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INVITED REVIEW

The effects of cardiac stretch on atrial fibroblasts: analysis of the evidence and potential role in atrial fibrillation

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Abstract

Atrial fibrillation (AF) is an important clinical problem. Chronic pressure/volume overload of the atria promotes AF, particularly via enhanced extracellular matrix (ECM) accumulation manifested as tissue fibrosis. Loading of cardiac cells causes cell stretch that is generally considered to promote fibrosis by directly activating fibroblasts, the key cell type responsible for ECM production. The primary purpose of this article is to review the evidence regarding direct effects of stretch on cardiac fibroblasts, specifically: (i) the similarities and differences among studies in observed effects of stretch on cardiac fibroblast function; (ii) the signalling pathways implicated; and (iii) the factors that affect stretch-related phenotypes. Our review summarizes the most important findings and limitations in this area and gives an overview of clinical data and animal models related to cardiac stretch, with particular emphasis on the atria. We suggest that the evidence regarding direct fibroblast activation by stretch is weak and inconsistent, in part because of variability among studies in key experimental conditions that govern the results. Further work is needed to clarify whether, in fact, stretch induces direct activation of cardiac fibroblasts and if so, to elucidate the determining factors to ensure reproducible results. If mechanical load on fibroblasts proves not to be clearly profibrotic by direct actions, other mechanisms like paracrine influences, the effects of systemic mediators and/or the direct consequences of myocardial injury or death, might account for the link between cardiac stretch and fibrosis. Clarity in this area is needed to improve our understanding of AF pathophysiology and assist in therapeutic development.

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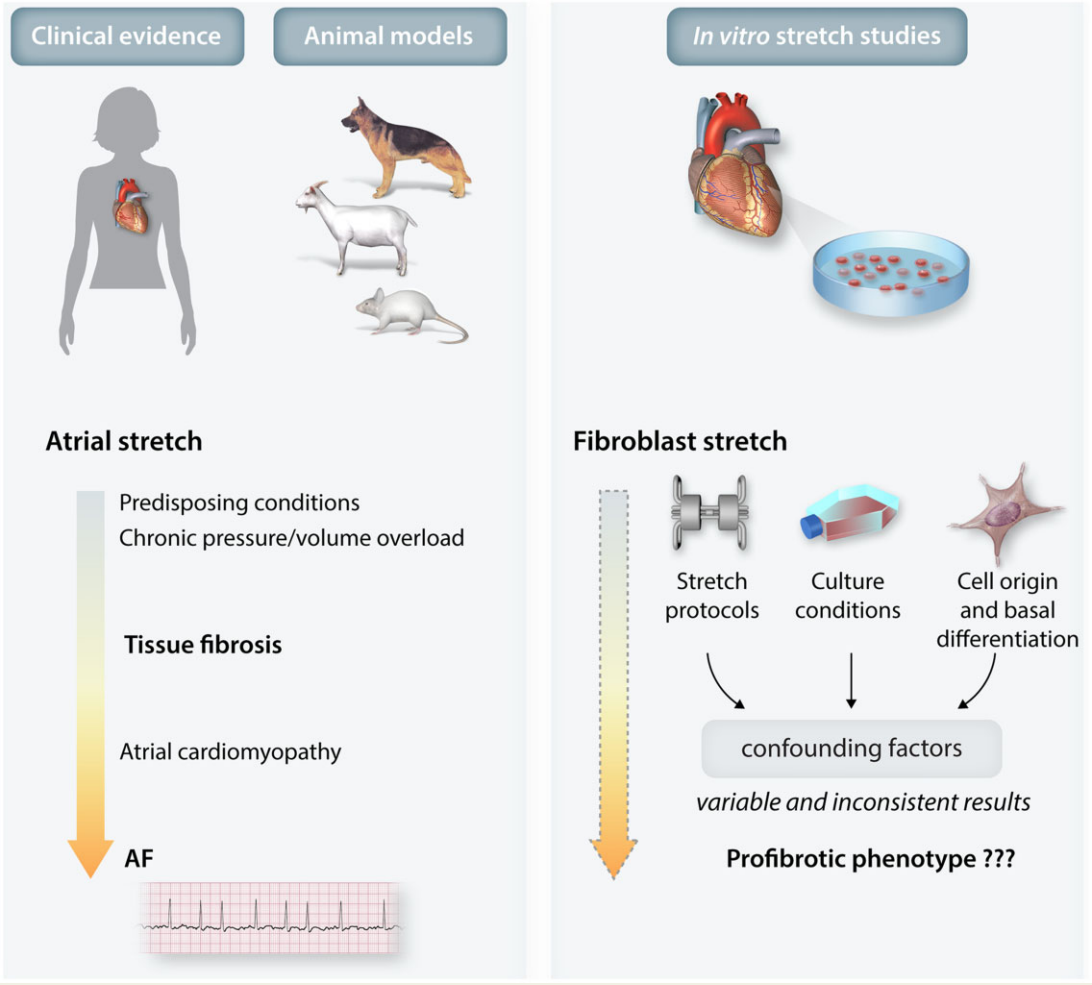
Graphical Abstract



Keywords Pressure overload • Stretch • Mechanical strain • Atrial fibrillation • Fibrosis • Cardiac fibroblast

1. Introduction

Mechanical stretch of the heart, typically associated with pressure and/or volume overload, is believed to be an important contributor to the development of cardiac fibrosis. Atrial fibrosis promotes the occurrence of atrial fibrillation (AF), the most common cardiac arrhythmia, and its associated risk of stroke, heart failure, and mortality.[1] The lifetime risk of AF is estimated to be 22–26%, meaning that 1 in 4 people will be affected over the course of their lives.[1] AF is the single most important risk factor for stroke in the elderly and is believed to be a potentially important contributor to cognitive decline and dementia.[1] Increased atrial pressure alters atrial electrophysiology and is associated with atrial fibrosis, thereby favouring AF occurrence.[2–5] Moreover, AF *per se* impairs atrial mechanical function, establishing a positive feedback loop that exacerbates the situation (*Figure 1*).[6,7] Animal models that recapitulate cardiac pressure overload show atrial remodelling that is characterized by conduction slowing, cellular calcium overload, fibrosis, fibroblast proliferation, and alterations in collagen degradation.[8] How mechanical stimuli are transduced at a cellular level and trigger fibrosis is still a matter of active investigation. Stretch and hemodynamic load modulate the function of many mechanosensitive ion channels and transmembrane proteins that, in tight interplay with the extracellular matrix (ECM), activate a range of signalling pathways to modify cellular function.[9–11]

Cardiac fibroblasts are widely distributed and are the primary cellular controllers of ECM homeostasis. Sustained hemodynamic loading is believed to cause fibroblasts to proliferate, migrate, and differentiate into myofibroblasts, which abundantly secrete ECM proteins. This process causes fibrosis that prompts the progression of many cardiac disorders by hampering myocardial excitation–contraction coupling and by disturbing impulse propagation and ECM-dependent signalling pathways.[12] The general molecular mechanisms and signalling pathways involved in fibroblast dysregulation and cellular mechanotransduction have been described extensively elsewhere.[13] Mechanical forces can promote fibroblast activation via a number of processes, including changes in the neurohumoral environment, the release of paracrine factors from cardiomyocytes, interactions with leucocytes and cytokines, and direct effects

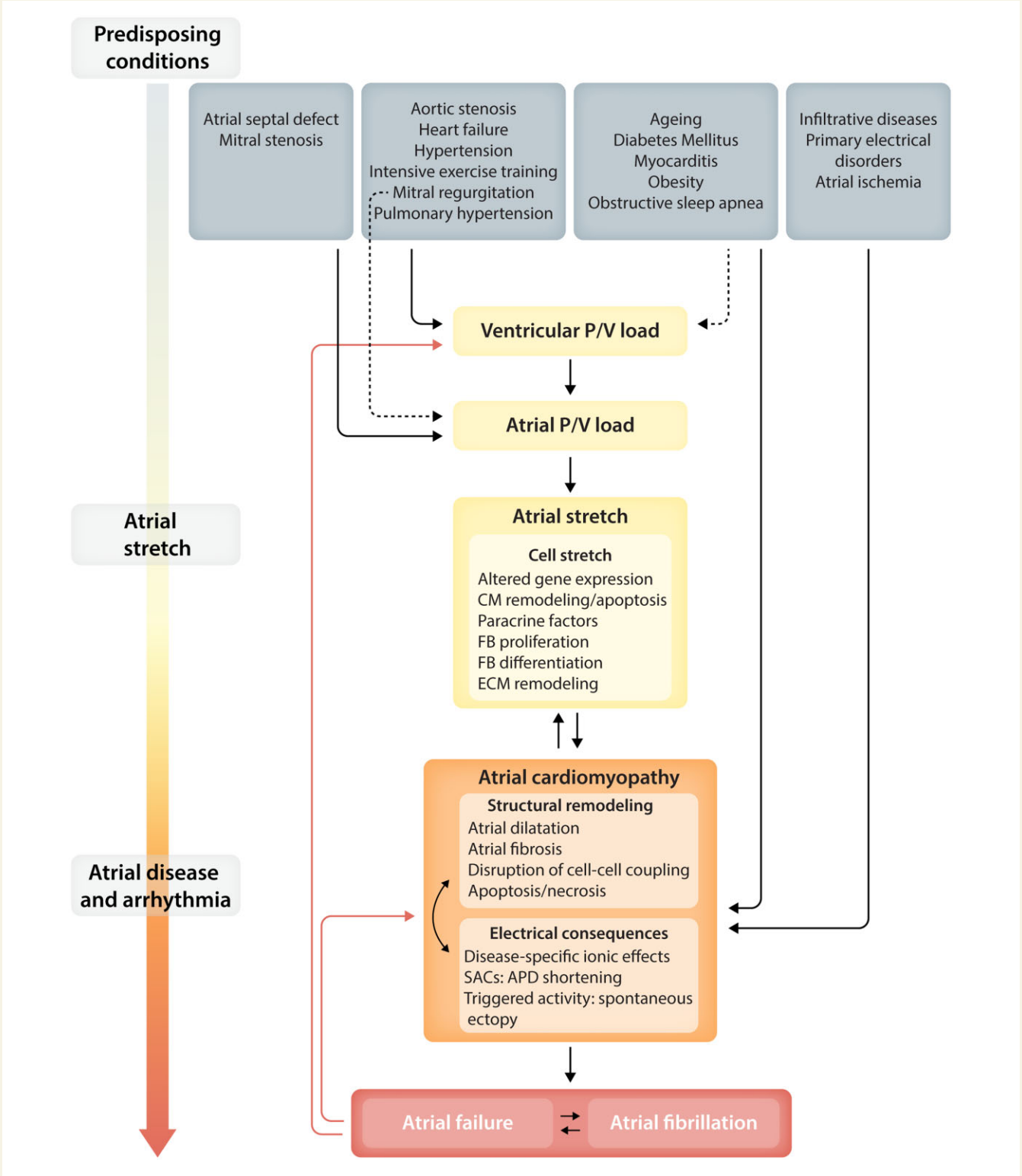


Figure 1 Schematic of processes believed to be involved in AF-promoting responses to stretch. Conditions leading to atrial stretch and its consequences are shown at the top. These lead to atrial stretch, either directly via altered atrial load (primary or secondary to ventricular overload) or indirectly by affecting atrial function and causing atrial cardiomyopathy. Atrial stretch in itself causes cellular consequences that lead to atrial cardiomyopathy. Atrial cardiomyopathy leads to AF and can impair atrial function sufficiently to lead to atrial failure. CM, cardiomyocyte; ECM, extracellular matrix; FB, fibroblast; P/V, pressure/volume; SAC, stretch-activated channel.

of mechanical stretch on fibroblasts. A direct profibrotic effect of fibroblast stretch is often invoked in the literature and has been studied extensively. Here, we will briefly review *in vivo* evidence from patients and experimental models and then focus on the results of studies examining the direct response of cardiac fibroblasts to stretch. The primary issues that we aim to address are as follows: (i) how cardiac fibroblasts respond to stretch, (ii) whether the profibrotic effect of hemodynamic load *in vivo* can be attributed to direct fibroblast activation, and if so, (iii) what molecular pathways are responsible for such activation.

2. Clinical evidence regarding electrophysiological consequences of atrial stretch

Atrial overload is manifested as increased atrial pressure and/or volume. Although acute stretch caused by acute atrial overload might have substantial short-term consequences, the clinical occurrence is quite limited. Acute mitral regurgitation due to tear or rupture of part of the mitral valve apparatus results in immediate increases in left atrial (LA) pressure and volume. Substantial valve disruption (e.g. due to papillary muscle rupture) is usually rapidly fatal. Less severe dysfunction (e.g. due to a ruptured corda tendinea) is often compensated by adaptive responses, which are followed by changes typical of chronic stretch. Extensive acute myocardial infarction or myocarditis can result in rapid increases in atrial stretch, but the severe associated ventricular dysfunction usually overshadows the atrial changes for which the main clinical manifestation is usually AF.

Chronic atrial overload inducing sustained increases in atrial stretch is much more frequent, and therefore relevant, in clinical practice. Chronic atrial selective stretch is present in conditions generating primarily atrial overload, such as mitral stenosis, the classical paradigm of atrial pressure overload,[14] or congenital heart defects causing atrial volume overload like atrial septal defects.[15] More commonly, chronic atrial stretch develops in response to primary ventricular overload, either pressure- or volume-related, in which, despite initial adaptive responses at the ventricular level, the haemodynamic load is eventually transmitted retrogradely to the atria. Typical settings include hypertension, heart failure, and valve disease other than mitral stenosis (*Figure 1*). Patients with hypertension often show features of diastolic dysfunction including increased LA pressure and atrial dilation,[16] a common surrogate of atrial stretch, and inadequate blood pressure control is associated with further atrial dilation.[17] Atrial overload is particularly important in heart failure, both with reduced and preserved ejection fraction, and in valvular heart diseases such as mitral regurgitation and aortic stenosis, where LA enlargement is common and portends a greater risk of ventricular decompensation and clinical morbidity/mortality.[18–21] Other clinical conditions like intensive exercise training,[22] obstructive sleep apnea,[23] and pulmonary hypertension[24] have also been associated with atrial dilation.

The clinical consequences of atrial stretch are very important. As shown in *Figure 1*, a variety of common clinical conditions lead to atrial stretch, either directly via altered atrial load (primary or secondary to ventricular overload) or indirectly by affecting atrial function and causing atrial cardiomyopathy. Atrial stretch itself produces remodelling that causes structural and electrical changes in the atria that, with time, lead to the development of atrial cardiomyopathy.[25] Atrial cardiomyopathy produces two principal clinically relevant manifestations, AF and the newly recognized entity of 'atrial failure'.[26,27] Atrial failure is characterized by cardiac dysfunction due to primary abnormalities in atrial function, in the absence of significant ventricular or valvular dysfunction.[27] AF and atrial failure are intrinsically related to each other (*Figure 1*), and both conditions feed back to each other and to atrial cardiomyopathy, directly and by inducing further ventricular overload. Atrial failure and AF have significant prognostic consequences, since both have potential deleterious effects on ventricular function and may facilitate the progression to heart failure, and both are believed to be associated with blood stasis and endothelial dysfunction, predisposing to thrombus formation and thromboembolic events.[26,27]

Clinical observations on the atrial remodelling consequences of atrial stretch are limited. Atrial pressure increases induced by ventricular pacing or initiation of AV node re-entrant tachycardia are associated with acute decreases in atrial effective refractory period (ERP) and monophasic action potential duration.[28] Acute atrial loading with atrial pacing causes exaggerated atrial responses among AF patients compared to sinus rhythm (SR) controls in terms of increased LA wall tension and decreased ERP, despite comparable increases in LA pressure.[29] In contrast with the direct effects of acute atrial stretch, patients with chronic atrial volume loading due to atrial septal defects show increased low-atrial ERP, P-wave duration, and conduction delay across the crista terminalis.[15] Inherited atrial cardiomyopathy due to mutation in the atrial natriuretic peptide gene causes extensive atrial fibrosis in association with atrial dilation and contractility impairment.[30]

3. Experimental observations relating to cardiac stretch in animal models

Common findings in pressure-overloaded hearts include the induction of the immediate early genes (IEG), c-Myc, c-Fos, and Fra-1 as the earliest response.[31] This is followed by increases in α-smooth muscle actin (αSMA) in the myocardium and fibroblasts from pressure-overloaded hearts,[32–34] although some studies found that the changes are time variant, first increasing and then declining slowly after several days.[35] Fibroblast activation and increased transforming growth factor-β (TGFβ) expression also occur in pressure-overloaded rat hearts,[36–38] followed by increased deposition of collagen and fibronectin, and reduced collagen degradation.[38–40] A study of systemic hypertension also showed early collagen remodelling, with collagen type3/type1 ratio increasing during the progression of systemic hypertension (4 weeks) while returning to basal levels thereafter (35–88 weeks).[41] However, another study reported unchanged collagen expression 1 week after the establishment of the model and decreased collagen type3/type1 ratios after 8 weeks because of an increase in the synthesis of collagen I.[42] Lipoma-preferred partner (LPP), a nucleo-cytoplasmic shuttling adaptor protein that is a mechanosensitive protein highly expressed in cardiac fibroblasts, is up-regulated in hearts from pressure-overloaded rats but unchanged in hearts from myocardial infarction rats.[43] The expression of chymase, an enzyme capable of converting angiotensin I to II as well as affecting a variety of ECM-localized enzyme systems, is increased in cardiac fibroblasts isolated from a pure volume-overload rat model.[44]

Enhanced collagen degradation and increased MMP levels are seen in patients with congestive heart failure (CHF).[45] Similarly, in the LA and left ventricle (LV) of dogs with tachypacing-induced CHF, apoptosis and white-cell infiltration are transiently increased after the imposition of

tachypacing, mitogen-activated protein kinases (MAPKs) are activated, and active TGFβ1 monomer and angiotensin II expression are increased compared to control dogs. In the same study, LA fibrous-tissue content was increased 20-fold with CHF.[46] Burstein *et al.* also reported much greater profibrotic responses in the LA vs. LV of CHF dogs, including increased fibroblast proliferation and activation, increased ECM gene expression, and induction of platelet-derived growth factor (PDGF) and its receptor.[47] A significant interaction between chamber (atrium vs. ventricle) and condition (CHF vs. control) indicates that these responses are chamber specific.[47] Dawson *et al.* also reported that atrial tissues and freshly isolated atrial fibroblasts from CHF dogs displayed significantly greater mRNA levels of ECM genes.[48] Atrial fibrosis is also produced in a pig model of mitral regurgitation and promotes AF development.[49] On the other hand, in a goat model of chronic atrial volume loading due to an aortic LA shunt, while AF susceptibility was increased, neither LA ERP nor collagen content was changed.[50] Work in an LV-LA shunt dog model also showed AF promotion, but with slightly decreased LA ERP, increased conduction heterogeneity, and fibrosis localized to the inferior pulmonary vein region.[51]

Thus, both clinical and experimental data indicate that cardiac fibrosis occurs in pressure/volume-overloaded contexts, along with characteristic molecular changes, and that the atria are particularly susceptible to fibrosis development.

4. Fibroblasts and the ECM

Cardiac fibroblasts control the ECM framework that creates the functional scaffold for the myocardium.[52–54] Far from a passive skeleton, the ECM in turn acts as a reservoir for multiple growth factors, chemokines, enzymes, and matricellular proteins that regulate fibroblast phenotype and function.[55–57] Cardiac fibrosis appears as a result of a series of phenotypic changes that include fibroblast proliferation, differentiation, and ECM remodelling. Fibrosis affects cardiac structure and electrical function in complex ways. In addition to direct disturbance of conduction pathways by interrupting muscle bundles,[58] cardiomyocyte electrical activity can be affected by electrical coupling between fibroblasts and cardiomyocytes,[59,60] although the extent to which this occurs *in vivo* remains controversial. Upon cardiac injury, quiescent fibroblasts get activated by growth factors, cytokines, and other stimuli and differentiate into myofibroblasts.[61] Myofibroblasts are normally eliminated by programmed cell death after completing wound healing or tissue remodelling.[62] However, under conditions like prolonged cardiac injury, inflammation, or aging, myofibroblasts become resistant to apoptosis, accumulate in fibrotic regions, and lay down large amounts of ECM.[63]

In comparison with fibroblasts, myofibroblasts possess greater ability to synthesize collagen, deposit and degrade ECM, recruit inflammatory cells, and promote inflammatory cell infiltration.[64–67] They also express larger quantities of αSMA that confers contractile capacity involved in wound retraction[68–70] and extra domain-A (ED-A) fibronectin, the biologically active splice variant that plays a key role in activation.[69,71] Myofibroblasts secrete collagen I and collagen III, which constitute 80% and 10% of the normal structural ECM, respectively.[72] The metabolism of these proteins is determined by the balance between deposition of newly synthesized molecules, degradation of existing collagen by matrix metalloproteinases (MMPs), and inhibition of MMPs by the tissue inhibitors of metalloproteinases (TIMPs).[72] Collagen strand assembly in mature fibres, which is controlled by the lysyl oxidase enzyme (LOX) family members that cross-link collagen fibrils, is also a determinant step.[73,74] The ECM is a highly dynamic structure, with constant turnover of collagens and other ECM proteins.

At the cellular level, αSMA, vimentin, and other filaments form cellular stress fibres that in consort with the ECM, membrane receptors, integrins, focal adhesions, and other membrane proteins, participate in transducing external mechanical forces into subcellular structures, particularly the nucleus. In turn, changes in gene expression induce ECM remodelling and release of growth factors and other biologically active molecules that contribute to the maintenance of cardiac mechanotransduction.[13,55,75,76] This system constitutes a feed-forward loop that can result in continuous myofibroblast activation and contribute to pathological remodelling of the heart. It is widely assumed that the occurrence of fibrosis in conditions inducing cardiac pressure/volume loading is due to direct fibroblast activation by stretch, as described for non-cardiac fibroblasts.[77] In the following section, we review evidence from studies examining the response of isolated cardiac fibroblasts to stretch to evaluate (i) the nature of their response and (ii) the mechanisms involved.

5. Stretch studies in cultured cardiac fibroblasts

For over 30 years, there has been an increasing interest in studying how mechanical forces act on cardiac cells.[78] It is difficult to investigate the effects of stretch *per se in vivo*, because most manipulations that alter stretch also affect many other functions, for example autonomic tone, neurohormone concentrations, cytokine release, etc. Therefore, the only way to study pure and isolated stretch effects on fibroblasts is to evaluate them with *in vitro* systems. Cell-stretching devices have been used extensively to generate *in vitro* mechanical strain in many isolated cell types, particularly fibroblasts, in order to determine the consequences of cellular mechanotransduction.[79–85] One of the first *in vitro* stretch studies performed on cardiac cells was performed by Terracio *et al.*, who applied cyclic uniaxial stretch to neonatal rat cardiac fibroblasts.[78] They demonstrated that stretched fibroblasts elongate and orient perpendicular to the direction of stretch, with vimentin intermediate filaments reorganizing parallel to the long axis of the cells. This was the first study showing that mechanical stretch has a direct effect on cardiac fibroblasts. Since then, many other researchers have investigated cardiac fibroblast mechanotransduction by using similar stretch paradigms. Relatively few studies have specifically investigated atrial fibroblasts. The discussion below addresses findings for cardiac fibroblasts in general and considers the issue of the specific considerations that apply to atrial fibroblasts.

5.1 Stretch-induced phenotypic changes in cardiac fibroblasts

5.1.1 Proliferation, apoptosis, and morphology

There is no clear consensus on whether stretch increases, decreases, or has no real effect on fibroblast proliferation (*Table 1*). Some investigators reported a decrease in fibroblast proliferation under mechanical loading,[86–90] while others have seen an increase.[87,90,91] Moreover, many of those studies showed that proliferation of the stretched fibroblasts could be modified by changing other parameters in the experimental system, such as oxygen content, serum, or surface rigidity, suggesting that if stretch influences fibroblast proliferation, it is not in a straightforward manner and may be modulated by the specific conditions existing at the time of stretch.

Table 1 Stretch-induced changes in fibroblast proliferation, apoptosis and morphology

Response	Main findings	Cell type	Coating and plate	Stretch machine	Stretch pattern	Stretch duration	Serum	Reference
Increased proliferation	Small proliferation increase with stretch	NRVF	Collagen-coated silicone membrane	Homemade stretch device	20% uniaxial	24 h	Serum-free	Sadoshima *et al.*, (1992)[132]
	Proliferation increased by stretch	NRVF	Collagen-coated silicone membrane plate	Flexcell*	1-Hz, 30%	24 h and 48 h	Serum-free	Cao and Gardner, (1995)[91]
	Increased fibroblast proliferation with low stretch (2%) for 24 or 72 h. In high-stretch conditions (8%), increased only at 24 h	Human atrial fibroblast	Fibronectin	Homemade stretch device	1-Hz, 2% or 8% uniaxial	24 h and 72 h	NS	Ugolini *et al.*, (2016)[92]
	Increase in proliferation of stretched fibroblasts seeded on low stiffness substrate (3 kPa), and in fibroblasts cultured with medium from stretched cardiomyocytes	Adult mouse ventricular fibroblast	Collagen I-coated polyacrylamide gel on PDMS membrane	Homemade stretch device	Static, 3% or 6% uniaxial	24 h and 48 h	10%-FBS	Herum *et al.*, (2017)[90]
	Increased proliferation with low stretch+hypoxia (1%-O_2), no stretch+hypoxia and low stretch+physoxia (6%-O_2)	Human ventricular fibroblast	Fibronectin	Homemade stretch device	1-Hz, 2% or 8% uniaxial	24 h	NS	Ugolini *et al.*, (2017)[87]
No change or decreased proliferation	Decreased proliferation after 12-h stretch in 10% FBS-containing medium. No change with longer stretch or 1% FBS	Foetal RCF	Elastin-coated plate	Flexercell* FX-2000	1.5-Hz, 20% equibiaxial	8–88 h	0%, 1%, or 10% FBS	Butt and Bishop, (1997)[89]
	Decreased proliferation after stretch. Fibroblasts stopped at G_2/M; increased p21, decreased cyclin-B1	Neonatal RCF	Collagen-coated silicone membrane	Modified from Lee *et al.*, 1996[131]	20% sustained stretch	1, 4, 12, 24 h	10% FBS	Liao *et al.*, (2004)[88]
	Decreased proliferation by 12-h stretch regardless of ECM substrate. Decrease most marked on randomly organized collagen	Neonatal RCF	Fibronectin-, laminin-, collagen-coated and uncoated charged silicone elastic membrane	Modified from Yost *et al.*, 2000[101]	0.08 Hz, 5% uniaxial	12 h	10% NBS + 5% FBS	Atance *et al.*, (2004)[86]
	Decreased proliferation with high strain (8% stretch) for 72 h	Human atrial fibroblast	Fibronectin	Homemade stretch device	1 Hz, 2% or 8% uniaxial	24 h and 72 h	NS	Ugolini *et al.*, (2016)[92]
	Decreased proliferation on high substrate stiffness (8 kPa)	Adult mouse ventricular fibroblast	Collagen I-coated polyacrylamide gel, polydimethylsiloxane membrane	Homemade stretch device	Static, 3% or 6% uniaxial	24 h and 48 h	10% FBS	Herum *et al.*, (2017)[90]
	No change with no-stretch+physoxia (6%-O_2) or high-strain stretch (8%)+physoxia	Human ventricular fibroblast	Fibronectin	Homemade device	1 Hz, 2% or 8% uniaxial	24 h	NS	Ugolini *et al.*, (2017)[87]

Continued

Table 1 *Continued*

Response	Main findings	Cell type	Coating and plate	Stretch machine	Stretch pattern	Stretch duration	Serum	Reference
Apoptosis	No cell death or apoptosis	NRVF	Collagen I	Flexercell FX-2000	1 Hz, 20% equiaxial	24, 48, 96 h	2.5% FBS	Persoon-Rothert *et al.*, (2002)[98]
	No apoptosis	Neonatal RCF	Rat tail collagen-coated silicone membrane	Modified from Lee *et al.*, 1996[131]	20% sustained stretch	1, 4, 12, 24 h	10% FBS	Liao *et al.*, (2004)[88]
	Few apoptotic genes differentially expressed in stretched fibroblasts; >15% apoptotic genes increased in myocyte-enriched fractions	NRVF	Collagen I	Flexercell FX-2000	1 Hz, 20% equiaxial	24 h	2.5% FBS	Boerma *et al.*, (2005)[99]
Morphology	Cells elongated, oriented, perpendicular to the direction of stretch	Neonatal RCF	Laminin-coated silicone elastomer membranes	Homemade stretch device	10 cycles/min, 10% uniaxial stretch	72 h	NS	Terracio *et al.*, (1988)[78]
	Cells rotated to align more perpendicular to the direction of stretch	Neonatal RCF	Aligned collagen-coated silicone membrane	Homemade stretch device	3%, 6%, 12% uniaxial; 0, 5, 10 cycles/min	12 h	5% FBS + 10% NBS	Yost *et al.*, (2000)[101]
	Cells surface area increased on all ECM substrates	Neonatal RCF	Fibronectin-, laminin-, collagen-coated; uncoated charged silicone elastic membrane	Modified from Yost *et al.*, 2000[101]	0.08 Hz, 5% uniaxial	1, 5, and 10 min	10% NBS + 5% FBS	Atance *et al.*, (2004)[86]
	Cell margin ruffling, disorganization of long actin stress fibres, retraction of cortical cytoplasm. Effects that are immediate, persisted over time	Neonatal RCF	Laminin-coated silastic membrane	Modified from MacKenna *et al.*, 2000[76]	5% equibiaxial	0, 1.25, 2.5, 5, 10, 20, and 40 min	10% FBS + 5% NBS	Fuseler *et al.*, (2007)[100]

ECM, extracellular matrix; FBS, foetal bovine serum; NBS, newborn bovine serum; NRVF, neonatal rat ventricular fibroblasts; NS, not stated; RCF, rat cardiac fibroblasts.
*Please note that this company has used both Flexcell and Flexercell as names; we have retained the usage in each paper cited.

Ugolini *et al.* reported an increase in proliferation of fibroblasts stretched under hypoxic conditions and their data suggest that proliferation may be more dependent on oxygen levels than stretch.[87] Moreover, in physiological conditions, described by the authors as 6% oxygen, stretch only increased proliferation under low strain levels.[92] Herum *et al.* reported that stretch-induced proliferation depended on the stiffness of the substrate for cell seeding, and although the profibrotic markers collagen and fibronectin were up-regulated after stretch regardless of the substrate, they saw increased proliferation when fibroblasts were seeded on low-stiffness membranes, and decreased proliferation upon seeding on high-stiffness membranes.[90] These authors observed paracrine signalling from stretched cardiomyocytes on fibroblast proliferation. Other studies have also suggested that paracrine factors are important in stretch-related cellular signalling,[55,93,94] which reinforces the idea that a proliferative response of fibroblasts in stretched myocardium may be dependent on cardiomyocyte-released factors rather than, or in addition to, the direct effects of stretch on fibroblasts themselves. Studies on human samples have shown a reduction in fibroblast proliferation and migration (but an increase in differentiation) in cultured atrial fibroblasts isolated from AF patients vs. SR controls.[95] In the scenario of atrial fibrosis, which is a common feature of AF patients,[96] an increase in the atrial content of secretory myofibroblasts is expected and might explain the reduction in overall proliferation.

Results are much more consistent in terms of apoptosis. Although it is well established that cardiomyocytes enter apoptosis when stretched or when exposed to pressure overload,[46,97] cardiac fibroblasts did not show significant signs of cell necrosis or apoptosis even after 96 h of high-strain stretch.[98] Boerma *et al.* reported that after stretch, up to 15% of the cardiomyocyte apoptotic genes were differentially regulated compared to non-stretched controls, while fibroblasts had very few genes differentially expressed.[99] These studies suggest that cultured cardiac fibroblasts are more resilient to stretch-induced apoptosis than cardiomyocytes.

Fibroblasts do change morphology and orientation when stretched, although these changes can also be modulated by ECM substrates.[86] Fuseler *et al.* observed ruffling of cell edges, disorganization of actin fibres, and cell shortening in all directions when fibroblasts were subjected to static equibiaxial stretch with 5% elongation.[100] In response to long-term uniaxial stretch, cardiac fibroblasts and vimentin filaments were elongated, polarized, and oriented perpendicular to the direction of stretch.[78,101] These studies suggest that fibroblast shape is sensitive to stretch but that change in cytoskeleton, cell morphology, and cell polarity depend on time and direction of the applied stretch, as well as on culture conditions, particularly the matrix.

5.1.2 Short-term responses at a molecular level

The initial responses of cultured fibroblasts to stretch are primarily the activation of the immediate early gene (IEG) pathways (*Figure 2*, *Table 2*). Activation of G proteins and kinases activates ERK2 and JNK1 and leads to the phosphorylation of the early response genes c-Jun, c-Fos, and Fra-1.[102,103] These enhance the transcriptional activity of active dimer activator protein-1 (AP1)-dependent genes,[102,103] which orchestrate longer-term responses.[104] In contrast to the unclear effects of stretch on proliferation, there seems to be a consensus that the phosphorylation and nuclear translocation of IEG are the first responses in stretched fibroblasts. MacKenna *et al.* demonstrated a rapid, matrix-dependent activation of the ERK pathway and the JNK pathway in response to 4% static stretch, which could also be induced, although to a lesser extent, by stretch-conditioned medium.[55] Rapid activation of JNK and p38 was confirmed by Papakrivopoulou *et al.*, who showed phosphorylation after 10 min of cyclical stretch.[105] Lal *et al.* demonstrated that the phosphorylation of JNK1 was fast and transient, appearing after 5 min of stretch, peaking at 15 min and disappearing immediately thereafter. In contrast, p38 also peaked at 15 min and returned to baseline but was phosphorylated again after 4 h of stretch and remained phosphorylated after 24 h.[106] Atance *et al.* also reported significant MAPK activation 1 min after starting cyclic stretch,[86] and G proteins were also activated in cells subjected to 1 min of equibiaxial stretch.[106] Similar increases in c-Jun and Fra-1 gene expression were also observed when non-stretched fibroblasts were cultured with medium obtained from stretched fibroblasts or cardiomyocytes.[93]

5.1.3 Long-term responses at a molecular level

Despite the consensus about the short-term responses being mediated by the activation of the IEG, the downstream long-term outcomes are much more variable among studies and seem to depend largely on the type of stretch and culture conditions. The transition to myofibroblast phenotype generally involves activation of the TGFβ pathway and is associated with up-regulation of αSMA and collagen production.[84,107]

Collagen metabolism is a complex process that is tightly regulated by many enzymes. Both increases in collagen secretion in pressure-overloaded hearts and in fibrotic cardiac regions, and enhanced collagen degradation, have been described in patients with CHF.[45] As seen in studies with cultured cardiac fibroblasts from patients and experimental animals, the responses of collagen expression/secretion to stretch are diverse (*Table 3*). Stretch significantly increased collagen I expression and/or secretion in some studies[86,89,105,108,109] and decreased it in others,[43,89,107,110] with some studies showing both increases and decreases depending on the conditions. As well, collagen III levels were increased after stretch in some papers[107,108,110,111] and decreased in others.[86,110] Differential behaviours in collagen expression occurred with variations in cell culture parameters like serum, membrane-coating, etc.[68,84,89,107,110,112] Furthermore, the relative myofibroblast vs. fibroblast content is likely important, given the different phenotypes they express.

Several studies have reported changes in αSMA expression in cardiac fibroblasts exposed to sustained mechanical forces (*Table 4*). Some studies suggest that stretch has opposite effects on αSMA depending on the basal level of αSMA expression, enhancing expression when basal levels were low and decreasing expression in fibroblasts with high basal levels.[68,112] However, this finding is not universal.[90,108] Moreover, levels of αSMA have also been seen to fluctuate in an *in vivo* pressure-overloaded model, first increasing and then declining slowly after abdominal aortic constriction.[35]

There are also discrepancies in upstream regulation of αSMA. Wang *et al.* and Papakrivopoulou *et al.* reported that the stretch-induced increase in αSMA and procollagen 1A1 mRNA expression were dependent on ERK phosphorylation, while their reduction was dependent on p38;[68,105,112] another study reported the opposite, showing that the stretch-induced increase in αSMA expression was prevented in p38-deficient cells.[113]

Similarly, TGFβ, the most known driver of fibroblast differentiation, has been shown to act differently in several stretch studies (*Table 5*). Some studies reported that stretch induced an increase in the expression and activity of TGFβ1,[84,107] depending on the type of stretch applied. Exposing fibroblasts to exogenous TGFβ exerted a profibrotic effect by inducing collagen and αSMA expression, although interestingly this effect was attenuated in the presence of stretch.[93,112,113] Further

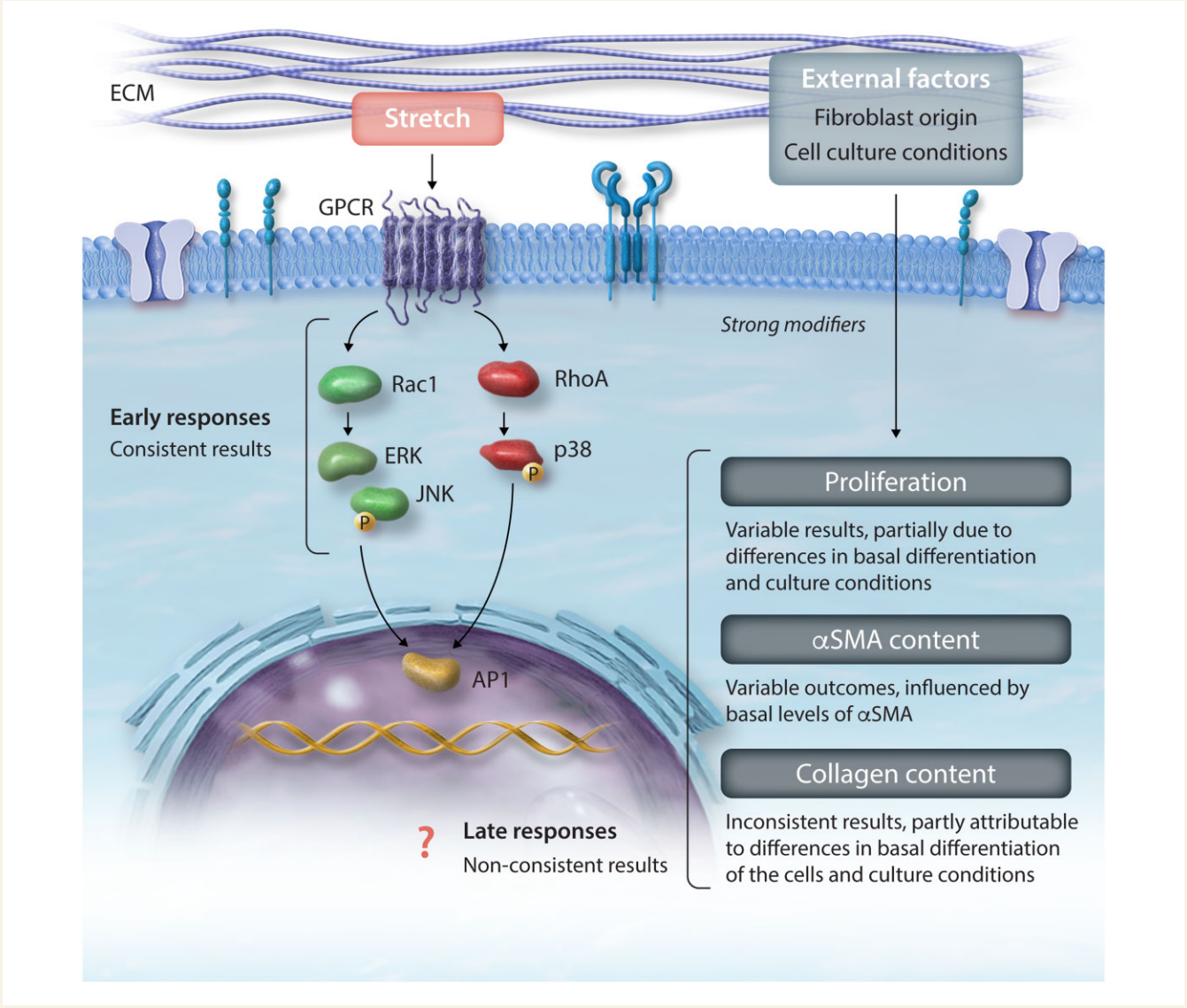


Figure 2 Schematic of biochemical and functional changes in response to stretch and modifying factors. αSMA, alpha-smooth muscle actin; AP1, activator protein 1; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog family member A.

studies also demonstrate that promoter activity of collagen 1A1 was dependent on TGFβ.[114] Although many studies have focussed on the role of TGFβ1, not all have seen an increase in its activity with stretch[84] and little is known on the effects on cardiac fibroblasts of other TGFβ cytokines like TGFβ2 and β3.

Several papers have reported that stretch affects the expression of angiotensin genes or tumour necrosis factor (TNF). Lal *et al*. observed that p38 phosphorylation increased angiotensinogen expression while JNK1/2 decreased it; they noted time-dependent regulation of angiotensin expression by static stretch, decreasing it at 4 h and increasing it after 8 h.[115] Another study from the same group characterized the upstream regulators of these events and saw that stretch activated Rac1 and RhoA within 5 min. Rac1 activity returned to control levels after 4 h, whereas RhoA remained high throughout the whole period of stretch (24 h). Rac1 inhibited the expression of angiotensin through both JNK-dependent and JNK-independent mechanisms, while RhoA stimulated it through a p38-dependent mechanism.[116] Exposing cardiac fibroblasts to exogenous angiotensin increased collagen I and αSMA expression, indicating a profibrotic effect.[112,117] In fact, angiotensin exposure also increased TNF to a similar extent as stretch alone.[118]

In this section, we have tried to summarize key observations regarding the main effects of stretch on cardiac fibroblasts. A great deal of variability in responses was seen among studies; in the section below, we identify some of the technical factors that might explain this apparent lack of consistency.

5.2 Factors that govern the response of fibroblast to stretch

There is substantial variability in the published results of studies evaluation *in vitro* stretch on cardiac fibroblasts. Many factors are likely involved

Table 2 Immediate responses of signalling molecules in fibroblasts to stretch

Response	Main findings	Cell type	Coating and plate	Stretch machine	Stretch pattern	Stretch duration	Serum	Reference
Immediate early gene activation	Quick and transient phosphorylation of c-fos	NRVF	Collagen-coated silicone membrane	Homemade stretch device	20% uniaxial	24 h	Serum-free	Sadoshima *et al.*, (1992)[132]
	ERK2 and JNK1 activated by stretch in matrix-dependent manner. Stretch-conditioned medium also activated	ARVF	Collagen, laminin, vitronectin, and fibronectin	Modified from Lee *et al.*, 1996[131]	4% static equibiaxial	1, 5, 10 min	NS	MacKenna *et al.*, (1998)[55]
	G-proteins activated by 1 min of stretch, several GTP-binding response depending on stretch pattern	Adult male RCF	Collagen I-coated silicone elastic membrane	Modified from Lee *et al.*, 1996[131]	5-s, 10-s, or 60-s stretch, 3% or 6% equibiaxial	5–60 s	10% FBS	Gudi *et al.*, (1998)[106]
	C-fos and fra-1 mRNA increased. C-Jun mRNA unchanged	NRVF	Collagen I-coated flexible plate	Flexercell	15% biaxial static stretch	0–60 min	Serum-free	Van Wamel *et al.*, (2000)[93]
	P38-phosphorylation increased	ARVF	Collagen I, plasma fibronectin, or fibronectin ED-A domain peptide-coated magnetite beads	Ceramic permanent magnet	Static force perpendicular to cell surface	15 min to 8 h	Serum-free	Wang *et al.*, (2000)[112]
	ERK and p38 phosphorylation increased	NRVF	Collagen I-coated magnetite beads	Ceramic permanent magnet	Static force perpendicular to cell surface	0.5, 1, 2, and 4 h	Serum-free	Wang *et al.*, (2003)[68]
	MAPK activated in time-dependent and coating-dependent manner; greater and faster on collagen and charged membrane	Neonatal RCF	Fibronectin-, laminin-, collagen-coated; uncoated charged silicone elastic membrane	Modified from Yost *et al.*, 2000[101]	0.08 Hz, 5% uniaxial	1, 5, and 10 min	10% NBS + 5% FBS	Atance *et al.*, (2004)[86]
	ERK1/2 and p38 phosphorylation increased	Foetal RCF	Elastin-coated Flex-I plate	Flexercell FX-3000	1.5 Hz, 20%	From 10 min to 24 h	10% FBS	Papakrivopoulou *et al.*, (2004)[105]
	Rapid and transient phosphorylation of JNK. Long-term stable phosphorylation of p38 (4–24 h). p38α increased angiotensinogen expression; JNK1/2 phosphorylation decreased it	NRVF	Collagen IV-coated BioFlex plate	Flexercell FX-3000	20% equiaxial static stretch	2–30 min and 1–24 h	Serum-free	Lal *et al.*, (2008)[115]
	Stretch activated Rac1 and RhoA within 5 min. Rac1 activity returned to control after 4 h; RhoA remained high throughout 24 h of stretch. Rac1 inhibited angiotensin expression through JNK-dependent and independent mechanisms; RhoA stimulated it through p38-dependent mechanism	NRVF	Collagen IV-coated BioFlex plate	Flexercell FX-3000	20% equiaxial static stretch	From 2 min to 24 h	Serum-free	Verma *et al.*, (2011)[116]

ARVF, adult rat ventricular fibroblasts; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NBS, newborn bovine serum; NRVF, neonatal rat ventricular fibroblasts; NS, not stated; p38, p38 mitogen-activated protein kinase; Rac1, Ras-related C3 botulinum-toxin substrate-1; RCF, rat cardiac fibroblasts; RhoA, Ras homolog family member A.

Table 3 Stretch-induced changes in fibroblast collagen synthesis

Response	Main findings	Cell type	Coating and Plate	Stretch Machine	Stretch Pattern	Stretch Duration	Serum	Reference
Increase	Collagen-1A1 promoter activated by stretch	Foetal RCF	Collagen I	Flexercell* FX-3000	1 Hz, 10%	24–48 h	Serum-free	Lindahl *et al.*, (2002)[114]
	Procollagen-1A1 mRNA increased in ERK1/2- and p38-phosphorylation-dependent manner	Foetal RCF	Elastin-coated Flex I plate	Flexercell FX-3000	1.5 Hz, 20%	10 min to 24 h	10% FBS	Papakrivopoulou *et al.*, (2004)[105]
	Collagen-1A1 mRNA increased	Human cardiac fibroblasts	BioFlex plate	Flexcell* strain-unit	1 Hz, 5%	24 h	10% FBS	Prante *et al.*, (2007)[130]
No change or dual modulation	Collagen III/I-ratio increased; mRNA-level of collagen III increased, not collagen I	Neonatal RCF	Laminin-coated silicone elastomer membranes	Modified from Terracio *et al.*, 1988[78]	5% static uniaxial; 0.33 Hz, 5% cyclic uniaxial	6, 12, and 24 h	5% FBS + 10% NBS	Carver *et al.*, (1991)[111]
	Collagen protein increased with 48-h stretch in 10% FBS, unchanged at 24 h or after 48-h stretch in 1% FBS	RCF	Elastin-coated flexible or rigid plate	Flexercell strain-unit	1.5 Hz, 20%	24 h and 48 h	1% or 10% FBS	Butt *et al.*, (1995)[109]
	Procollagen-1A1 mRNA, protein synthesis and degradation increased after stretch in 10% FBS. Procollagen synthesis reduced in 0% FBS	Foetal RCF	Elastin-coated plate	Flexercell FX-2000	1.5 Hz, 20% equibiaxial	8–88 h	0%, 1%, or 10% FBS	Butt and Bishop, (1997)[89]
	Fibronectin, collagen I, collagen III mRNA increased by 10% uniaxial strain and 3% equibiaxial strain Decrease or no change with 20% uniaxial or 6% equibiaxial strain.	ARVF	Collagen I-coated silicone elastic membrane	Homemade uniaxial and equibiaxial stretchers	10%, 20% uniaxial; -6%, -3%, 3%, 6% equibiaxial	24 h	NS	Lee *et al.*, (1999)[84]
	Collagen I mRNA and protein secretion increased on all substrates, particularly on collagen substrate Collagen III unchanged	Neonatal RCF	Fibronectin-, laminin-, collagen-coated; uncoated charged silicone elastic membrane	Modified from Yost *et al.*, 2000[101]	0.08 Hz, 5% uniaxial	1, 5, 10 min, and 12 h	5% FBS + 10% NBS	Atance *et al.*, (2004)[86]
	Collagen I and Collagen III mRNA increased after 24-h stretch in 0% FBS, decreased with stretched in 10% FBS	Male ARVF	Collagen I-coated Bioflex culture plate	Flexercell	0.33 Hz, 3%, 6%, 9% equibiaxial	24 h	0%, 0.5%, 5%, or 10% FBS	Husse *et al.*, (2007)[110]
	Stretch reduced TGFβ-induced up-regulation of collagen I mRNA, enhanced TGFβ-induction of collagen III expression	HVF	Fibronectin-coated BioFlex plate	Flexercell FX-4000	1 Hz, 10%	72 h	Serum-free	Watson *et al.*, (2012)[107]

Continued

Table 3 *Continued*

Response	Main findings	Cell type	Coating and Plate	Stretch Machine	Stretch Pattern	Stretch Duration	Serum	Reference
	Collagen-1A1 and -3A1 mRNA increased by stretch on collagen-VI and laminin matrix. No change on collagen IV matrix or collagen I matrix	HVF	BioFlex plate coated with collagens I, IV, VI, or laminin	Flexercell FX-4000T	1 Hz, 10% equibiaxial	72 h	NS	Watson *et al.*, (2014)[108]
	Collagen protein increased in all hypoxic (1% O_2) conditions regardless of stretch. Under physoxia (6% O_2), collagen protein increased only with high-strain stretch (8%)	HVF	Fibronectin-coated	Homemade device	1 Hz, 2% or 8% uniaxial	24 h	NS	Ugolini *et al.*, (2017)[87]
	Collagen-1A1, -1A2, -3A1, and fibronectin mRNA increased but only statistically significant on high-stiffness matrix	Adult mouse ventricular fibroblast	Collagen I-coated polyacrylamide gel on polydimethylsiloxane membrane	Homemade stretch device	Static, 3% or 6% uniaxial	24 and 48 h	10% FBS	Herum *et al.*, (2017)[90]
Decrease	Procollagen protein decreased by long-term cyclic stretch	NRVF	NS	Flexcell FX-4000	Short term: 1 Hz, 10%, 2 h. Long term: 1 Hz, 5%	2 and 48 h	Serum-free	Hooper *et al.*, (2012)[43]

ARVF, adult rat ventricular fibroblasts; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; HVF, human ventricular fibroblasts; NBS, newborn bovine serum; NRVF, neonatal rat ventricular fibroblasts; NS, not stated; p38, p38 mitogen-activated protein kinase; RCF, rat cardiac fibroblasts; TGFβ, transforming growth-factor β.
*Please note that this company has used both Flexcell and Flexercell as names; we have retained the usage in each paper cited.

Table 4 Stretch-induced changes in fibroblast alpha-smooth muscle actin expression

Response	Main findings	Cell type	Coating and plate	Stretch machine	Stretch pattern	Stretch duration	Serum	Reference
Increase	αSMA mRNA and protein increased with stretch of one-day cultured fibroblasts (low basal αSMA level)	NRVF	Collagen I-coated magnetite beads	Ceramic permanent magnet	Static force perpendicular to cell surface	0.5, 1, 2, and 4 h	Serum-free	Wang *et al.*, (2003)[68]
	αSMA-mRNA increased on collagen-VI, collagen IV, and laminin matrix	AHVF	BioFlex plate coated with collagen I, IV, VI, or laminin	Flexcell* FX-4000T	1 Hz, 10% equibiaxial	72 h	NS	Watson *et al.*, (2014)[108]
	αSMA-mRNA increased by stretch. αSMA fibre formation with matrix stiffening	Adult mouse ventricular fibroblast	Collagen I-coated polyacrylamide gel adherent on PDMS membrane	Modified home-made stretch device	Static, 3% or 6% uniaxial	24 h and 48 h	10% FBS	Herum *et al.*, (2017)[90]
	αSMA-positive cells increased after 48-h stretch at 0.5 and 1 Hz; not observed in p38-deficient fibroblasts	Mouse embryonic fibroblasts	NS	Flexcell	0.5 and 1.0 Hz	48 h	10% FBS	Molkentin *et al.* (2017)[113]
No change	αSMA mRNA unchanged with stretch on collagen I matrix	AHVF	BioFlex plate coated with collagen I, IV, VI, or laminin	Flexcell FX-4000T	1 Hz, 10% equibiaxial	72 h	NS	Watson *et al.*, (2014)[108]
Decrease	αSMA mRNA and protein decreased with stretch on collagen I matrix, p38 dependent	ARVF	Collagen I, fibronectin, or fibronectin ED-A domain peptide-coated magnetite beads	Ceramic permanent magnet	Static force perpendicular to cell surface	15 min to 8 h	Serum-free	Wang *et al.*, (2000)[112]
	αSMA mRNA and protein decreased with stretch of three-day cultured fibroblasts (high basal αSMA level), p38-dependent	NRVF	Collagen I-coated magnetite beads	Ceramic permanent magnet	Static force perpendicular to cell surface	0.5, 1, 2, and 4 h	Serum-free	Wang *et al.*, (2003)[68]
	αSMA protein decreased by long-term stretch	NRVF	NS	Flexcell 4000	Short term: 1 Hz, 10%. Long term: 1 Hz, 5%.	2 h and 48 h	Serum-free	Hooper *et al.*, (2012)[43]
	TGFβ-induced up-regulation of αSMA mRNA and protein. Stretch reduced TGFβ induction of αSMA	AHVF	Fibronectin-coated BioFlex plate	Flexercell* FX-4000 T	1 Hz, 10%	72 h	Serum-free	Watson *et al.*, (2012)[107]

AHVF, adult human ventricular fibroblasts; ARVF, adult rat ventricular fibroblasts; αSMA, alpha-smooth muscle actin; FBS, foetal bovine serum; NRVF, neonatal rat ventricular fibroblasts; NS, not stated; p38, p38 mitogen-activated protein kinase; TGFβ, transforming growth-factor β.

*Please note that this company has used both Flexcell and Flexercell as names; we have retained the usage in each paper cited.

Table 5 Stretch-induced changes in fibroblast cytokine-regulation and other molecular effects

Response	Main findings	Cell type	Coating and plate	Stretch machine	Stretch pattern	Stretch duration	Serum	Reference
Cytokine regulation	Uniaxial stretch stimulated TGFβ1 activity, effect dependent on strain magnitude	ARVF	Collagen I-coated silicone elastic membrane	Homemade uniaxial and equibiaxial stretchers	10%, 20% uniaxial; -6%, -3%, 3%, 6% equibiaxial	24 h	NS	Lee *et al.*, (1999)[84]
	Stretch increased TGFβ mRNA, BNP protein, and receptor. Production and secretion of TGFβ protein, TGFβ receptor expression unchanged. Profibrotic effects of TGFβ attenuated	AHVF	Fibronectin-coated BioFlex plate	Flexercell* FX-4000 T	1 Hz, 10%	72 h	Serum-free	Watson *et al.*, (2012)[107]
General gene expression	Protein synthesis enhanced at 2 h of stretch, persisted to 24-h stretch. Increased protein/DNA ratio in cardiomyocytes incubated with conditioned medium from stretched fibroblasts	NRVF	Collagen I-coated plate	Flexercell	1 Hz, 20%	2, 4, 6, 18, and 24 h	Serum-free	Ruwhof *et al.*, (2001)[94]
Other	MMP increased; tissue plasminogen activator secretion increased	AHVF	Collagen I-coated silicone membrane	Flexcell* 3000	0.42 Hz, 25–35%	24 h	Serum-free and 5% FBS	Tyagi *et al.*, (1998)[120]
	TNF production increased by stretch in fibroblasts but not in cardiomyocytes	NRVF	Collagen-coated silicone membrane	Flexcell	0.1 Hz, elongation < 18%	6 h	NS	Yokoyama *et al.*, (1999)[118]
	Dual regulation of integrin β1-protein: expression increased under 3%, and decreased under 6% and 12% stretch	NRVF	Collagen-coated silicone membrane	Homemade stretcher	3%, 6%, 12% uniaxial at 0, 5, and 10 cycles/min	12 h	5% FBS + 10% NBS	Yost *et al.*, (2000)[101]
	XT-I mRNA and activity, total CS-GAG content increased by stretch. Stretch-induced XT-I expression reduced with anti-TGFβ1 antibody	AHVF	BioFlex plate	Flexcell	1 Hz, 5%	24 h	10% FBS	Prante *et al.*, (2007)[130]
	Angiotensinogen mRNA decreased after 4-h stretch, JNK-phosphorylation-dependent; p38-dependent increase after 8–24 h of stretch	NRVF	Collagen-IV-coated BioFlex plate	Flexercell FX-3000	Static, 20% equiaxial	From 2 min to 24 h	Serum-free	Lal *et al.*, (2008)[115]
	Rac1 controlled JNK-dependent decrease of angiotensin genes with stretch, RhoA acted upstream of p38 and mediated its increase	NRVF	Collagen-IV-coated BioFlex plate	Flexercell FX-3000	Static, 20% equiaxial	From 2 min to 24 h	Serum-free	Verma *et al.*, (2011)[116]

Continued

Table 5 *Continued*

Response	Main findings	Cell type	Coating and plate	Stretch machine	Stretch pattern	Stretch duration	Serum	Reference
	Membrane LPP protein decreased by short-term, increased by long-term, cyclic stretch	NRVF	NS	Flexcell 4000	Short term: 1 Hz, 10%; Long term: 1 Hz, 5%.	2 and 48 h	Serum-free	Hooper *et al.*, (2012)[43]
	SGK1 mRNA increased in time-dependent manner. Phospho-SGK1 and total SGK1 proteins increased in stretch elongation-dependent manner. SGK1-dependent chemokine release controlled by NF-κB pathway promoted macrophage migration. Effects lost with SGK1 knockout	Ventricular fibroblasts from adult WT and SGK1 knock-out mice	Silicone elastomer-bottomed plate	Flexcell FX-5000	1 Hz, 5% or 18%	1, 4, 6, 12, 24 h	NS	Gan *et al.*, (2018)[119]

AHVF, adult human ventricular fibroblasts; ARVF, adult rat ventricular fibroblasts; CS-GAG, chondroitin sulphate-glycosaminoglycan; FBS, foetal bovine serum; JNK, c-Jun N-terminal kinase; LPP, lipoma-preferred partner; MMP, matrix metalloproteinase; NBS, newborn bovine serum; NF-κB, nuclear factor kappa light-chain enhancer of activated B-cells; NRVF, neonatal rat ventricular fibroblasts; NS, not stated; p38, p38 mitogen-activated protein kinase; Rac1, Ras-related C3 botulinum-toxin substrate-1; RhoA, Ras homolog family member A; SGK1, serum-glucocorticoid-regulated kinase 1; TGFβ, transforming growth factor-β; TNF, tumour necrosis factor; XT-I, xylosyltransferase-I.

*Please note that this company has used both Flexcell and Flexercell as names; we have retained the usage in each paper cited.

but differences in experimental conditions likely play a major role (*Figure 3*). The key factors can be classified into: (i) differences in fibroblast origin, (ii) culture conditions, and (iii) type of stretch.

5.2.1 Fibroblast origin

An important source of variability between studies is the origin of cardiac fibroblasts. Three central factors have to be considered: species, age of the animal, and cardiac chamber of origin. The most commonly used species is the rat, although stretch studies have been also reported using mouse[90,119] and human[107,120] fibroblasts (*Figure 4A*). In terms of subject age, cells can be isolated from foetal, neonatal, or adult animals (*Figures 3 and 4B*). Whether stretch responses can be affected by age remains unknown, but as with cardiomyocytes that present greater surface adherence and reproductive abilities at the foetal and neonatal stage,[121] fibroblasts present stage/age-related phenotypic differences that could condition their behaviour towards stretch. For example, foetal human cardiac fibroblasts are smaller and proliferate faster than adult and differently influence neighbouring cardiomyocytes.[122,123] Foetal fibroblasts also have distinct epigenetic and transcriptomic features including chromatin accessibility, histone marks, motifs, and corresponding transcription factors, suggesting that their responses to stretch or other stimuli could differ.[122]

Another crucial consideration is the heart chamber from which fibroblasts are isolated. Most stretch studies use either isolated ventricular fibroblasts or pool atrial and ventricular fibroblasts together. The latter approach introduces additional variability, since atria and ventricle fibroblasts have different phenotypes[124] and respond differently to external stimuli.[47] While stretch responses of atrial fibroblasts have not been extensively investigated *in vitro*, it is known that atria and atrial fibroblasts exposed to pressure overload exhibit greater fibrotic responses compared to ventricular fibroblasts.[35,68,84,125,126] Moreover, atrial fibroblasts from patients with chronic AF showed significant changes in gene expression, proliferation, migration, and myofibroblast differentiation, suggesting they might respond differently to external stimuli compared to ventricles.[95]

5.2.2 Coating materials and membrane stiffness

Silicone membranes are often used in stretch devices because they are flexible, transparent, and non-deformable. However, they are hydrophobic and unsuitable for cell culture if not treated.[127] Researchers generally coat the membranes with ECM proteins, as they improve cell adhesion and mimic the *in vivo* environment around cardiac fibroblasts. Laminin, collagen, and fibronectin are the most common coating materials, followed by elastin and vitronectin. As an alternative, Lateef *et al.* used arginylglycylaspartic acid peptide, the binding sequence for integrins found in most ECM proteins, and also showed stable cell adhesion throughout the stretch protocol.[127]

Although the coating step might seem trivial, the ECM component used for coating is a critical determinant of the cellular responses induced by mechanical loading. Identical stretch protocols applied to fibroblasts seeded on different coatings induce differential responses in cell morphology, cell proliferation, signalling pathways, and gene expression.[17,18,98,108] The most plausible explanation is that different coatings differentially affect different cell contact proteins like integrins and produce differential activation of signalling pathways.[55] For example, Watson *et al.* reported that fibroblasts cultured on fibronectin-coated membranes decreased collagen I and increased collagen III expression under stretch conditions.[107] The same stretch protocol applied to cells cultured on collagen VI or laminin increased both collagen I and collagen III expressions,

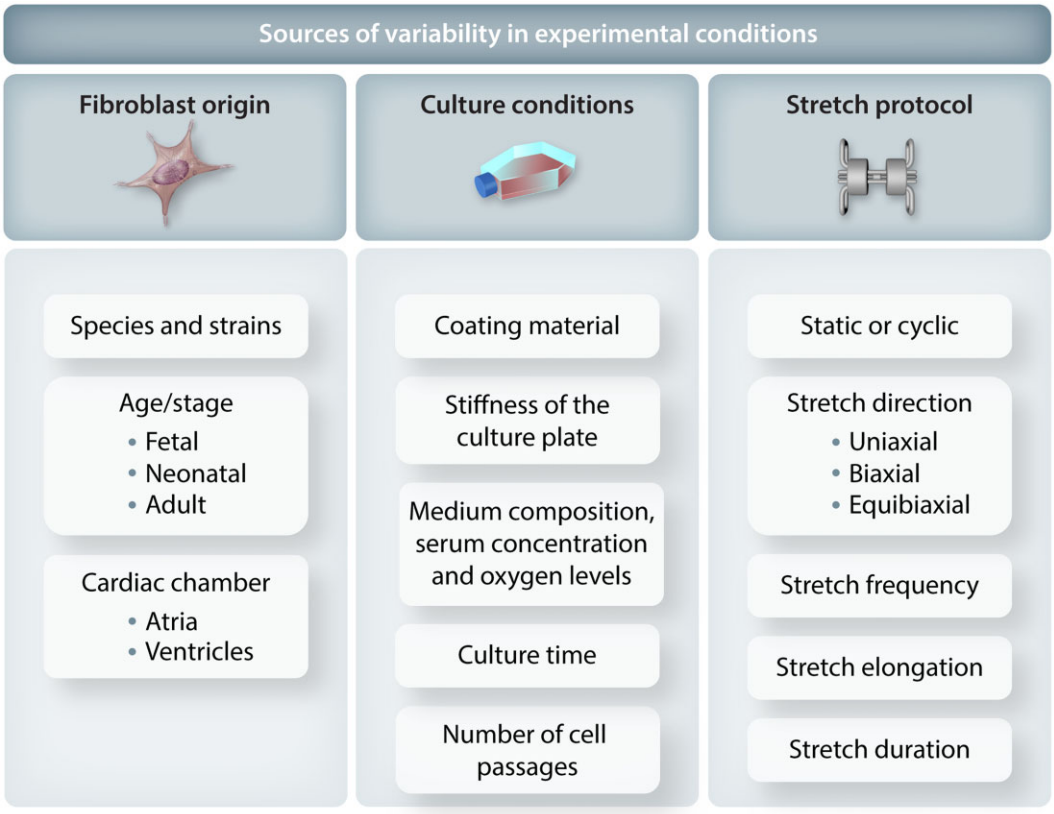


Figure 3 Factors that can affect the results of cardiac fibroblast stretch studies.

and when cells were seeded on collagen IV or I, there was no change.[108] The αSMA expression in those studies was also different depending on the coating: decreased by stretch on fibronectin coating but increased on collagen VI or laminin matrix.[108] Another study reported that stretch decreased αSMA expression when cells were cultured on collagen I matrix but not on fibronectin or fibronectin ED-A domain polypeptide.[112]

These observations highlight the crucial role of ECM components in mechanotransduction and suggest that stretch-induced effects might be ligand specific. The ECM is a complex structure containing and regulated by many proteins and molecules. Which component reproduces the 'correct' *in vivo* response is unclear and it is possible that *in vivo* responses result from a complex blend of ECM protein regulated effects that may be very difficult to reconstitute in an *in vitro* system.

Substrate stiffness can also influence the basal expression levels of profibrotic genes and cell proliferation.[90,107] Herum *et al.* reported that as substrate stiffness increased, more profibrotic responses were seen in the seeded fibroblasts and suggested 8 kPa as the suitable stiffness to avoid differentiation. This is considered the stiffness of a healthy myocardium, as opposed to 20–100 kPa values in fibrotic cardiac tissue.[90]

5.2.3 Culture time and cell passage

It is well known that cell culture and passage affect fibroblast properties, fostering their differentiation into myofibroblasts.[48,128] The state of fibroblast differentiation can have important consequences on the outcome of a stretch experiment and must be considered when comparing studies.[68] For example, freshly isolated fibroblasts from CHF dog atria differed from fibroblasts isolated from control animals in cell morphology and ECM gene expression (reflecting the *in vivo* profibrotic state), while after two days in culture these differences disappeared because control fibroblasts increased their collagen and fibronectin expression levels to a greater extent through the response to culture.[129] These culture/passage-dependent effects, seen in both neonatal and adult fibroblasts, modify collagen deposition, growth factor production, membrane receptor expression, focal adhesion proteins, and key myofibroblast marker proteins including vimentin, αSMA, and smooth muscle myosin heavy chain. In some studies, primary cardiac fibroblasts were stretched after short periods of culture,[110] while in others fibroblasts underwent up to more than 10 passages before stretch;[108] clearly these differences can importantly affect outcomes. Differences in basal levels of profibrotic genes due to culture conditions might contribute to discrepancies between studies. As mentioned above, stretch increased αSMA expression in cells cultured for only 1 day before stretch, while decreasing αSMA expression when cells were cultured for 3 days pre-stretch and had higher basal αSMA expression.[68,112] To avoid excessive cell differentiation because of culture, cells should not be cultured for prolonged periods and should be kept at very low passages.

5.2.4 Serum concentration

Serum is used in fibroblast culture and serum concentration plays a critical role in regulating fibroblast responses in general, but its specific

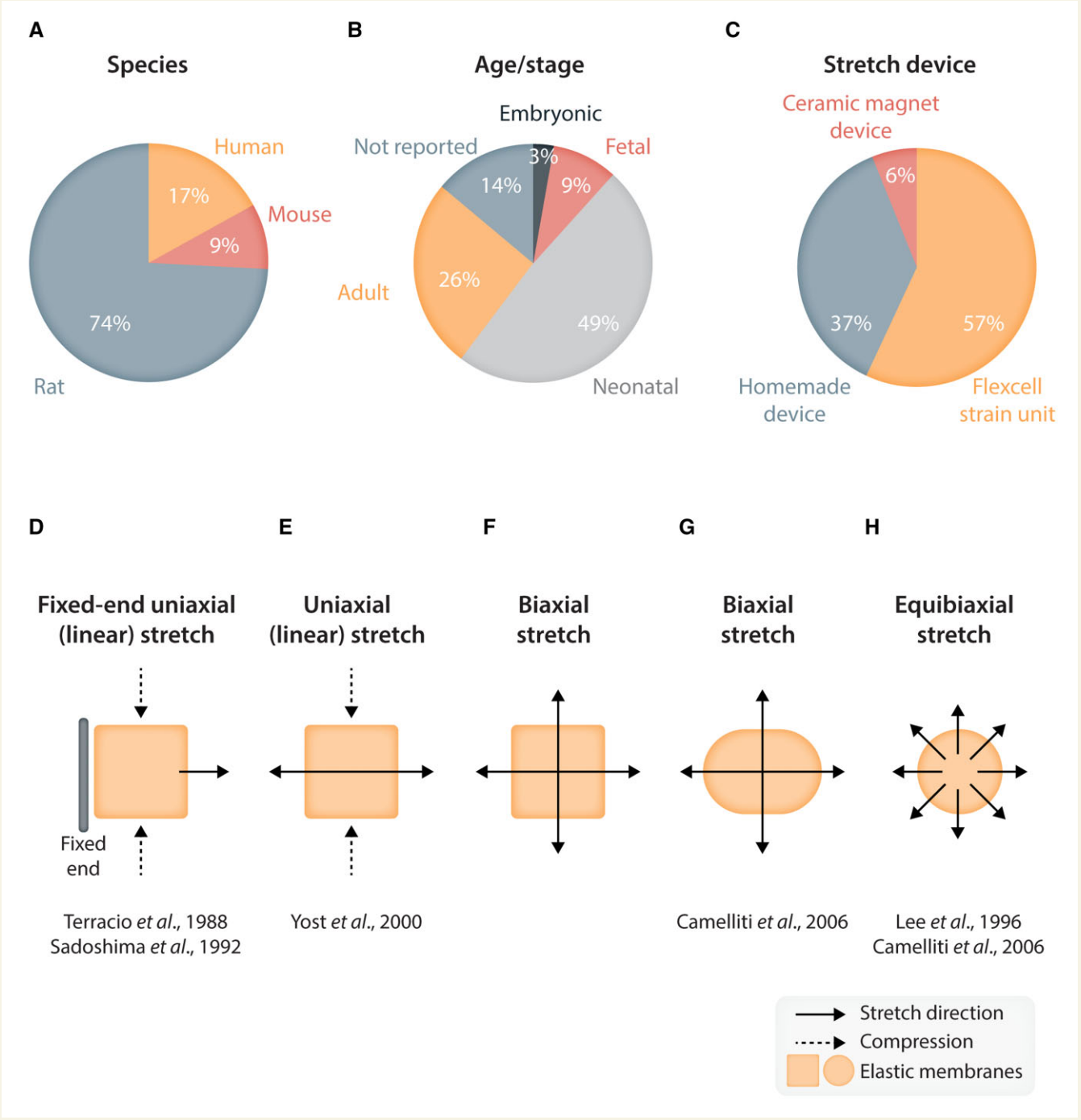


Figure 4 Schematic representing (top) the relative percentage use of different key conditions (species, age/stage and stretch device) across different studies reviewed in this paper; and (bottom) different types of stretch pattern used in research on stretch effects on fibroblasts. (A–C) Pie charts indicating the percentage of various species (A), age/stage of fibroblasts (B), and type of stretch devices (C) that were used in the *in vitro* stretch studies reviewed in the present paper. (D–H) Schematics showing the types of stretch patterns used for *in vitro* studies of stretch effects on fibroblasts. The solid arrows indicate the direction of applied stretch. (D, E) Uniaxial (linear) stretch. (F, G) Biaxial stretch. (H) Isotropic, equibiaxial stretch.

effect on stretch is not clear. Two studies by Butt *et al.* demonstrated a synergistic effect on collagen expression between serum concentrations and stretch. They reported that cells stretched over 48 h and cultured in 10% FBS increased procollagen expression and procollagen degradation, while when cultured in 1% FBS, collagen production did not change,[109] and in serum-free medium, procollagen synthesis decreased.[89] However, Husse *et al.* reported opposite results: a decrease in collagen I and III in 10% FBS medium, and an increase when cells were stretched in serum-free medium.[110] Although both teams used the same stretch device, they applied different stretch amplitudes and coating materials, which could explain the discrepancies. Other studies reported similar results to Butt *et al.*,[43,105,130] while Lindahl *et al.* reported a result

similar to Husse *et al.*[114] These apparent inconsistencies reinforce the complexity and limitations of these stretch studies and the need to clarify their basis.

5.2.5 Stretch protocol

One of the main constraints in studying mechanotransduction in a monolayer system is the difficulty in mimicking the complex and dynamic forces that mechanical stimuli exert in the intact heart. Static stretch mimics a chronic hemodynamic overload such as an increase in heart volume,[84,90,131–133] while cyclical stretch reflects regular cardiac contraction better.[92,101,131,134] However, performing *in vitro* stretch over extended periods at the frequencies of heart rates of a rat (~6 Hz) or a mouse (~10 Hz) is technically difficult and hard on the cells.

The strain generated by stretch devices can be classified as uniaxial, biaxial, and equibiaxial (*Figures 3 and 4D–H*). Uniaxial strain distribution is anisotropic and non-homogeneous on the elastic membrane;[135] the devices generate concomitant compression perpendicular to the axis of stretch and shear forces near membrane edges.[92,134] In contrast, biaxial stretch allows cells to be stretched without shear stress,[101] and equibiaxial stretch generates homogeneous and isotropic strains to the cell cultures.[133,136] Ugolini *et al.* designed a microdevice with a cell culture area of 5 mm^2, allowing the seeding of 1,000 cells, which generates uniform strain fields in a high-throughput fashion.[87,92] However, these small numbers of cells are often not compatible with expression assays like western blot. Another system designed by Wang *et al.* applies continuous static perpendicular forces to cardiac fibroblasts cultured on magnetic beads, but strain is not homogeneously distributed[68,112] (*Figure 4C*). Mechanical strain in intact hearts are multiaxial and non-uniform, so even the most sophisticated devices fail to fully recapitulate what happens in an intact heart.[84]

In principle, the elongation and frequency of stretch should mimic the natural mechanical forces exerted in the heart, which are very hard to quantify. Researchers have applied levels of stretch sufficient to produce 4% to 20% elongation. In their natural environment, fibroblasts stretch because of the contraction and relaxation of adjacent cardiomyocytes; therefore, myocyte stretch has been taken as a reference. Sadoshima *et al.* claimed that in the intact heart, a 20% increase in cell length is within the physiological range and suggested that cardiomyocytes were not injured or detached during such stretch.[132] Wang *et al.* also used 10% and 20% uniaxial stretch to mimic myocardial hypertrophy,[137] and MacKenna *et al.* claimed that the area change generated by 4% equibiaxial stretch was comparable to the changes observed *in vivo*.[55] Gan *et al.* also reported that equibiaxial stretch with a 5% elongation could simulate the physiological *in vivo* condition but that 18% equibiaxial stretch was deleterious.[119] Lee *et al.* showed that changing elongation extent caused collagen expression to change in different directions, although these findings were not reproduced by others.[84] When 10% uniaxial or 3% equibiaxial stretch was applied, collagen mRNA and fibronectin mRNA expression increased, but when the elongation was increased to 20% and 6% respectively, they decreased.[84] This observation implies that cardiac fibroblasts are able to distinguish and react differently to different types of mechanical forces. The range between physiological and pathological stretch is still a matter of debate. Overall, it is generally assumed that a uniaxial stretch between 10% and 20% cell elongation and an equibiaxial stretch of 3%–6% are within the physiological range of both cardiomyocytes and fibroblasts.

In terms of frequency, 1 Hz is most commonly used, although studies have used frequencies between 0.1 Hz and 1.5 Hz. Rodent cardiac fibroblasts in an intact heart are exposed to rates 6 to 10 times higher than the frequencies recapitulated by the stretch devices. There is no information available regarding differential cellular responses due to changes in frequencies.

The duration of stretch may also condition outcomes. Stretch-induced responses occur in a specific temporal sequence. Stretch protocols in the literature have been applied for periods varying from 5 s to 96 h.[98,106] The earliest response to stretch reported is the activation of the IEG and MAPKs and this does not seem to be affected by different experimental conditions (*Table 2*). However, changes in profibrotic genes like αSMA and collagen are only observed after long-term stretch (of at least 2 h for αSMA and at least 12 h for collagen)[68,111] and the reported responses vary widely (*Tables 3* and *4*). For example, angiotensinogen gene expression changes were biphasic, with a decrease after 4 h of static stretch and an increase between 8 h and 24 h of static stretch.[115] Another example is LPP, for which 2 h of cyclical stretch at 1 Hz with 10% elongation only affected subcellular localization, but a 48-h stretch period at 1 Hz with 5% elongation up-regulated protein expression.[43] The expression of serum-glucocorticoid-regulated kinase 1 (SGK1), a kinase that contributes to cardiac remodelling and the development of heart failure, is similarly regulated by stretch in a time-dependent fashion.[119]

The influence of the wide range of technical factors detailed above makes this literature particularly difficult to analyse. It is rare for different studies not to vary in important technical determinants and therefore when discrepant results are obtained, it becomes very difficult to know whether the results are truly in conflict or differ because of technical determinants, and if so which (if any) of the obvious technical differences are responsible for the differing outcomes. There seems to be a clear need to obtain a clearer consensus about the effects of stretch on fibroblast function and to understand better the determinants of the response.

6. Conclusions

It is clear that fibrosis occurs in many paradigms of cardiac pressure and/or volume overload. In atria subjected to chronic pressure/volume loading conditions, fibrosis is common and appears to play an important role in AF. However, contrary to widespread assumptions, the evidence regarding direct cardiac fibroblast activation by mechanical forces is weak and unclear, with key responses like fibroblast proliferation, collagen production, and indices of differentiation to myofibroblasts showing divergent and sometimes directionally opposite changes among studies in response to stretch. While the differences in response may be due to technical differences in terms of fibroblast origin, state of differentiation, degree of stretch, seeding medium, etc., it is not possible to identify clear patterns in the published work. Further research is needed to obtain reproducible results that answer the question of whether, and if so how, mechanical stretch directly activates fibroblasts.

There is great interest in developing novel therapeutic approaches that prevent fibrosis development in the treatment of heart disease, particularly AF.[138] In order to accomplish this goal, the key mechanistic determinants need to be understood. It is therefore important to determine definitively whether the association between pressure/volume loading and cardiac fibrosis is due to a direct activating effect on fibroblasts, to profibrotic substances produced by cardiomyocytes, leucocytes, or neurohormonal responses, or to some combination of these factors. Important new tools have become available to help in this effort.

For example, single-cell RNA sequencing allows for the identification of changes in cell populations identified by their transcriptomic signature, rather than by classical, somewhat arbitrary criteria. This approach has recently been used to evaluate changes in specific fibroblast cell populations associated with AF.[139] Clarifying these issues may provide the key to developing new and more effective therapies to prevent the development and progression of atrial fibrosis and positively influence the natural history of AF.

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