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Review

Structural and functional characterisation of cardiac fibroblasts

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Abstract

Cardiac fibroblasts form one of the largest cell populations, in terms of cell numbers, in the heart. They contribute to structural, biochemical, mechanical and electrical properties of the myocardium. Nonetheless, they are often disregarded by in vivo and in vitro studies into cardiac function. This review summarizes our understanding of fibroblast origin and identity, their structural organization and role in myocardial architecture, as well as functional aspects related to cell signalling and electro-mechanical function in the heart.

Keywords: Connective tissue; Myocardium; Remodelling; Gap junction; Heart

1. Introduction

Cardiac myocytes occupy approximately 75% of normal myocardial tissue volume, but they account for only 30–40% of cell numbers [1]. The majority of the remaining cells are non-myocytes, predominantly fibroblasts. Other cell types, such as endothelial or vascular smooth muscle cells, represent comparatively small populations [2].

Fibroblasts are found throughout the cardiac tissue, surrounding myocytes and bridging 'the voids' between myocardial tissue layers, so that, in essence, every cardiomyocyte is closely related to a fibroblast in normal cardiac tissue (Fig. 1). Pathological states are frequently associated with myocardial remodelling involving fibrosis. This is observed in ischemic and rheumatic heart disease, inflammation, hypertrophy, and infarction. The growth in fibrous tissue content is based on the maintained proliferative potential of fibroblasts (largely absent in myocytes of the adult heart [3]), and the synthesis of extra-cellular matrix (ECM) proteins, predominantly by fibroblasts [4].

Fibroblasts contribute to cardiac development, myocardial structure, cell signalling, and electro-mechanical function in healthy and diseased myocardium [4–6]. In the following sections, we summarise current insight into cardiac fibroblast origin and identity, their structural and functional contribution to cardiac function, and highlight emerging hypotheses and targets for further research.

2. What is a fibroblast?

2.1. Terminology and scope

Fibroblasts are obligatory components of the ECM. They are widely distributed in most vertebrate organisms, and associated with the various forms of connective tissue [7].

Fibroblasts are traditionally defined as cells of mesenchymal origin that produce interstitial collagen (in contrast to myocytes that form collagen type IV as part of their basement membrane, fibroblasts also produce types I, III and VI). Collagen synthesis or deposition is, however, not usually assessed in the context of fibroblast identification (i.e. via in situ hybridization, or via immuno-cytochemical localization). Instead, cell classifi-

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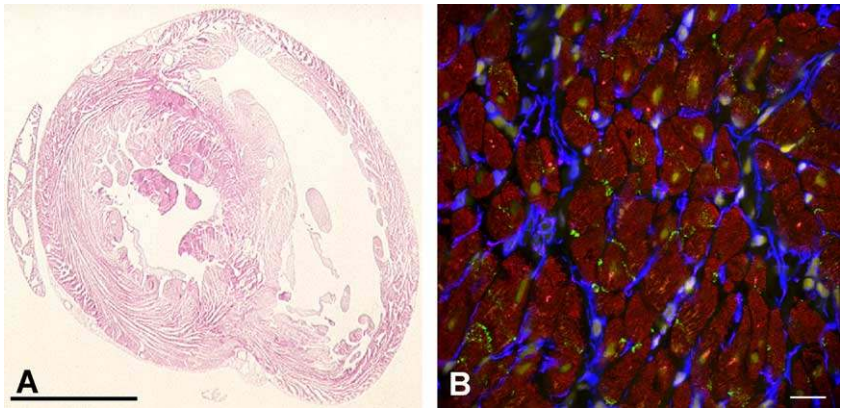

Fig. 1. Cardiac tissue structure overview. (A) Gross histological view (transversal cross-section) of rabbit ventricular myocardium, stained with Hematoxylin and Eosin, shows layered sheets of cardiac muscle cells (pink), separated by 'voids' that are filled with (un-labelled) non-myocytes. (B) Larger magnification view of ventricular myocardium (confocal microscopy tissue cross-section), immuno-stained with anti-myomesin to mark myocytes (red), anti-vimentin to mark fibroblasts (blue), anti-connexin43 (bright green dots) and with DAPI (4′ -6-Diamidino-2-phenylindole) to label nuclei (pale yellow–green patches), showing the dense network of fibroblasts that surrounds myocyte clusters of 2–4 cells. Scale bars in A and B: 5 mm and 20 μm, respectively.

cation is often based on morphological characteristics and/or proliferative potential.

These 'substitute identifiers' can vary widely with location and metabolic activity of individual cells, organs, or organisms. In general, though, fibroblasts lack a basement membrane and tend to have multiple processes or sheet-like extensions. They contain an oval nucleus (with 1 or 2 nucleoli), extensive rough endoplasmic reticulum, a prominent Golgi apparatus, and abundant cytoplasmic granular material.

The lack of a truly specific marker has long been a limiting factor in studying fibroblasts in vivo. A useful label for cardiac fibroblasts are anti-vimentin antibodies that react with the abundant intermediate filaments of fibroblasts [8]. This marker also labels other cell types, for example vascular endothelial cells and neurones, that contain intermediate filaments. Given the characteristic cyto-morphological differences between these cell types, however, anti-vimentin has been a suitable tool for reliable identification of cardiac fibroblasts.

A more specific marker for cardiac fibroblasts is the collagen receptor *Discoidin Domain Receptor 2* (DDR2 [9]). DDR1 and DDR2 represent a relatively novel family of collagen specific receptor tyrosine kinases [10,11]. Receptor tyrosine kinases are a family of proteins involved in the conversion of extracellular stimuli into cellular responses [12]. These receptors mediate a variety of cell functions, including growth, migration, morphology and differentiation. The tissue distribution of DDR1 and DDR2 varies (and can be mutually exclusive [13]), and DDR2 expression has been detected in both rat and mouse heart [14]. Originally defined as a collagen receptor on mesenchymal cells, DDR2 has also been found on leukocytes, as well as in tumours, but not on cardiomyocytes or cardiac endothelial and smooth muscle cells [9]. Interestingly, DDR2 gives a labelling pattern that is very similar to that obtained using vimentin antibodies (Fig. 2).

It is important to note that fibroblasts are principally motile cells that contain actin (mainly α-smooth muscle actin) and myosin, so that their identification as 'cells

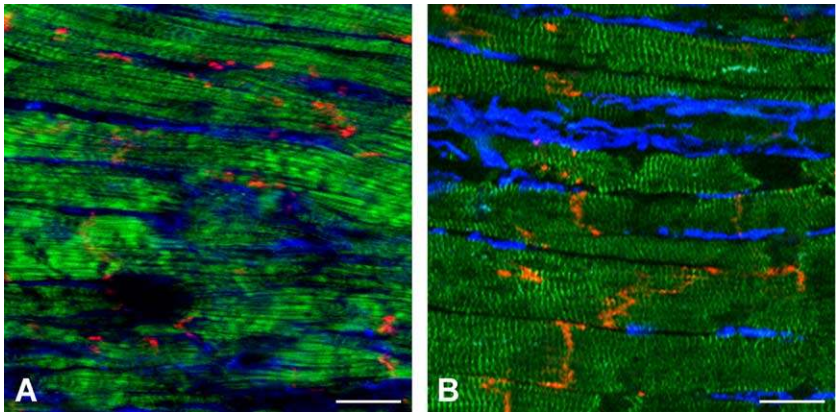

Fig. 2. Fibroblast myocyte interrelation in cardiac tissue. (A) Fibroblasts labelled with anti-DDR2 (blue) separate phalloidin-labelled myocytes (green) in mouse ventricular tissue (red label: connexin43). (B) Fibroblasts labelled with anti-vimentin (blue) form layers between anti-myomesin labelled myocytes (green) in sheep ventricular tissue (red: connexin43). Note the principal similarity of cell type interrelation identified by the two antibodies. Scale bar: 20 μm.

void of contractile proteins' may cause false-negative findings.

In addition, the term 'myofibroblast'—if applied solely on the basis of actin or myosin presence—may be more misleading than helpful. Fibroblasts are pleiomorphic, and their actin or myosin content and arrangement are affected by the environment, in particular mechanical parameters. An increased contractile filament content does not necessarily transform a fibroblast into a new cell type, but may merely represent a distinctive phenotype. In the absence of a clear and consistent definition, myofibroblasts will therefore not be addressed as a separate entity in this review.

3. Origin of cardiac fibroblasts

3.1. Physiology

The mesenchymal cells that form the cardiac fibroblast population are believed to be derived from two principal sources: (1) the pro-epicardial organ, and (2) the epithelial–mesenchymal transformation during the formation of cardiac valves [15,16]. Other sources, such as the developing bone marrow and neural crest, differentiation from the vascular walls, or circulating progenitor cells, are conceivable, but we are far from an understanding of their relevance in the healthy heart.

Fibroblast content increases with normal development and aging [2,17]. During early human development, myocyte and connective tissue cell numbers increase at a similar rate, from about 0.5×10^9 at 28 weeks of foetal development to $2–3\times10^9$ several weeks post partum. Thereafter, myocyte cell numbers remain stable, while the connective tissue cell count rises with cardiac weight to $\sim7\times10^9$ at 2 months of age [2]. This is mirrored by an increase in the volume fraction of connective tissue, which reaches about 5–6% in normal adult myocardium, but may exceed 50% of the adult human sino-atrial node (SAN) [18,19].

Similar dynamics are observed in other mammals, such as rat and hamster, where little connective tissue is observed in the early embryonic heart [20,21]. Most of the connective tissue is involved, at that stage, with the formation of the cardiac skeleton and the various valvular structures. The three-dimensional collagen network, composed of the epimysium, perimysium and endomysium, begins to form in late foetal development. It is largely laid down during neonatal growth [22], accompanied by rapid proliferation of fibroblasts and substantial deposition of collagen [23]. Following neonatal development, fibroblast cell division returns to a very low level, unless stimulated by either physiological or pathological signals.

Thus, fibroblasts form a majority cell population in the normal adult heart (up to two-thirds), which is largely interspersed in the collagen network. Their physiological origin remains to be elucidated more comprehensively.

3.2. Pathology

Net collagen deposition in adult hearts is normally very low. Aging is associated with increased cross-linking of collagen, contributing to tissue stiffening. In disease states, however, such as cardiac hypertrophy, heart failure, and infarction, collagen deposition is dramatically increased [24]. The associated proliferation of fibrous tissue has been divided by Weber and colleagues into two categories: (1) reparative fibrosis, which occurs dispersed through the myocardium, and (2) reactive fibrosis, which occurs initially associated with capillaries, and then spreads to the myocardium [25].

Fibroblasts are sensitive to circulating hormones [26,27], which affect their proliferative response to pathological stimuli. Investigations using H^3 to mark dividing cell populations in cardiac hypertrophy showed that only a fraction of fibroblasts, mostly located near blood vessels, contain the label [28]. This is taken to indicate that tissue fibrosis may involve only a minority of fibroblasts that proliferate rapidly.

It is conceivable that the proliferating, perivascular fibroblast population in the heart stems from bone marrow-derived circulating progenitors [29]. Cossu and Bianco [30] have described mesoangioblasts as a progenitor of mesodermal tissues. These multipotent cells have the ability to differentiate into a variety of both vascular and mesodermal tissues [31], including cell populations such as endothelial cells and fibroblasts. Their origin is thought to be the hematopoietic stem cell population in bone marrow. Analysis of mesoangioblasts by lineage markers indicates that they have early endothelial markers, Flk1, CD34 and VE cadherin, as well as α-smooth muscle actin, which is in keeping with the suggestion that they can be precursors of pericytes and fibroblasts.

A further progenitor population may lie in the intima of vascular walls [32]. These cells have the potential to form a variety of cells including fibroblasts, smooth muscle and endothelial cells. However, little is known about the stimulus that controls cell differentiation or interrelation with circulating progenitor cells, and applicability of these concepts to the heart.

The origin of proliferating cardiac fibroblasts in pathological situations is, therefore, not established beyond doubt. Still, the fact that cardiac fibroblasts, isolated from saline-perfused hearts, proliferate vividly suggests that circulating progenitor cells are not an absolute requirement.

It should further be noted that fibroblasts are more than just collagen producing cells of the stroma. They have been termed "sentinel cells" that function as local immune modulators [31,33]. They may also contribute to cardiac electrophysiology (see recent editorials on this topic [34,35]), as discussed below. Thus, fibroblasts do more than proliferate and contribute collagen to the ECM. Their precise role in cardiac patho-physiology remains to be elucidated.

4. Fibroblast structural organization

4.1. Physiology

There are pronounced regional differences in the organization and content of connective tissue in the heart. Ventricular myocardium is arranged in highly oriented layers that are about two to four cells thick. These layers are embedded into a dense connective tissue network (Fig. 3A), and are interconnected by branches of myocytes [36,37] and fibroblasts [38].

SAN pacemaker tissue has a higher relative fibroblast content than ventricle, occupying some 45% to 75% of SAN volume in man [18,19]. Cells in the SAN, in particular in its central region, are less regularly organised than in ventricular tissue (Fig. 3B). Fibroblasts are found either interspersed with pacemaker myocytes, or forming islands consisting largely of connective tissue only. The age-related increase in SAN connective tissue coincides, in man, with a significant reduction in myocyte content [18,19]. These changes have been suggested to contribute to SAN dysfunction in the elderly [18].

In all cardiac tissue areas, fibroblasts form a complex 3-D network within the connective tissue matrix that they occupy [8,9]. There are abundant anatomical contacts, including extensive membrane appositions between neighbouring fibroblasts and myocytes. These have recently been shown to be site of homogeneous and heterogeneous gap-junctional coupling in rabbit right atrium [8].

4.2. Pathology

Fibroblast proliferation and tissue remodelling are features of many of cardiac pathologies, and their detailed discussion would go beyond the scope of this paper.

As an illustration of some key structural re-arrangements during pathological remodelling, we present findings from a recent study of sheep coronary occlusion, where fibroblast infiltration of the damaged tissue occurred within hours of myocardial infarction. Fibroblast density increased with time post-infarction, reaching a maximum after 1 week, followed by a subsequent decrease. Interestingly, fibroblast content increases even in remote tissue regions that are not directly affected by the infarct. Fig. 4 illustrates the characteristic appearance of viable tissue remote from the infarct zone, with a highly organized myocyte and fibroblast pattern (Fig. 4A), compared to the infarct border with a mix of disrupted and healthy myocytes and fibroblasts (Fig. 4B), and the central infarct zone that is densely packed with fibroblasts (Fig. 4C) at 1 week after infarction [39].

In general, pathologies cause fibroblast proliferation and mobilisation, supporting their spread into apparently unaffected tissue, as well as fibroblast phenotype changes (as reviewed elsewhere [5,25,40]). The potential contribution of fibroblasts to pathologically altered cardiac function is discussed below.

4.3. In vitro models

Standard in vitro cardiac cell culture models do not reproduce the in vivo structural organization of cardiac fibroblasts, or their interrelation with myocytes. Most cardiac cell cultures actually try to actively restrict fibroblast content.

Using advanced micro-structuring techniques it is, however, possible to create more in vivo-like models of cardiac tissue [41]. In particular, by growing cells on spatially restricted patterns, myocytes and fibroblasts can be prompted to acquire more in vivo-like phenotypes and spatial interrelation. Thus, using micro-fluidic deposition of ECM proteins to guide cell attachment on elastic mem-

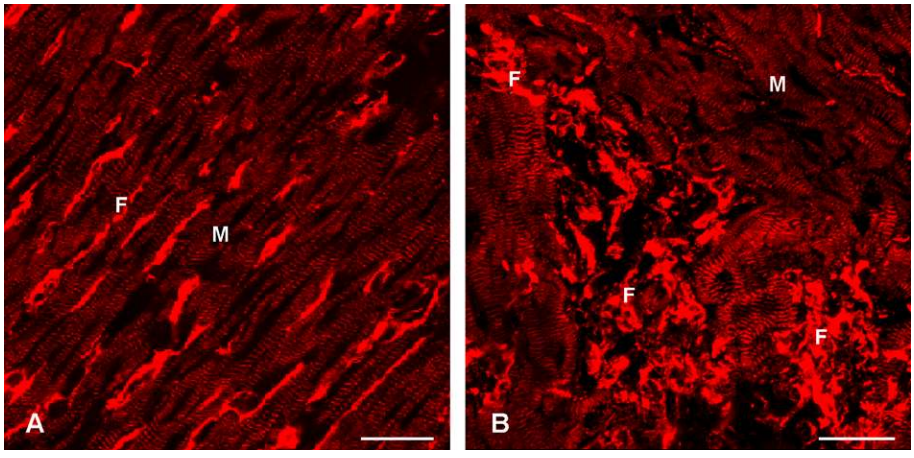

Fig. 3. Fibroblast organization in rabbit normal ventricular tissue (A) and rabbit SAN (B). Labelling for fibroblasts (anti-vimentin, brightly stained elongated cells) and myocytes (anti-myomesin, striated cells) identifies the spatial interrelation of cell types. Note that the SAN contains regions consisting almost exclusively of fibroblasts, while in normal ventricle, fibroblasts and myocytes are interspersed in a regular pattern. F: fibroblast; M: myocyte. Scale bars: 20 μm.

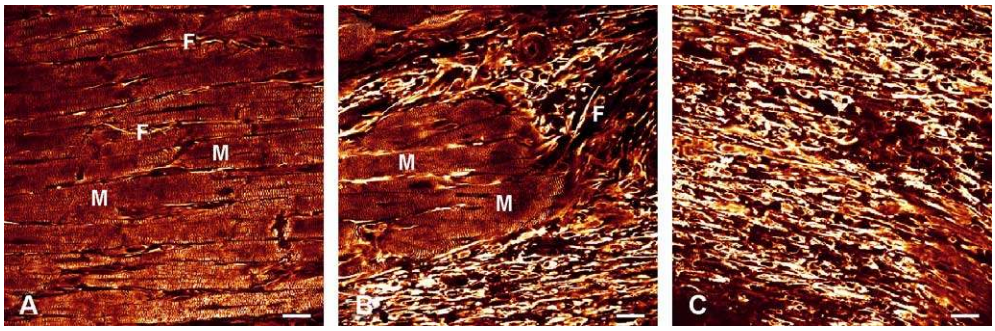

Fig. 4. Fibroblast organization in sheep normal ventricular myocardium (A), infarct border zone (B) and centre (C), 1 week after infarction. Fibroblasts were stained with anti-vimentin (F, bright elongated cells) and myocytes with anti-myomesin (M, striated cells). Scale bars: 20 μm.

branes, structured co-cultures of cardiomyocytes and fibroblasts have recently replicated important features of cardiac tissue-architecture [42]. Strands of aligned myocytes show cross striation that is 'in register' among neighbouring cells. These strands can be surrounded by parallel threads of fibroblasts, with abundant contacts between the cell types [38] (Fig. 5A), as seen in vivo.

More complex, three-dimensional cultures can be devised by the culturing of myocytes on spatially aligned collagen threads [43], where multiple layers of myocytes and fibroblasts organize in an in vivo-like manner (Fig. 5B).

These advanced in vitro models are still being fully characterized, but it is already evident that they will offer improved tools for studying cardiac fibroblast function in normal and pathological conditions.

5. Fibroblast function

Fibroblasts contribute to structural, biochemical, mechanical and electrical properties of the myocardium [4,8,39,40,44]. The following sections will address relevant aspects of their function.

5.1. Structural and biochemical function

Fibroblasts are involved in the maintenance of myocardial tissue structure, including ECM homeostasis and production of factors involved in maintaining a balance between synthesis and degradation of connective tissue components, for example cytokines, growth factors and matrix metalloproteinases (MMP).

In cardiovascular diseases, fibroblasts play a central and dynamic role in the myocardial remodelling process, which includes hypertrophy of cardiomyocytes, migration and proliferation of fibroblasts, and changes in the extent and composition of the cardiac ECM [5,40]. Excessive fibroblast proliferation and increase in ECM protein content (fibrosis) induce myocardial stiffening—an important patho-physiological facet of cardiac dysfunction [45,46]. Fibrotic tissue remodelling is associated with increased expression of MMP and humoral factors, such as transforming growth factor TGF-β, angiotensin II, endothelin-1 and tumour necrosis factor-α.

MMP, by degrading interstitial fibrillar collagen, act as key players in the ECM remodelling process after myocardial infarction, and in infarct healing [47]. In the early phase

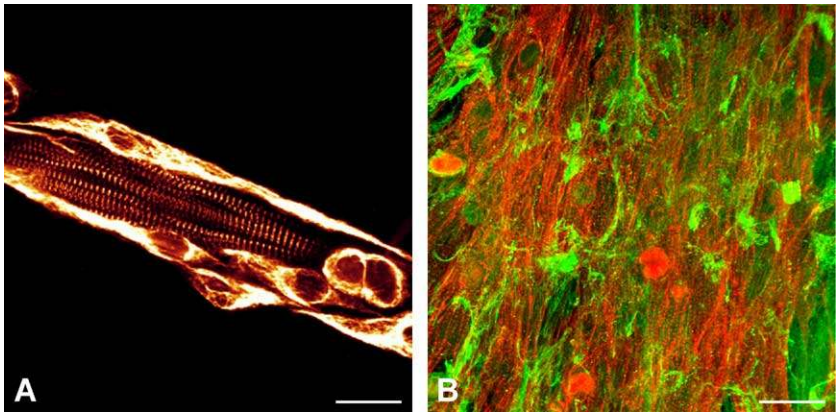

Fig. 5. In vitro cardiac cell culture models. (A) Structured co-cultures of neonatal rat fibroblasts (bright elongated cells, labelled with anti-vimentin antibodies) and myocytes (striated cells, labelled with anti-myomesin antibodies). (B) Three-dimensional co-cultures of neonatal myocytes (phalloidin-stained red striated cells) and fibroblasts (DDR2-stained, green). Fibroblast show long processes that envelope the myocytes, similar to in vivo cardiac tissue organization. Scale bar: 20 μm.

of myocardial repair, MMP disrupt the collagen ECM network. This enhances inflammatory cell infiltration that, by producing cytokines, promotes subsequent fibroblast migration, proliferation and differentiation, followed by deposition of new ECM and scar formation. Increased expression and activation of MMP leads to excessive ECM degradation, impairment of infarct healing and potentially cardiac rupture [47].

MMP are not exclusively expressed by fibroblasts, but also other cardiac cells (like myocytes and endothelial cells) and by inflammatory cells. They are also involved in the regulation of cell growth and migration, cell survival/death and angiogenesis. The details of MMP regulation in normal and pathological conditions go beyond the scope of this review, and we refer the reader to previous publications [47,48].

Angiotensin II, TGF-β and tumour necrosis factor-α are involved in autocrine and paracrine regulation of myocyte hypertrophy, fibroblast proliferation and ECM protein turnover [40]. Angiotensin II further stimulates collagen gene expression and collagen synthesis, and it reduces collagen degradation (by attenuating MMP activity in cardiac fibroblasts) [45], while endothelin-1 induces hypertrophy in myocytes and stimulates collagen synthesis [40].

Myocardial tissue remodelling may also be promoted by chronic adrenergic stimulation, which is an important feature of heart failure. This has been shown to affect not only myocyte hypertrophy and cell death [49], but also to increase proliferation of cultured human cardiac fibroblasts via secretion of autocrine factors [50].

Interestingly, statins, normally prescribed for lowering cholesterol, have recently been shown to directly inhibit fibroblast proliferation, an effect that may contribute to the prevention of adverse cardiac remodelling [51].

Thus, fibroblast activity may give rise to, at times unexpected, effects on cardiac structure and function. Given their susceptibility to a large range of humoral factors, they may foster a novel target for pharmacological interventions.

5.2. Mechanics

Cardiac tissue is continuously subjected to changes in segment length and tension. Many pathologies are accompanied by disturbances in the mechanical environment and myocyte contractility. These changes affect fibroblast structure and function.

The cardiac ECM is a stretch-sensitive network and is actively involved in the transduction of mechanical signals to cardiac cells. Fibroblasts sense mechanical stress via multiple pathways, including integrins, ion channels and second messenger responses. Stretch activates various signal transduction pathways that lead to cell proliferation, enhanced deposition of ECM protein and release of growth factors and MMP (see above). ECM protein synthesis may be indirectly induced by paracrine growth factors, or directly stimulated by an intracellular signalling pathway that activates ECM genes [52], like the mitogen-activated protein kinase-mediated pathway recently suggested to regulate procollagen gene expression in cultured cardiac fibroblasts [53].

Fibroblasts and myocytes appear to be closely interrelated in their response to mechanical stimulation, at least in vitro. Stretch of cultured cardiac myocytes and fibroblasts induces growth responses, mediated by factors released from the stretched cells [54]. For example, the stretch-induced release of growth factors from myocytes, cultured on elastic membranes, is sufficient to induce fibroblast responses that are normally associated with mechanical stimulation [55], including cell proliferation [4], collagen synthesis [56], and changes in gene expression [57]. In turn, fibroblasts co-cultured with myocytes can mediate the hypertrophic response of myocytes to stretch, by increasing endothelin or angiotensin II production [58].

Thus mechanically modulated myocyte–fibroblast cross-talk is important for myocyte growth [59], fibroblast proliferation and ECM turnover [60].

5.3. Electrical signalling

Fibroblasts are not only involved in structural aspects of tissue maintenance or remodelling, but they are also direct contributors to cardiac electrophysiological properties [61].

Fibroblasts can affect electrophysiology passively, for example by acting as obstacles to the orderly spread of electrical excitation (e.g. fibroblasts, separating groups of muscle cells, may reduce regional electrical coupling, causing slow or discontinuous conduction). Interstitial fibrosis and collagen accumulation are furthermore an important source of local anisotropy in myocardial ischaemia and hypertrophy, which in turn enhances predisposition to cardiac arrhythmogenesis [62,63].

The possibility that fibroblasts may *actively* contribute to cardiac electrophysiology has been considered only recently.

Cardiac fibroblasts are electrically ‘non-excitable’ cells (i.e. they do not respond to an electrical stimulus with generation of an action potential), but they are efficient mechano-electrical transducers [64]. Fibroblasts respond to mechanical stimuli, such as imposed by the contractile activity of the surrounding myocardium, or by external stretch, with changes in their membrane potential [61]. These mechano-sensitive potentials are attributed to stretch-activated ion channels, permeable to Na^+, K^+ and Ca^{2+} [65].

The mechanisms that link changes in fibroblast electrophysiology to cardiomyocyte activity are only beginning to emerge. One hypothesis involves direct gap-junctional coupling of fibroblasts and myocytes. Early studies, conducted using floating double-barrelled micro-electrodes inserted into isolated beating rat atrial tissue, suggested that there is both capacitive and electrotonic coupling of fibroblasts and myocytes [66].

After myocardial infarction, right atrial fibroblasts show several electrical abnormalities, such as a negative shift of

their resting membrane potential, an increase in membrane resistance, altered mechanically induced potentials, and enhanced sensitivity to mechanical stress [61]. These altered electrical properties of fibroblasts could, if electrically coupled to myocytes, lead to changes in pacemaker activity [67], such as the depression of heart rate observed in this model [61]. Furthermore, heterogeneous coupling of fibroblasts and myocytes in the infarct border zone would affect the spread of excitation in such areas and could contribute to the highly irregular and arrhythmogenic electrical properties of cardiac scar tissue [6,68].

Thus, fibroblasts may affect electrical signalling in the heart passively and, if electrically coupled to cardiomyocytes, actively. The extent of gap-junctional coupling of cardiac fibroblasts is discussed next.

6. Fibroblast gap-junctional coupling

6.1. Fibroblast–fibroblast coupling

To date, fibroblast gap junction coupling has mainly been studied in other organs, including skin, kidney, periodontal ligaments, gastrointestinal musculature and eye [69–72]. Cardiac fibroblast gap junctions have been investigated mainly in cell cultures, where they express connexin43 (Cx43) [73–75]. A contemporary study by Gaudesius et al. [76] identified both Cx43 and Cx45 between fibroblasts in neonatal rat heart cultures, and showed that fibroblasts are able to conduct electrical signals over extensive distances (up to 300 μm) in vitro. Junctions between fibroblasts tend to be much smaller than those involving cardiomyocytes.

This is in keeping with electron microscopy of serial sections of rabbit SAN, which revealed that fibroblasts are regularly coupled by small gap junctions [77].

More recent work has focussed on in vivo fibroblast–fibroblast structural and functional coupling in rabbit SAN, using a combination of cell type identification, connexin labelling, and *Lucifer yellow* scrape loading techniques [8,38]. Cardiac fibroblasts have been shown to express Cx40 and Cx45, but not Cx43, in rabbit SAN. Fibroblasts are coupled via Cx40 in fibroblast-rich areas devoid of myocytes (Fig. 6A,B), and by Cx45 in regions of the node where fibroblasts intermingle with myocytes (Fig. 6C,D). Thus, there are two spatially distinct fibroblast populations in the SAN, which express different connexins. Scrape

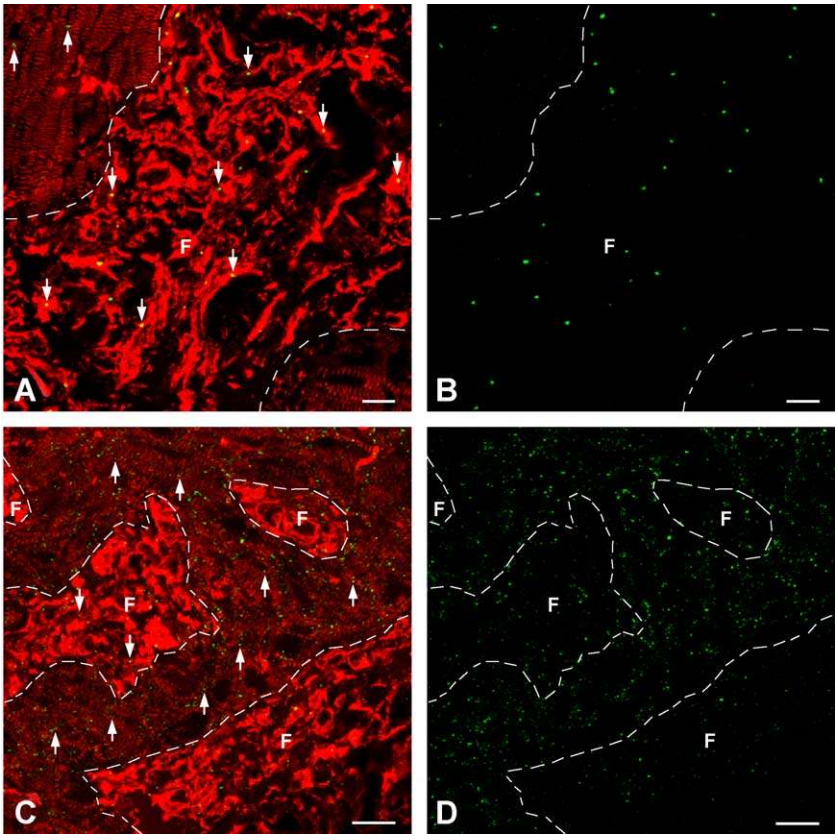

Fig. 6. Gap junctions and cell types in rabbit SAN. (A and B) Connexin40 (green dots, arrows) expression pattern. (A) Triple labelling for connexin40, myocytes and fibroblasts, showing that fibroblasts (F) express connexin40 in fibroblast-rich areas devoid of myocytes. (B) Connexin40 labelling from panel A, relative to areas of preferential cell content. Connexin40 is predominantly localized in fibroblast rich regions (F). (C and D) Connexin45 (green dots, arrows) expression pattern. (C) Fibroblasts express connexin45 in regions where they intermingle with myocytes. (D) Connexin45 labelling from panel C, relative to areas of preferential cell content. Connexin45 is present in fibroblast-only (F), but more prominently in fibroblast–myocyte mixed tissue. Therefore, SAN fibroblasts express multiple connexin isoforms in spatially distinct patterns. Scale bars: 20 μm. Modified from Ref. [8], with permission.

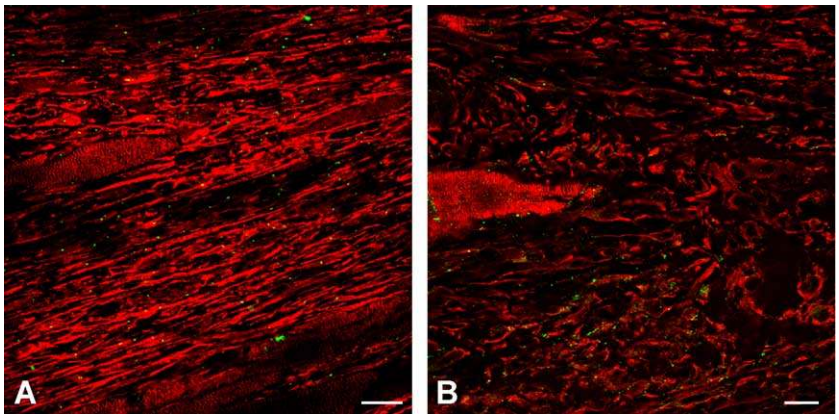

Fig. 7. Fibroblasts and gap junctions in sheep infarct tissue. (A) Connexin45-expressing fibroblasts in 1 week old infarct (green dots: connexin45). (B) Connexin43-expressing fibroblasts in 2 weeks old infarct (green dots: connexin43). Fibroblasts in the infarct express multiple connexins isoforms in spatially and temporally distinct patterns. Scale bars: 20 μm.

loading of *Lucifer yellow*, a gap junction permeable dye, revealed extensive threads of labelled fibroblasts, confirming their functional coupling [8].

Additional research into in vivo fibroblast gap junction expression and coupling during pathological conditions revealed that fibroblasts, in sheep infarct ventricular tissue, express Cx45 (Fig. 7A) or Cx43 (Fig. 7B), but not Cx40, again with spatially and temporally distinct patterns [39]. Cx45-expressing fibroblasts appear in the damaged tissue within a few hours after infarction and reach their peak density after 1 week. Cx43-expressing fibroblasts emerge later and their numbers continue to rise until at least 4 weeks after infarction. Both Cx43 and Cx45 are clearly involved in fibroblast coupling, although with different dynamics during progressive myocardial infarction [39].

Such coupling could contribute to several processes, including electrical, chemical and mechanical signalling. The dynamic nature of these signals could be critical for normal homeostasis, as well as for the response to injury. The highly coupled fibroblast network in the diseased myocardium could also be involved in the pronounced inflammatory response and lesion expansion that occur immediately after myocardial infarction and continue for days and weeks post-infarction [39].

Thus, cardiac fibroblasts form a complex network of cells, interconnected by different gap junctions, depending on tissue region and patho-physiological state.

6.2. Fibroblast–myocyte coupling

Cultured cardiac myocytes and fibroblasts readily form functional gap junctions (see Fig. 8A) [66,73]. Single gap-junctional channels in such fibroblast–myocyte cell pairs have a conductance of 29pS [73], a value close to that of Cx45. These heterogeneous junctions support synchronization of spontaneous activity in distant cardiac myocytes,

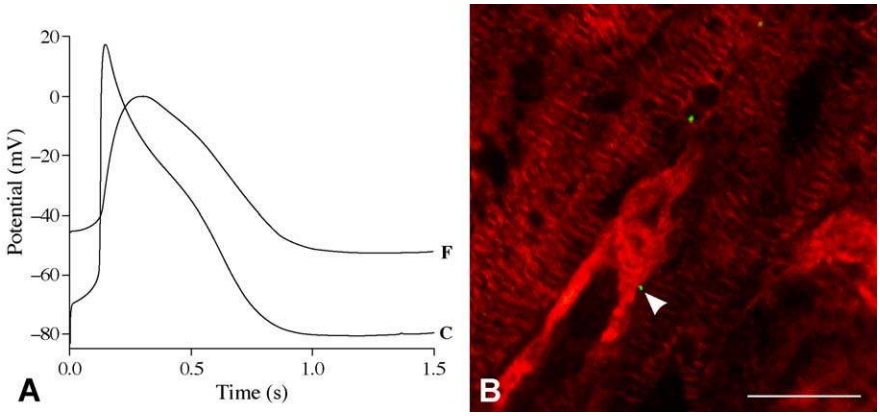

Fig. 8. Myocyte–fibroblast functional (in vitro) and structural coupling (in vivo). (A) Neonatal myocyte–fibroblast pair in cell culture: the action potential of the cardiomyocyte (C) affects the membrane potential in an electrically coupled neighbouring fibroblast (F). Given the fibroblast's high membrane resistance, this impulse can be transmitted to other cells and, potentially, serve as a trigger for excitation in distant cardiomyocytes. From Ref. [66] with permission. (B) Confocal image of rabbit sino-atrial node, immuno-histochemically stained for myocytes (red striated cells), fibroblasts (brightly stained solid red cells) and connexin45 (green dots). One of the connexin45 spots in the given optical plane is located at the point of contact of a myocyte and a fibroblast (arrow). Scale bar: 10 μm. From front cover of Ref. [8], with permission.

interconnected by fibroblasts only [76,78]. This behaviour is promoted by the fact that fibroblasts have a very high membrane resistance, making them excellent long-distance conductors [35].

In addition, cultured cardiac fibroblasts may act as a 'current sink' for connected myocytes. Thus, transfection of fibroblasts with the voltage-sensitive potassium channel Kv1.3 changes cardiomyocyte excitability in neonatal rat heart culture, illustrating the pronounced effect of electrically coupled fibroblasts on cardiomyocyte electrophysiology in vitro [79].

Immuno-histochemical identification of connexins in co-cultures of cardiac fibroblasts and myocytes revealed Cx43 and Cx45 at points of heterogeneous cell contact. These junctions are generally considerably smaller than cardiomyocyte nexus [76].

In vitro evidence therefore suggests that myocytes and fibroblasts are able to form functional gap junctions, which could underlie the ability of fibroblasts to serve as conductors for electrical excitation and to affect myocyte electrophysiology.

Functional evidence of myocyte–fibroblast coupling in vivo is more difficult to obtain, since cell types need to be distinguished on the basis of electrophysiological recordings (no optical control). This is easy for cardiomyocytes, and in the case of fibroblasts that are not (or only very weakly) coupled to adjacent cardiomyocytes. Unfortunately, these fibroblasts do not form the object of interest in the given context. Fibroblasts that are electrically well-coupled to cardiomyocytes mimic the electrophysiological behaviour of the latter cells (because of the high membrane resistance of fibroblasts, see Fig. 8A for illustration). Myocyte-coupled fibroblasts are, thus, difficult to identify by electrophysiological means [66], and related studies have remained inconclusive regarding the presence of heterogeneous fibroblast myocyte coupling in vivo.

Investigations of native tissue by transmission electron microscopy (TEM) yielded only one tiny "gap junction-like contact" between a myocyte and a fibroblast in rabbit SAN [77], while extended areas of close myocyte–fibroblast membrane appositions were regularly observed. It is possible that tissue preparation for TEM (involving fixation, dehydration, transversal cutting of thin sections, etc.) does not favour the identification of the comparably small heterogeneous connections, which may furthermore lack some elements of mechanical and structural support commonly present in myocyte nexus. Also, heterogeneous cell coupling could occur via dispersed gap-junctional channels that do not form a sufficiently electron-dense substrate for TEM detection (as reported for pig coronary arteries [80]).

More recently, the structural and functional interrelation of fibroblasts and myocytes was studied in native tissue, using immuno-histochemical techniques. Identification of gap-junctional coupling was combined with positive identification of coupled cell-types in rabbit SAN. Preparations were cut in the plane of the node to expose large areas of intact tissue. This revealed that fibroblast–myocyte coupling occurs in regions of the SAN where both cell types express Cx45. Here, 10% of the total Cx45 was located at points of fibroblast–myocyte contact (Fig. 8B). Interestingly, it is assumed that immuno-histochemically identifiable gap junctions should contain 75 to 100 individual channels—an aggregation of electron-dense material that one would normally expect to be able to identify in TEM. The discrepancy in the related observations is, at present, unresolved. The functionality of the heterogeneous gap junctions in rabbit SAN was confirmed via *Lucifer yellow* dye transfer, which revealed coupling of heterogeneous fibroblast/myocyte cells strands [8].

Thus, gap-junctional coupling in the heart is not restricted to cardiomyocytes. Fibroblasts show homo- and heterogeneous coupling, which would allow them to play an active role in cardiac electrophysiology.

7. Relevance and potential

Cardiac fibroblasts are important determinants of both structure and function of the myocardium. They contribute to structural, biochemical, mechanical and electrical characteristics of cardiac function [4,5,44].

Connective tissue and fibroblasts form a structural support-framework for myocytes and blood vessels, guiding cardiac tissue cytoarchitecture. In the normal myocardium, fibroblasts are largely quiescent cells, responsible for homeostasis of ECM. In diseased myocardium, fibroblasts are major players of cardiac remodelling, including fibroblast proliferation, migration, increased ECM turnover and enhanced release of humoral factors [44]. Release of cytokines and growth factors has both auto- and paracrine effects on the activity and phenotype of myocytes and fibroblasts.

In normal myocardium, fibroblasts form a highly coupled network [8] that may contribute to metabolite regulation, waste removal and biochemical signalling, similar perhaps to astrocytes in the nervous system [81]. Fibroblasts also form a coupled network in infarct tissue [39], where they may play a role in nutrient transfer, metabolite regulation, inflammatory responses, infarct lesion spread, and myocardial remodelling [82]. Direct fibroblast–fibroblast and fibroblast–myocyte coupling may provide a substrate for a bystander effect, where fibroblasts pass information, including 'death signals', from infarcted myocytes to the surrounding tissue, again potentially similar to the spread of neuronal damage via coupled astrocytes [83].

Connective tissue and fibroblasts have been generally considered as an obstacle to electrical excitation, contributing to discontinuous conduction and arrhythmogenesis [62,84]. Recent evidence suggests, however, that fibroblasts may, in addition, act as a substrate for electrical coupling of cardiomyocytes.

In cell culture, fibroblasts are known to form gap junctions with myocytes [73,74], influencing their electrophysiology and providing a substrate for electrical conduction between separated myocytes over extended distances [76]. In vivo, fibroblasts are structurally and functionally coupled to myocytes via gap junctions, at least in the rabbit SAN [8]. Fibroblasts could, therefore, be involved in short-range impulse conduction in the heart, by interconnecting groups of SAN cells, or potentially layers of myocardial tissue in the ventricular wall. Furthermore, fibroblasts may contribute to long-range conduction, such as in the case of synchronization of electrical activity in recipient and donor myocardium across transplantation scar tissue, or by connecting islands of surviving myocytes inside an infarct with surrounding healthy myocardium. As an in-between effect, fibroblast–myocyte coupling in patho-physiological conditions may cause electrotonic depression of viable tissue by the bulk of fibroblasts of the ischaemic zone (fibroblasts acting as a current sink), contributing to slowed conduction or block of excitation [85].

Computational models predict that, in addition to effects on excitation and conduction [6], fibroblast mechano-sensitivity may provide an alternative mechano-electric transducer and contribute to cardiac mechano-electric feedback [6,68]. Being located outside the contractile machinery (myocytes), fibroblasts would offer a different modality of mechano-receptive signals and, by direct gap-junctional coupling with other fibroblasts and/or adjacent myocytes, have the potential to affect electrophysiology both in the context of physiological feedback or patho-physiological responses, including arrhythmogenesis [85].

In conclusion, fibroblasts are perhaps the most under-estimated cell population in the heart. They contribute to cardiac development, structure, and function. They are sources and targets of bio-chemical and electro-mechanical signalling pathways. Future research focussing on cardiac fibroblasts will be required to improve our understanding of cardiac function in normal and patho-physiological states.

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