale relationships and accelerate the development of therapeutics that control how mechanical stimuli remodel the heart.

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Competing interests
A.D.M. and J.H.O. are co-founders of and have an equity interest in Insilicomed, and A.D.M. has an equity interest in Vektor Medical. A.D.M. and J.H.O. serve on the scientific advisory board of Insilicomed, and A.D.M. is a scientific adviser to both companies. Some of their research grants have been identified for conflict of interest management on the basis of the overall scope of the project and its potential benefit to these companies. The authors are required to disclose this relationship in publications acknowledging the grant support; however, the research subject and findings reported in this Review did not involve the companies in any way and have no specific relationship with the business activities or scientific interests of either company. The terms of this arrangement have been reviewed and approved by the University of California San Diego in accordance with its conflict of interest policies. J.J.S., P.M.T. and K.S.B. declare no competing interests.

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