Functional crosstalk between cardiac fibroblasts and adult cardiomyocytes by soluble mediators

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Received 14 September 2014; revised 6 December 2014; accepted 10 December 2014; online publish-ahead-of-print 5 January 2015

Time for primary review: 35 days

Aims
Crosstalk between cardiomyocytes and fibroblasts in physiological conditions and during disease remains poorly defined. Previous studies have shown that fibroblasts and myocytes interact via paracrine communication, but several experimental confounding factors, including the use of immature myocytes and the induction of alpha-smooth muscle actin (α-SMA) expression in fibroblasts by prolonged culture, have hindered our understanding of this phenomenon. We hypothesize that fibroblasts and myofibroblasts differentially affect cardiomyocytes viability, volume, and Ca²⁺ handling via soluble mediators. More specifically here: (i) we compare the effects of freshly isolated fibroblasts and cultured fibroblasts from normal rat hearts on adult cardiomyocytes; (ii) we compare the effects of (freshly isolated) normal fibroblasts and myofibroblasts from pressure-overloaded hearts; and (iii) we study the contribution of TGF-β and the importance of the crosstalk between the two cell types.

Methods and results
We used co-culture methods and conditioned medium to investigate paracrine interaction between fibroblasts and cardiomyocytes. All fibroblast types reduce cardiomyocyte viability and increase cardiomyocyte volume but α-SMA-negative fibroblasts increase cardiomyocyte Ca²⁺ transient amplitude, whereas cultured fibroblasts and myofibroblasts from pressure-overloaded hearts decrease Ca²⁺ transient amplitude. In turn, cardiomyocytes release soluble mediators that affect fibroblast proliferation. Using SB431542 to block TGF-β type 1 receptors, we determined that TGF-β directly causes cardiomyocyte hypertrophy and participates in bi-directional regulatory signalling between fibroblasts and cardiomyocytes.

Conclusions
Fibroblasts have different roles during physiology and disease in regulating myocardial function via soluble mediators. A crosstalk between fibroblasts and cardiomyocytes, controlled by TGF-β, is crucial in this interaction.

Keywords
Fibroblasts ● Paracrine communication ● Calcium handling ● TGF-β

1. Introduction
‘Fibroblasts’ are numerous within the healthy myocardium and are essential in the regulation of the extracellular matrix to maintain correct functioning of the heart.¹² In cardiac pathology, fibroblasts take on an activated phenotype characterized by the expression of alpha-smooth muscle actin (α-SMA) and associated with the development and maintenance of myocardial fibrosis, and are termed ‘myofibroblasts’.³ Growing evidence shows that fibroblasts and myofibroblasts actively modulate the structure and function of cardiomyocytes through direct cell contact, indirectly via the extracellular matrix, and by the release of soluble mediators.⁴⁵. The latter modality, involving a paracrine interaction, has been widely postulated, with several studies showing that conditioned medium obtained from fibroblast cultures affects cardiomyocyte biology in vitro; effects observed include neonatal cardiomyocyte hypertrophy and detrimental effects on cardiomyocyte contractility and electrophysiology.⁶–¹⁰ These findings suggest novel pathways of physiological and pathophysiological modulation of myocardial performance, particularly relevant for cardiac mechanosensitivity and electrophysiology. However, several important details...
are still missing and the precise nature of the fibroblast–cardiomyocyte interaction through soluble mediators remains unknown.

An important confounding factor is that previous studies into paracrine communication have been performed using fibroblasts isolated from normal hearts and activated to express α-SMA by culture to simulate the pathological phenotype. However, cultured fibroblasts are likely to be distinct from both normal fibroblasts and myofibroblasts and, despite the expression of α-SMA, it is unclear whether they offer a reliable representation of myofibroblasts from pathological specimens. Furthermore, most studies have used neonatal cardiomyocytes, with immature characteristics in gene expression, structure, and function, and different responses to paracrine signals compared with adult cardiomyocytes. For these reasons, the applicability of the current knowledge of fibroblast–cardiomyocyte interaction through soluble mediators to the physiology and pathophysiology in the adult heart remains limited.

The identification of the soluble mediators involved in the paracrine interaction is another important, but poorly defined aspect. The network of mediators is likely complex as both fibroblasts and cardiomyocytes can release and respond to a variety of substances. Various mediators, including TGF-β, angiotensin II, IL-6, and endothelin-1, have been linked to the paracrine effects of cultured fibroblasts. TGF-β may particularly be important, as it is readily released by fibroblasts; several studies have shown that cultured fibroblast-derived or exogenous TGF-β induces neonatal cardiomyocyte hypertrophy and affects cardiomyocyte electrophysiology and contractility.

Fibroblasts are not only a source but also a target for TGF-β as this molecule is the best-known stimulus for myofibroblast differentiation as well as a signal for the release of downstream mediators. Given the local release of TGF-β from both fibroblasts and cardiomyocytes, and the ability of both cell types to respond to this mediator, it is possible to hypothesize that TGF-β is involved in regulatory crosstalk between the two cell types, an aspect that has not been properly investigated.

Here, we tested the hypothesis that fibroblasts and myofibroblasts differentially affect adult cardiomyocyte viability, volume, and Ca2+ handling via soluble mediators. More specifically, (i) we compare the effects of freshly isolated fibroblasts and cultured fibroblasts from normal rat hearts on adult rat cardiomyocytes; (ii) we compare the effects of (freshly isolated) normal fibroblasts and myofibroblasts from pressure-overloaded hearts; and (iii) we study the contribution of TGF-β using specific co-culture methods and conditioned medium to determine whether it acts via direct effects on cardiomyocytes or through downstream mediators.

2. Methods

Animal procedures were approved by the Harefield Heart Science Centre Local Ethical Review panel, were performed according to the UK Home Office regulations, and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.1 Animal models

A 10-week model of pressure overload in Lewis rats (180–200 g; Harlan UK) was the source of myofibroblasts. About 1.5–5% isofluorane in O2 was used for anaesthesia. A combination of 10–15 mg/kg amoxicillin trihydrate (Clamoxyl LA, Pfizer, New York, NY, USA) and 10–20 μg/kg buprenorphine hydrochloride (Vetergesic, Reckitt & Colman, UK) were used as prophylactic antibiotics and for pain relief. Pressure overload of the left ventricle was induced by thoracic aortic constriction (TAC), as previously described. The ascending aorta was constricted to the size of a cannula with a 0.9 mm external diameter. Sham-operated animals were produced by performing identical surgery except for constricting the aorta. At least three animals were used per group for each experiment.

2.2 Cell isolation

Rats were sacrificed by cervical dislocation after anaesthesia with 5% isoflurane. Myocytes were isolated by enzymatic digestion of the left ventricle and kept in buffer solution [20 NaCl, 5.4 KCl, 5 MgSO4, 5 NaHCO3, 20 glucose, 20 taurine, 10 HEPES (free acid), and 0.2 CaCl2; pH 7.4] until used when they were transferred to culture medium. Myocytes were put into the experimental set-ups within 2 h of isolation. For fibroblast isolation, the left ventricle was digested as described previously. Fibroblasts were collected from the cell suspension by spinning at 620 g for 4 min. Fibroblasts were plated in culture in maintenance medium (DMEM, 15% fetal bovine serum, 4 mM l-arginine, 100 U/mL penicillin, and 100 μg/mL streptomycin). The medium was changed after 2 h to remove unattached cells and leave a highly pure population of fibroblasts. For cultured fibroblasts, the cells were cultured for 2 weeks and used between passages 2–5, at which point they express α-SMA. Freshly isolated fibroblasts and myofibroblasts were kept in maintenance medium for 20 h before being used.

2.3 Immunofluorescence

IF of isolated, fixed fibroblasts was used to characterize α-SMA development in culture. Cells were fixed with ice-cold methanol for 5 min. Monoclonal Mouse Anti-Human (Dako, Denmark) primary antibody and goat anti-mouse Alexa488 (Molecular Probes, Eugene, OR, USA) secondary antibody were used. Nuclei were marked with propidium iodide. Cells were isolated from three hearts and five images per isolation were recorded. The number of α-SMA-positive cells was calculated as a percentage of total cells.

2.4 Co-culture and conditioned medium

The co-culture used Transwells with 0.4 μm pores (Corning, Inc., Corning, NY, USA) to physically separate the cells within the same medium, allowing paracrine communication but preventing direct cell contact. Fibroblasts were collected from culture using 0.25% Trypsin–EDTA and plated in 6.5 mm Transwells in 200 μL of medium. These were left for 4 h to settle and recover from the trypsin exposure before use. The number of fibroblasts and medium used are detailed below.

Cardiomyocytes were transferred from buffer solution to medium and plated at a density of 5000 cells/dish on laminin (Sigma-Aldrich, UK)-coated glass bottom dishes (MatTek, Ashland, MA, USA). The myocytes were left to attach for 5 min and then 2 mL of medium was added. Thirty minutes later, this was replaced with 2 mL of fresh medium at which point the myocytes were ready to use. The Transwells containing the fibroblasts were suspended over the myocytes in the glass bottom dish, so that the base of the Transwell sat within the culture medium on the myocytes but did not touch the base of the dish. As a control, cardiomyocytes were co-cultured with 5000 further cardiomyocytes in the Transwells. The co-cultures were incubated at 37°C and 5% CO2 for 24 h before analysis.

Initial experiments with cultured fibroblasts used 20 000 cultured fibroblasts per set-up and serum-supplemented medium for both the Transwell and glass bottom dish (67% DMEM, 16% medium-199, 10% horse serum, 4% fetal bovine serum, 20 mM HEPES, 100 U/mL of penicillin, and 100 μg/mL of streptomycin). Experiments with freshly isolated fibroblasts and myofibroblasts used 10 000 cells per set-up and ITS medium for both the Transwell and glass bottom dish (medium-199, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 1:1000 dilution ITS supplement (PAA, Austria; Cat. No. F01-015)). The details of the parameters for each set-up used are summarized in Supplementary material online, Table S1.
Conditioned medium was produced using the same set-ups, but excluding one cell type. After 24 h, the medium was collected and frozen until required. Target cells were then exposed to conditioned medium for 24 h.

To investigate the effects of TGF-β, 10 μM SB431542 (Sigma-Aldrich, UK) was added to the co-culture or conditioned medium for the 24 h of co-culture.

2.5 Cardiomyocyte viability and structure

The viability of cardiomyocytes was assessed by cell morphology. Cells in the field of view were counted as either rod shaped (normal) or abnormally shaped (hypercontracted, considered non-viable). Abnormally shaped cardiomyocytes were not used for measurements of volume, transverse tubule (t-tubule) structure, or Ca\(^{2+}\) handling.

To measure changes in cell volume and t-tubule structure after co-culture, cells were incubated with 10 μM di-8-ANEPPS for 10 min and measured as previously described. Non-co-cultured cells from the same animals were used as controls to account for baseline variation in cell volume and structure.

2.6 Myocyte Ca\(^{2+}\) transients and sarcoplasmic reticulum Ca\(^{2+}\) content

To assess stimulated Ca\(^{2+}\) transients, cardiomyocytes were stained with the Ca\(^{2+}\)-sensitive dye Fluo-4-AM (5 μM for 10 min) and field-stimulated at 1 Hz. Ca\(^{2+}\) transients were recorded with repeat line scan images with a Zeiss Axiocam microscope on a LSM-510 laser scanning confocal module (Zeiss, Germany). The images were analysed using the MATLAB R2006b software (MathWorks, Inc., Natick, MA, USA).

Sarcoplasmic reticulum (SR) Ca\(^{2+}\) content was assessed with Indo-1 AM (Invitrogen), and measuring the rise in cytoplasmic Ca\(^{2+}\) in response to the rapid application of caffeine.

2.7 Cultured fibroblast cell number and cytotoxicity

In this experiment, 5000 cultured fibroblasts were cultured in 100 μL of cardiomyocyte-conditioned medium or normal medium for 48 h. The number of cells was analysed using the CellTiter 96® AQueous proliferation assay (Promega, Madison, WI, USA), and cytotoxicity was analysed using the CellTiter 96® AQueous cytotoxicity assay (Promega). Ideally, these experiments would use freshly isolated fibroblasts/myofibroblasts, but due to requirement of 48 h in culture for the experiment this was not possible and cultured fibroblasts had to be used.

2.8 Statistical analysis

Statistical analysis used a Mann–Whitney test for two data sets and Kruskal–Wallis test for multiple groups of data. The analysis was carried out with the Prism4 software (GraphPad Software, San Diego, CA, USA). Data are represented as mean ± S.E.M. and a value of P < 0.05 was taken as significant. N represents the number of individual cells with the number of cultures in brackets, except for viability, ELISA data, and fibroblast cell number and cytotoxicity where N represents the number of experimental set-ups. *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1 Differential effects of cultured fibroblasts and freshly isolated fibroblasts on cardiomyocyte viability, size, and Ca\(^{2+}\) transients

In an initial experiment, we co-cultured 20 000 cultured fibroblasts with 5000 adult rat ventricular cardiomyocytes. After 24 h, cardiomyocyte viability was reduced in co-culture and the viable cardiomyocytes were larger than control (see Supplementary material online, Figure S1). Cardiomyocyte Ca\(^{2+}\) transient amplitude was reduced without effects on the time to peak and time to decay of the transient (Figure 1A–D). T-tubule density was unaffected (see Supplementary material online, Figure S1). SR Ca\(^{2+}\) content was reduced (see Supplementary material online, Figure S1) after co-culture and may underlie the reduced Ca\(^{2+}\) transient amplitude.

As the number of freshly isolated fibroblasts and myofibroblasts available was less, we tested the effect of different numbers of cultured fibroblasts and ITS (serum-free) medium. Similar results on Ca\(^{2+}\) transient amplitude were obtained using different culture media and 10 000, 20 000, and 40 000 cultured fibroblasts.

We then determined that, 48 h after isolation, fibroblasts are still α-SMA-negative, despite developing α-SMA over 7 days in culture as previously shown21,22 (see Supplementary material online, Figure S2), and we performed a new series of co-culture experiments with cardiomyocytes. As observed with cultured fibroblasts, co-culture with α-SMA-negative fibroblasts reduced the viability of cardiomyocytes and increased the volume of the remaining cardiomyocytes (see Supplementary material online, Figure S3). The effect of freshly isolated α-SMA-negative fibroblasts on the cardiomyocyte Ca\(^{2+}\) transient was different from cultured fibroblasts with a resultant increase in the Ca\(^{2+}\) transient amplitude (Figure 1E and F). There were no changes in the time course of the transient (Figure 1G and H), and t-tubule density was unaffected (see Supplementary material online, Figure S3).

3.2 Differential effects of freshly isolated normal fibroblasts and myofibroblasts from pressure-overloaded hearts on cardiomyocyte Ca\(^{2+}\) transients, viability, and size

We then compared the effects of myofibroblasts isolated from the heart of a 10-week model of pressure-overload (TAC) with fibroblasts isolated from the heart of control animals. Thoracic aortic constriction induced a compensated hypertrophy, characterized by an increased ejection fraction measured by echocardiography and an increase in the left ventricle to body weight ratio. IF of the isolated fibroblasts from the TAC model showed α-SMA expression after 48 h in culture, and are therefore, by definition, myofibroblasts (Figure 2A).

Freshly isolated fibroblasts from normal hearts also increased Ca\(^{2+}\) transient amplitude, whereas myofibroblasts from TAC heart reduced Ca\(^{2+}\) transient amplitude (Figure 2B and C). The time to peak was unaffected, but the time to decay was reduced in both co-cultures (Figure 2D and E). Both fibroblasts and myofibroblasts reduced the viability (Figure 2G) and increased the volume of the cardiomyocytes to similar values (Figure 2F–H) with no effects on the t-tubule density (data not shown).

3.3 Blockade of the TGF-β type 1 receptor prevented the effects of co-culture on cardiomyocytes but not all the effects of conditioned medium

TGF-β1 was measured in the media collected from the different experimental conditions. TGF-β1 was increased in both fibroblast–cardiomyocyte and myofibroblast–cardiomyocyte co-culture media compared with cardiomyocyte-only culture, and was higher in the fibroblast group compared with the myofibroblast group (Figure 3).
TGF-β1 level measured in the fibroblast–cardiomyocyte co-culture medium was significantly higher than that measured in the medium collected from a culture of only fibroblasts (Figure 3). No difference was found between TGF-β1 levels measured in media from myofibroblasts either in co-culture or on their own (Figure 3).

When added to the co-culture media, the TGF-β type 1 receptor antagonist, SB431542, blocked the increase in cardiomyocyte Ca²⁺ transient amplitude in the fibroblast–cardiomyocyte co-cultures and the decrease in Ca²⁺ transient amplitude in the myofibroblast–cardiomyocyte co-cultures, although it did not affect the increased speed of the Ca²⁺ transient decay in either group (Figure 4A–D). In addition, SB431542 reduced the time to peak in the fibroblast co-culture group (Figure 4C). It also prevented the loss in cell viability (Figure 4F) and the increase in cardiomyocyte volume in both fibroblast and myofibroblast co-cultures (Figure 4E and G). T-tubule density was not changed in any group (data not shown).

Blocking TGF-β type 1 receptors in co-culture does not differentiate between direct effects of TGF-β on the cardiomyocytes and indirect effects through mediators released from fibroblasts or myofibroblasts, downstream of TGF-β signalling. Therefore, we added SB431542 to fibroblast or myofibroblast-conditioned medium to target specifically TGF-β secreted by fibroblasts and acting on cardiomyocytes.
No changes in the cardiomyocyte Ca^{2+} transient were observed with fibroblast or myofibroblast-conditioned medium (Figure 5A–D). However, addition of SB431542 to myofibroblast-conditioned medium increased the time to peak compared with myocyte-only culture (Figure 5C). Both fibroblast- and myofibroblast-conditioned media reduced the viability of cardiomyocytes, but unlike in co-culture, this effect was not significantly affected by SB431542 (Figure 5F). Only myofibroblast-conditioned medium induced cardiomyocyte hypertrophy, and this was blocked by the addition of SB431542 (Figure 5E–G). T-tubule density was unchanged in any group (data not shown).
Fibroblast–myocyte paracrine interaction

The role of a direct effect of TGF-β was tested by adding TGF-β directly to myocytes in culture. At 10, 50, and 100 pg/mL, levels similar to those measured in our experiments, there were no effects on the Ca²⁺ transients (see Supplementary material online, Figure S4). Upon repeating the experiment, increasing the level of TGF-β using 0.1, 1, and 10 ng/mL, the time to decay of the Ca²⁺ transient was increased by 1 and 10 ng/mL of TGF-β, but time to peak and amplitude were unaffected (see Supplementary material online, Figure S4).

3.4 Cardiomyocyte-derived paracrine mediators affect cultured fibroblast proliferation

Owing to the observed differences between the effects of co-culture and conditioned medium, we investigated whether adult cardiomyocytes release soluble mediators that could affect fibroblasts and may be involved in the dynamic regulation of the paracrine interaction. Cardiomyocyte-conditioned medium increased the number of cultured fibroblasts after 24 h (Figure 6A), despite an increase in cell death (Figure 6B), which is consistent with an increase in cultured fibroblast proliferation.

4. Discussion

The results presented here show that α-SMA-negative fibroblasts affect adult cardiomyocytes when co-cultured in a system which only allows paracrine communication, and that some of these effects are different when using cultured fibroblasts or myofibroblasts isolated from pressure-overloaded rat hearts. Furthermore, the consequences on cardiomyocyte structure and function of co-culturing with fibroblasts were different from those obtained using fibroblast-conditioned medium alone, suggesting a dynamic paracrine relationship between co-cultured fibroblasts and myocytes. By using freshly isolated fibroblasts/myofibroblasts and adult cardiomyocytes, this study aimed to overcome problems associated with using neonatal cardiomyocytes as well as fibroblasts isolated from normal hearts and activated by culture as in other studies.\(^\text{9,10,26,27}\) Previously, a single study has compared fibroblasts and myofibroblasts, although these were kept in long-term culture\(^\text{6}\) and two studies have used adult cardiomyocytes.\(^\text{8,28}\) This study compared the effects of freshly isolated fibroblasts and myofibroblasts on adult cardiomyocyte viability, size, and Ca²⁺ transients.

The most regularly reported effect of cultured fibroblast-derived mediators on neonatal cardiomyocytes is cardiomyocyte hypertrophy.\(^\text{9,10,27,29,30}\) and this study has demonstrated that this effect is also evident with adult cardiomyocytes. We have shown that this hypertrophic response also results from co-culture with α-SMA-negative fibroblast and myofibroblasts and with myofibroblast-conditioned medium. Cardiomyocyte volume was not different from freshly isolated cardiomyocytes in either serum-supplemented or ITS medium, suggesting that growth of myocytes when exposed to fibroblasts/myofibroblasts is not caused by culture medium factors. TGF-β has previously been linked to paracrine induction of hypertrophy,\(^\text{14}\) and TGF-β was responsible for the hypertrophic response observed here. Fibroblast-conditioned medium did not induce hypertrophy, potentially due to lower levels of TGF-β in the conditioned medium compared with co-culture. This also suggests that, in co-culture, signals from the cardiomyocytes may trigger increased TGF-β release from the fibroblasts.

We demonstrated that cardiomyocytes signal to fibroblasts via soluble mediators, leading to increased fibroblast proliferation. A different proliferative capacity of the fibroblasts and myofibroblasts resulting in different cell numbers could cause the different effects observed. However, this is unlikely to explain the results as we have shown that a wide variation of fibroblast number does not produce significant differences in the Ca²⁺ cycling response (see Results). The identity of the signal arising from the myocytes has not been elucidated, but previous studies have shown that cardiomyocyte-derived angiotensin II can increase TGF-β release from fibroblasts,\(^\text{31}\) and other signals including vascular endothelium growth factor and ATP signal from cardiomyocytes to fibroblasts.\(^\text{2,3}\) This dynamic interaction between cardiomyocytes and fibroblasts has been highlighted previously and demonstrates the importance of co-culture rather than relying on conditioned medium to understand the effects of paracrine communication.\(^\text{26}\)

All fibroblast types tested here reduced the viability of cardiomyocytes, either in co-culture or via conditioned medium. In co-culture, but not with conditioned medium, this was prevented by blockade of the TGF-β type 1 receptor. Further studies to investigate the direct effects of TGF-β on cell survival are warranted. However, our studies suggest that the reduction in viability involves a factor/s downstream of TGF-β and released from the fibroblasts through the activation of myocytes. Although, to our knowledge, a complete examination of the effects of TGF-β on the secretory profile of cardiac fibroblasts is not available, TGF-β stimulates the release of various factors including connective tissue growth factor and endothelin-1 from fibroblasts.\(^\text{19,24}\) Endothelin-1 has previously been reported to be pro-apoptotic and may warrant further investigation.\(^\text{25}\) Cultured fibroblasts have previously been shown to reduce adult cardiomyocyte viability by stimulating apoptosis, but only when exposed to hypoxia.\(^\text{28}\) However, these findings were observed after 5 h and measurements at 24 h, like in our study, were not reported. A recent study has suggested that fibroblasts may protect against cardiomyocyte death.\(^\text{34}\) This difference may be due to the use of neonatal cells or the use of ischaemia – reperfusion to stress the cardiomyocytes.

Figure 3 TGF-β1 levels increase in both single fibroblast culture and co-culture with cardiomyocytes, but to different levels. The level of TGF-β1 was increased in fibroblast (FB) and myofibroblast (MFB) co-cultures, and was significantly higher in FB compared with MFB co-culture. In the FB-alone group, TGF-β1 levels were lower than in FB co-cultured with cardiomyocytes (M), whereas MFB alone has similar levels to MFB co-cultured with M (M + M n = 8; FB + M n = 10; MFB + FM n = 10; FB n = 6; MFB n = 6).

Figure 6B

Figure S4

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Fibroblast-derived mediators have been shown to reduce contractility and electrophysiological changes in neonatal rat cardiomyocytes.\textsuperscript{6,7,9,10} Cultured fibroblasts also affect the electrophysiology of adult cardiomyocytes, although the effects were different from those in neonatal cells, highlighting the importance of using adult cells.\textsuperscript{8} The results here show that co-culture with cultured fibroblasts and myofibroblasts has negative effects on the Ca\textsuperscript{2+} transient, reducing its amplitude with an associated reduction in SR Ca\textsuperscript{2+} content. Conversely, co-culture with \textalpha-SMA-negative fibroblasts increased the Ca\textsuperscript{2+} transient amplitude. SR Ca\textsuperscript{2+} content is a major determinant of Ca\textsuperscript{2+} transient amplitude. Although measurements of SR Ca\textsuperscript{2+} were not achieved in the experiments with freshly isolated fibroblasts/myofibroblasts, it was reduced after co-culture with cultured fibroblasts and may represent a mechanism by which the effects on Ca\textsuperscript{2+} handling are mediated. SR Ca\textsuperscript{2+} content is controlled by the balance between SERCA and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in the removal of Ca\textsuperscript{2+} from the cytoplasm. It has previously been shown that SERCA expression is sensitive to soluble mediators and may represent a mechanism underlying these changes.\textsuperscript{37,38} Changes in SERCA would also be expected to alter the dynamics of the Ca\textsuperscript{2+} transient decay, which was variably changed in these experiments, and further experiments are therefore required to investigate these potential mechanisms.
The effects of both fibroblasts and myofibroblasts co-culture on Ca^{2+} transient amplitude were blocked by the TGF-β type 1 receptor antagonist. However, when using conditioned medium only or when TGF-β was added directly to myocytes, these effects were not seen. This suggests a dynamic communication that requires crosstalk between the two cell types. That the same paracrine pathway was responsible for producing opposing effects in different groups highlights the importance of the context in which these interactions take place, and may therefore call into question the physiological relevance of the effects of isolated factors. An alternative explanation is that the mediators responsible

Figure 5 Fibroblast- or myofibroblast-conditioned medium does not affect cardiomyocyte Ca^{2+} transient amplitude; TGF-β type 1 is only involved in the hypertrophic response induced by myofibroblast condition medium. (A–D) Neither fibroblast (FB), nor myofibroblast (MFB), -conditioned medium affected Ca^{2+} transient of cardiomyocytes (M) [M + M n = 49(8); FB + M n = 43(7); FB + M + SB n = 49(8); MFB + SB + M n = 52(8); MFB + M + SB n = 47(8)]. (E) FB- and MFB-conditioned medium reduced cardiomyocyte viability, but this effect was not blocked by the TGF-β type 1 receptor antagonist (SB; M + M n = 9; FB + M n = 12; FB + M + SB n = 9; MFB + SB + M n = 10; MFB + M + SB n = 6). (E and G) Only MFB-conditioned medium increased the volume of M and this was blocked by the TGF-β antagonist [M + M n = 49(7); FB + M n = 48(7); FB + M + SB n = 49(8); MFB + SB + M n = 50(8); MFB + M + SB n = 50(8)]. Scale bar = 10 μm.
may be unstable and broken down in the conditioned medium; for example, it has previously been reported that the level of mediators in cultured fibroblast-conditioned medium is reduced by up to 50% after 24 h.39

Adding TGF-β directly to myocytes failed to recapitulate the effects of co-culture on cardiomyocyte Ca^{2+} transients, despite the TGF-β antagonist blocking the effect in co-culture. Previously, cultured fibroblast-conditioned medium has been shown to affect cardiomyocyte electrophysiology via TGF-β, but direct addition of TGF-β only had effects at when higher concentrations were used.8 Even at higher concentrations of TGF-β, we did not see effects on the Ca^{2+} transient supporting the conclusion that these effects are not due to direct TGF-β signalling but rather due to dynamic signalling between the fibroblasts and cardiomyocytes (Figure 6C). Other factors that have been investigated in the interaction between cultured fibroblasts and myocytes include endothelin, IL-6, and insulin-like growth factor,10,26,27 and a recent study has shown a paracrine role of miRNAs,40 which could also pass through the Transwell. These factors may be involved in the dynamic paracrine interaction highlighted here.

### 4.1 Similarities between cultured fibroblasts and freshly isolated myofibroblasts

An issue facing the field of fibroblast research is what fibroblasts activated to express α-SMA in culture actually represent. Generally, these have been used to represent myofibroblasts in pathology, yet some studies have shown that they have different properties.6,12 The results presented here show that cultured fibroblasts and myofibroblasts have similar paracrine effects on adult cardiomyocytes and, although more work is required to investigate whether common mechanisms are involved, these data support the similarity between the cell types.

### 4.2 A role for myofibroblasts in disease progression

The paracrine interaction between fibroblasts and cardiomyocytes is important in the whole heart response to pressure overload.41,42 Fibroblast-specific Krueppel-like factor 5 knockout prevented the hypertrophic response of the heart to overload, in part due to the removal of paracrine signalling.41 However, these studies have not looked at the effects on cardiomyocyte function. We have shown that myofibroblasts from pressure overload and fibroblasts from non-diseased animals have differential effects on cardiomyocyte Ca^{2+} transient amplitudes, and that TGF-β is important in mediating these effects. Under pressure overload, TGF-β is increased in the myocardium43 and may represent a mechanism of local regulation of myocyte function. Ca^{2+} transient amplitude is increased in our model of compensated pressure overload, suggesting that in vivo this effect is overpowered by other signalling pathways.20 However, Ca^{2+} transient amplitude is consistently observed to fall when this compensatory response is exhausted,20,44 and our data suggest that paracrine signals from

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**Figure 6** Cardiomyocyte-conditioned medium increases fibroblast proliferation. Cardiomyocyte (M)-conditioned medium was added to cultured fibroblasts (cFB). After 24 h, M-conditioned medium increased the number (A) of cFB compared with controls without altering cytotoxicity (B), suggesting that M affect cFB proliferation (cFB n = 8; cFB + M n = 8). (C) Diagrammatic representation of potential paracrine networks between fibroblasts and cardiomyocytes.
myofibroblasts may contribute to the decline in cardiomyocyte function in pathological conditions. Alternatively, it is also possible that the reduced Ca\textsuperscript{2+} transient amplitude is a protective effect to prevent further Ca\textsuperscript{2+} hypertrophy and loss of cardiomyocyte viability. The myofibroblasts could therefore be acting in a cardioprotective manner. These suggestions need to be tested in an in vivo setting.

It is important to note that although the time in culture was limited and controlled to use fibroblasts before they were activated by culture to express \( \alpha \)-SMA, it cannot be excluded that other more subtle changes had occurred. Early changes from fibroblasts into protomofibroblasts have been suggested\(^{39} \) \( ^{39} \) and more subtle changes in fibroblast phenotype cannot be ruled out representing a possible limitation to our study. Furthermore, the presence of contaminating cells in freshly isolated fibroblasts cannot be completely excluded. Using IF, smooth muscle and endothelial cells were not identified within the isolated cell population, and the technique used here has previously been used to provide a highly purified population of fibroblasts.\(^{46} \) Another limitation of this study is that adult cardiomyocytes from healthy hearts were used and further studies aimed at testing the effects of fibroblasts/myofibroblasts on diseased cardiomyocytes are warranted.

In addition, hypertrophy can also be defined as physiological or pathophysiological, which mechanism underlies the changes measured here has not been elucidated. Further studies investigating this point are warranted. The mechanisms underlying changes in the Ca\textsuperscript{2+} transient also represent an interesting area of further investigation. Finally, although we have investigated the role of TGF-\( \beta \), this is a complex signalling network and identification of other mediators involved continues to be of interest and importance.

### 5. Summary and conclusion

This study has shown that (i) \( \alpha \)-SMA-negative fibroblasts from normal hearts reduce adult cardiomyocyte viability, increase cardiomyocyte volume, and increase Ca\textsuperscript{2+} transient amplitude through paracrine signalling; (ii) despite similar effects on cardiomyocyte size and viability, cultured fibroblasts and myofibroblasts from pressure-overloaded hearts have an opposite effect on Ca\textsuperscript{2+} transients, leading to a smaller amplitude; (iii) cardiomyocytes also release soluble mediators that affect fibroblast proliferation. TGF-\( \beta \) is important, participating in a bi-directional regulatory signalling between fibroblasts and cardiomyocytes.

We conclude that fibroblasts have different roles during physiology or disease in the regulation of myocardial function via soluble mediators, and that a crosstalk between the two cell types operated by TGF-\( \beta \) is crucial in this interaction.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

### Conflict of interest

none declared.

### Funding

The work was supported by the NHLI Foundation, the Magdi Yacoub Institute, and an Imperial College Fellowship (to P.C.).

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