Expression of fibulin-6 in failing hearts and its role for cardiac fibroblast migration

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Abstract

The cardiac extracellular matrix (ECM) undergoes a dynamic transition following myocardial infarction. Fibulin-6 is expressed in cell junctions particularly in tissues subjected to significant mechanical stress. Fibulin-6 deficiency results in defective cell migration in nematodes and early embryonic lethality in mice. The role of fibulin-6 in healthy and failing myocardium is unknown. We have examined the expression and distribution pattern of fibulin-6 during myocardial remodelling (MR) and detailed its effect on the migratory function of cardiac fibroblasts (CFs) in response to TGF-β1.

Methods and results

In healthy murine myocardium, fibulin-6 expression is largely confined to larger coronary arteries. It is induced during the early and the late phase of remodelling after infarction in murine hearts predominantly in the scar–muscle junction. Similar results are obtained in human ischaemic cardiomyopathy. Fibulin-6 is mostly expressed in close vicinity to vimentin-positive cells and is also abundantly expressed in vitro in cultured neonatal CF. TGF-β1 does not induce smooth muscle actin in fibroblasts deficient of fibulin-6, which also compromised their migration. Cells that had migrated expressed more fibulin-6 compared with stationary cells. Plated on fibulin-6-depleted matrix, stress fibre induction in fibroblast in response to TGF-β1 was impaired. In ex vivo explant cultures from post-infarct myocardium, the number of emigrating fibroblasts was also significantly reduced by fibulin-6 siRNA knockdown.

Conclusion

Fibulin-6, a fibroblast-released ECM protein, may play an important role during MR by imparting an effect on CF migration in close and complementary interplay with TGF-β1 signalling.

Keywords

Myocardial ischaemia • Myocardial remodelling • Fibulin-6 • Fibroblast • Migration

1. Introduction

Myocardial infarction (MI) is the leading cause of cardiac failure. Its prognosis is determined by ventricular remodelling that entails vigorous changes in the architecture and composition of myocardial extracellular matrix (ECM) leading to progressive fibrosis and scar formation. Therefore, matrix remodelling is one of the critical determinants of the outcome following MI. Though the complete picture of matrix remodelling remains elusive, the dynamic state of cardiac ECM governed by cardiac fibroblast (CF) has been widely described.1,2 ECM does not act as a silent scaffold, but is a rather active player in modulating various cell behavioural patterns like proliferation, growth, survival, migration, and differentiation not only during cardiac development but also during myocardial remodelling (MR).2,3

Fibulin-6 is an evolutionarily highly conserved ECM protein belonging to the fibulin family of secreted glycoproteins5 with prominent effects on cellular function. Fibulin-6 was first described in Caenorhabditis elegans as its orthologue hemicentin-1 (Hmcn1). Hmcn1 is encoded by him-4. Due to the presence of the characteristic fibulin-like domain in Hmcn1, it belongs to the fibulin family and has been named fibulin-6. Studies in nematodes and zebrafish demonstrated its essential roles in the formation of transient cell contacts that are required for tissue...
organization, migration, basement membrane invasion, as well as in the formation of stable cell—cell and cell—ECM contacts, mainly in epithelial tissues. C. elegans mutants show defective cell migration, tissue fragility, and chromosomal instability. In a mouse, a prominent expression in various epithelial cells, in the pericellular ECM of endothelial cells, and vascular smooth muscle cells (VSMCs) has been observed. It is involved in the maintenance of the architecture of epithelial cell attachment in tissues exposed to significant mechanical strain. Fibulin-6 contributes to the formation of contractile rings of the cleavage furrow during cytokinesis in both C. elegans and mouse embryos. Its genetic targeting in mice results in sterility with multiple nuclei and early embryonic lethality at blastocyst stage. Hence, this remains a hindrance to functionally characterize the role of the protein in mammals. Distribution and expression pattern of fibulin-6 in the cardiovascular system and its effect in pathophysiological myocardium remains uncharacterized. However, other members of the fibulin family, like fibulin-1, 2, 4, 14 and 5, are reported to have a functional relevance in cardiovascular diseases. Structurally (depending on the length and domain structure), fibulin-6 is closest to fibulin-1, 2, and 8. Fibulin-1 and -2 are amorphous fibrous proteins, but fibulin-6 is minimally oligomeric. Fibulin-6 is widely expressed in the extracellular matrix of tissues and is involved in various cellular processes such as adhesion, migration, and differentiation. It is also involved in the regulation of vascular smooth muscle cell proliferation and migration. Fibulin-6 has been implicated in the prevention of atherosclerosis and the repair of damaged blood vessels. It has also been shown to be involved in the regulation of the extracellular matrix during tissue repair and regeneration. Its role in the cardiovascular system is not fully understood, but it is believed to play a key role in the maintenance of the architecture of epithelial cell attachment in tissues exposed to significant mechanical strain. Fibulin-6 contributes to the formation of contractile rings of the cleavage furrow during cytokinesis in both C. elegans and mouse embryos. Its genetic targeting in mice results in sterility with multiple nuclei and early embryonic lethality at blastocyst stage. Hence, this remains a hindrance to functionally characterize the role of the protein in mammals.

In recent years, several proteomic and transcriptomic studies in MI and MI/reperfusion (MI/R) models have revealed regulation of various ECM proteins like fibronectin, laminin, fibrillin, fibulin, and decorin apart from several types of collagen that are transcriptionally up-regulated. A study in a porcine MI/R model demonstrated a signature of more than 100 vital ECM proteins during early and late MI, among which fibulin-6 was identified.

We have therefore examined the distribution, localization, and expression of fibulin-6 in healthy and ischaemic myocardium after permanent MI in murine and human heart failure. To examine its functional relevance during myocardial damage and infarct healing, we explored the role of fibulin-6 for migratory properties of fibroblasts with respect to TGF-β1-dependent function in vitro and in vivo models of MR after infarction.

2. Methods

2.1 Mouse model of permanent MI injury

This study was performed conforming to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and was cleared by the institutional ethical committee and the regional authorities (LAVES) for animal care and experiments under the signature 13/1243 and 07/1307. Permanent MI by ligating the left anterior descending coronary artery was induced in C57BL/6 wild-type (WT) mice as described (see Supplementary material online, Methods). Anaesthesia was induced by inhalational isoflurane anaesthesia (1.5–2.0% in air) and maintained with isoflurane (1.5% Vol%) after tracheal intubation and ventilation. Analgesia was provided using metamizol administered with the drinking water for a dose of ~200 mg/kg/day. Sham control animals were treated exactly the same way but without tying the ligation. The hearts were excised at indicated times after the animals were deeply anaesthetized. Animals were euthanized by cervical dislocation in inhalational isoflurane anaesthesia. For molecular and histological analysis, hearts were harvested at 24 h, 3, 7, and 14 days after surgery. Area at risk and infarct area were localized histologically on haematoxylin and eosin (H&E) stainings or by the loss of autofluorescence at Ex/Em 489/506 (cy2) on immunofluorescent stainings.

2.2 Myocardial samples from left ventricular assist device implantation

Sampling of LV tissue conformed to the principles outlined in the Declaration of Helsinki and with approval of the local ethical committee. Following informed consent, apical LV tissue was obtained from 25 patients receiving left ventricular assist device (LVAD) for decompensating ischaemic cardiomyopathy. The tissue was removed for the insertion of the device’s inflow cannula. Five non-failing hearts that had been rejected for transplantation were used as comparators.

2.3 Neonatal mouse ventricular CF isolation and culture

Neonatal mouse ventricular CFs were isolated as detailed in Supplementary material online, Methods. Fibroblasts were grown and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal bovine serum. 3T3 cell lines of CF were also established from neonatal CFs using published protocols.

2.4 Matrix production from CF

Cell-free ECM (cardiogel) from 7 days of cultured neonatal CF was produced using previously published protocols (see Supplementary material online, Methods).

2.5 Stimulation of neonatal mouse ventricular CF with TGF-β1

For TGF-β1 (Cell Signaling Technology, Inc.) stimulation, 1 × 10⁶ neonatal CFs/well were plated on a six-well plate. The media was gradually replaced with 5% serum to medium containing 1% insulin/transferrin/selenium-A-supplement (Gibco, Life Technologies) for 2 days, then 10 nM murine TGF-β1 was added for 48 h.

2.6 siRNA transfection

Fibulin-6 silencer select in vivo siRNA was synthesized by Ambion Life Technologies (s233951). As negative control, scrambled (scr) siRNA was used (Silencer Select negative control siRNA, Ambion). 3T3 CFs or neonatal CFs were transfected with 20 nM siRNA via reverse transfection as described (see Supplementary material online, Methods).

2.7 Scratch wound-healing assay

A fluorescence-based quantitative scratch wound-healing assay was employed as previously published to study fibroblast migration after fibulin-6 siRNA knockdown (KD) compared with scr siRNA-transfected cells as control (see Supplementary material online, Methods).

2.8 Isolation of migrating and non-migrating cells

Isolation of migrating and non-migrating cells was performed as described (see Supplementary material online, Methods).

2.9 Quantitative real-time PCR

Using TRiZide reagent (Applichem, Darmstadt, Germany), total RNA was extracted from non-ischaemic and ischaemic myocardium: LVAD and non-failing heart tissue; neonatal CF and 3T3 cells. Primers and reagents were purchased from Eurogentec (Cologne, Germany; see Supplementary material online, Table S1). All the samples were run in duplicates on a real-time RT-PCR cycler (Rotorgene 3000, Cor-bett Life Science, Hilden, Germany) using SYBR-GREEN (SensiFAST™ SYBR, Bioline GmbH, Germany) and normalized to HPRT gene expression. Data are expressed as 2⁻ΔΔC_T.
2.10 Fibulin-6 and -8 mouse polyclonal antibody generation

Polyclonal antibodies against an N-terminal fragment of mouse Hemicentin-1 (Hmcn1N) i.e. fibulin-6 and against a C-terminal fragment of mouse Hemicentin-2 (Hmcn2C) i.e. fibulin-8 were generated, lacking domains shared with other fibulin family members. cDNAs encoding amino acid residues 1653–2275 (Ig-like domain 14–20) of Hmcn1N, or residues 4429–4753 (G2 domain and epidermal growth factor domain 1 and 2) of Hmcn2C, were generated by RT-PCR on total RNA from mouse skin and cloned with S′-terminal Nhel and 3′-terminal Xhol restriction sites. Amplified PCR products were inserted into a modified pCEP-Pu vector containing an N-terminal BM-40 signal peptide and a C-terminal strep-tag downstream of the restriction sites, and recombinant plasmids were introduced into HEK293-EBNA cells (Invitrogen) using FuGENE 6 transfection reagents (Roche). Cells were selected with puromycin (1 μg/mL) and the recombinant protein was purified directly from serum containing cell culture medium. After filtration and centrifugation (30 min, 10 000 g), cell culture supernatants were applied to a Streptactin column (1.5 mL, IBA GmbH) and eluted with 2.5 mM desthiobiotin, 10 mM Tris–HCl, pH 8.0.

The purified recombinant Hmcn1 and 2 fragments were used to immunize rabbits (Pineda Antikörper Service, Berlin, Germany). Obtained antisera were purified by affinity chromatography on columns with purified Hmcn1N and Hmcn2C fragments, respectively, coupled with CNBr-activated Sepharose (GE Healthcare). Specific antibodies were eluted with 3 M KSCN, and the eluate was dialysed against PBS, pH 7.4.

2.11 Immunofluorescence, immunohistochemistry, and immunoblotting

Immunofluorescence, immunohistochemistry, and immunoblotting were performed using standard protocols (see Supplementary material online, Methods).

2.12 RNA in situ hybridization analysis

RNA in situ hybridization analysis on paraffin sections with digoxigenin-labelled antisense riboprobes was performed as described elsewhere (see Supplementary material online, Methods for probe information).

2.13 Fibroblast migration from murine ventricular explants

Ex vivo mouse ventricular explants from 14 days MI mice hearts were harvested (as described above in Section 2.1) and were subjected to explant migration studies as described (see Supplementary material online, Methods).

2.14 Statistical analysis

Unless otherwise stated, data are presented as median (horizontal bar) and interquartile (box) and 5/95% (whiskers) ranges. Since the data mostly had non-Gaussian distributions or significantly different SDs, Kruskal–Wallis test followed by Mann–Whitney U-test for groupwise comparisons with Holm’s correction for multiple testing was employed. P-values <0.05 were considered statistically significant.

3. Results

3.1 Fibulin-6 is expressed in endocardium and blood vessels of mouse and human heart

Expression patterns of fibulin-6 in the cardiovascular system of adult mammals have not been investigated before. We studied its expression and localization pattern in myocardium from sham mice and in samples from non-failing hearts that had been rejected for transplantation. Using immunofluorescence, we detected expression of fibulin-6 in vascular endothelial cells in coronary arteries (Figure 1A–C) and sparsely in endocardial endothelium (Figure 1I). Pericellular expression of fibulin-6 in the vessels was also observed in the human mammary artery (see Supplementary material online, Figure S1A and B).

The specificity of the antibody was verified employing peptide competition and comparative stainings (see Supplementary material online, Figure S2A–I) as well as fibulin-6 siRNA KD CEs and embryonic mouse skin preparations (see Supplementary material online, Figures S1B and S2C and D).

3.2 Fibulin-6 expression increases transcriptionally and translationally after permanent MI in mouse and in human ischaemic cardiomyopathy

To investigate potential changes in the expression pattern of fibulin-6 in failing mouse hearts, permanent MI in C57Bl/6 WT mice was inflicted. As we observed a prominent expression of fibulin-6 in blood vessels, we determined whether fibulin-6 expression changes in coronary arteries in remote and ischaemic myocardium when subjected to MI. No significant alteration of fibulin-6 expression in infarcted and remote regions was observed, since the fraction of fibulin-6/α-SMA-positive vessels remained unchanged (Figure 1D). Moreover, the transcriptional expression of fibulin-6 in ischaemic myocardium compared with sham and remote, which displayed unaltered expression at all investigated time points (Figure 1E), was 4.2-fold higher at 24 h after MI, returned to baseline levels at 3 and 7 days after MI, but increased 1.9-fold again at 14 days after MI. The specificity of the fibulin-6 KD was confirmed. Fibulin-6 KD did not affect the expression of other closely related fibulin members (see Supplementary material online, Figure S3A and B). Elevated expression of fibulin-6 at mRNA level was also found in human samples from patients suffering from ischaemic cardiomyopathy in comparison with non-failing hearts (Figure 1F). RNA in situ hybridization for fibulin-6 at 7 days MI revealed no fibulin-6 expression in sham (Figure 1G) but induction of its expression along the endocardial endothelial cell layer 7 days after MI (Figure 1H). Endocardial endothelial cells were clearly positive for mRNA signal for fibulin-6 after MI. Additionally, some signal was also detected in the cells beneath or further away from endocardial line in the myocardium.

Fibulin-6 is not accessible to western blotting due to its large size of 600 kDa as well as a lack of antibodies suited for blotting. We therefore resorted to immunofluorescence staining to analyse spatial expression and cell association of fibulin-6 protein. We found sparse and patchy expression of fibulin-6 in endocardium (Figure 1I) with no notable difference between sham and 24 h post-MI. Three days after MI, endocardial expression was more prominent and continued to increase until 7 days (Figure 1J) and 14 days (Figure 1K), eventually resulting in 100% of endocardial endothelial cells expressing fibulin-6 (Figure 1L). Furthermore, between Days 7 and 14, fibulin-6 expression extended from the endocardial line into the inner myocardial layers, far beyond the endocardial endothelial lining of the scar region (Figure 1M).

Additionally, RNA in situ hybridization of fibulin-6 in 7 days MI tissue also revealed its expression in the interstitial cellular mass of the peri-infarct zone near the remote–infarct junction (Figure 2A). Immunostaining showed similar results. Mechanical strain is highest in the peri-infarct zone at the insertion sites of the scars in surviving myocardium, which we termed here as the infarct–remote junction. During the early remodeling phase (3 days), small fibulin-6-positive trajectories emerged from the infarct–remote junction towards the remote area, where fibulin-6 was
expressed. Seven and 14 days after MI, a further increase of fibulin-6 expression with the formation of connective tissue trajectories into the peri-infarct zone was observed (Figure 2B).

Triple staining of 7 days and 14 days MI sections with fibulin-6, vimentin (fibroblast marker), and α-SMA (myofibroblast marker) revealed the expression of fibulin-6 in close association with vimentin-positive cells in the infarct zone, demonstrating its association with fibroblasts. However, there rarely was any direct co-localization or deposition of fibulin-6 in close proximity to α-SMA-positive cells (Figure 2C). Human ischaemic cardiomyopathy sections revealed a very similar pattern of fibulin-6 expression (Figure 2D).

### 3.3 Secretion and deposition of fibulin-6 by fibroblasts in vitro

To examine whether fibulin-6 is produced by fibroblasts or myofibroblasts, we used neonatal mouse CF, allowing the cells to secrete ECM...
3.4 TGF-β1-mediated α-SMA expression in CF is attenuated when fibulin-6 is absent

Fibroblasts differentiate to myofibroblasts in response to TGF-β1. Compared with non-stimulated cells, we observed an abundant expression of α-SMA stress fibres in CF upon TGF-β1 stimulation. α-SMA stress fibre formation was majorly reduced in TGF-β1-stimulated fibulin-6 KD cells (Figure 3H). TGF-β1-induced α-SMA mRNA (Figure 3I) and protein (Figure 3J) levels were also reduced by 43 and 48%, respectively, in fibulin-6 KD cells.

3.5 Matrix deposited fibulin-6 rescues TGF-β1 response in fibulin-6 KD CF

We next asked whether the fibulin-6 in the matrix is sufficient for TGF-β1-induced stimulation of CF. 3T3 cells transfected with scr siRNA and fibulin-6 siRNA were plated on normal matrix (cardiogel, containing fibulin-6). TGF-β1-stimulated 3T3 cells with scr siRNA, induced extensive stress fibre formation compared with unstimulated cells (Figure 4A and B). Also, fibulin-6 KD CF formed as much stress fibres as scr transfected cells upon stimulation (Figure 4C and D). Scr siRNA-transfected 3T3 CF, stimulated with TGF-β1 when plated on fibulin-6-deficient cardiogel, displayed stress fibre formation (Figure 4E), whereas TGF-β1-stimulated 3T3 cells KD for fibulin-6 did not form stress fibres on fibulin-6-deficient matrix (Figure 4F).

3.6 Migrating 3T3 CFs induce fibulin-6 expression and fibulin-6 KD reduces TGF-β1-dependent migration

We examined the effect of fibulin-6 expression during the migration of fibroblasts. Fibulin-6 transcript levels were 1.4-fold up-regulated in migrating cells compared with control adherent cells (Figure 5A). To examine whether fibulin-6 is directly involved in fibroblast migration, we first used an in vitro migration assay using 3T3 CFs with suppressed proliferation. Migration index gradually increased in control fibroblasts until 24 h after scratching, while the migration index of the cells transfected with fibulin-6 siRNA was reduced by 53% (Figure 3B). TGF-β1 is known to induce migration, thus it can have a positive effect on fibroblast migration by inducing trans-differentiation into myofibroblasts. Consistent with the reduced α-SMA expression, TGF-β1-induced cell migration was also reduced in the fibulin-6 KD CF (Figure 5C).

3.7 Enhanced migration of fibroblasts from infarct explants depends on fibulin-6

To mimic the in vivo situation during remodelling after infarction as closely as possible and to examine the role of fibulin-6 for fibroblast migration in tissue, we adapted a model of tissue explant culture from 14 days MI mouse hearts and compared it with remote myocardium. After 72 h of culture, 46.5-fold more vimentin-positive fibroblasts (see Supplementary material online, Figure S4A) emerged from explants from the infarct compared with remote myocardial explants (Figure 6A and B). Fibroblasts from the infarct explants also migrated 81% longer distances compared with remote myocardium-borne fibroblasts (Figure 6C). Enhanced fibroblast migration from the ischaemic explants was dependent on fibulin-6, since fibulin-6 KD (KD = 53.2%), see Supplementary material online, Figure S4B) significantly decreased the number of cells migrating out, as well as their maximum migratory distance by 58 and 33.3%, respectively, compared with scr siRNA (Figure 6D–F).

4. Discussion

The expression and distribution pattern of fibulin-6 in healthy myocardium or in the process of MR is unknown. We identified fibulin-6 expression in healthy human and mouse myocardium. Its expression in selected intramyocardial coronary arteries is preserved in human ischaemic cardiomyopathy samples and in murine infarcted tissue. Sparse fibulin-6

Figure 1  Differential expression and localization of fibulin-6 in healthy myocardium and during MR following murine MI and in human ischaemic cardiomyopathy. Tissues were harvested from healthy mice or mice after MI and from LVAD explants (apex of the human failing heart) or from rejected donor hearts. RNA was extracted for RT-PCR to assess fibulin-6 expression. The tissues were also stained by immunofluorescence techniques for fibulin-6, Isolectin B4 (ILB4), von Willebrand factor (vWF), and α-SMA to discern differences in expression levels and in spatial or cellular distribution of fibulin-6. (A) Fibulin-6 expression in the subendothelium of larger intramyocardial coronary arteries of murine sham myocardium. Triple staining for α-SMA (blue) for VSMC, ILB4 (green) for endothelial cells, and fibulin-6 (red). (B) Vascular expression of fibulin-6 in non-failing human hearts, double-stained for fibulin-6 (red) and vWF (green) (C) or α-SMA (green) (bar = 50 μm). (D) Percentage of fibulin-6-positive coronary arteries in mouse ischaemic (I) and remote (R) myocardium relative to their total number (positive for αSMA) (n = 6, P = ns). (E) Cumulative mRNA expression of fibulin-6 in sham, remote, and ischaemic myocardium from 24 h to 14 days of MI in mice (n = 6, P < 0.0022 for 24 h and 14 days MI, P = ns in 3 days and 7 days). (F) RT-PCR analysis of human ischaemic cardiomyopathy samples compared with non-failing healthy myocardium, rejected for transplant (n = 5/25, P < 0.05). RNA in situ hybridization for fibulin-6 in (G) sham and in (H) 7 days MI (bar = 200 μm). The right panels and insets show the magnified image of area marked with rectangles (bar = 50 μm). Arrows indicate positive signal. Immunofluorescence staining of fibulin-6 expression in sham (I) following MI in mouse heart for 7 days (j) and 14 days (K). Immunofluorescence micrographs depict fibulin-6 (red), α-SMA (green), and nuclear (blue) staining (bar = 50 μm), accompanied by H&E stainings of immediately neighbouring sections (bar = 100 μm). (L) Morphometric analysis of fibulin-6 along the endocardial line relative to the total length of the endocardial line from 24 h to 14 days of MI. (M) The average thickness of the myocardial layer staining positive for fibulin-6 at 7 days and 14 days after MI (both n = 6, P < 0.0016).
Figure 2 Expression of fibulin-6 in the infarcted to remote myocardial junction. Mouse infarct and human failing heart tissue were subjected to in situ hybridization for fibulin-6 mRNA or immunofluorescently stained for fibulin-6 and vimentin to identify fibroblasts expressing the protein. (A) Fibulin-6 mRNA expression detected via in situ hybridization in 7 days MI sections at the infarct–remote junction (bar = 200 μm). The right panel is the magnified image of the area marked with a rectangle (bar = 50 μm). Arrows indicate positive signal. (B) Expression of fibulin-6 (red) in the trajectories forming at the junction between remote (R) and infarct (I) myocardium from 3 to 14 days of MI. Vital myocardium is depicted by its green autofluorescence in the FITC channel and delineates the remote myocardium. DAPI nuclear staining is blue. (C) Ischaemic myocardium of 7 and 14 days of MI stained with vimentin (green, arrows), α-SMA (blue), and fibulin-6 (red). Co-localization signals are displayed in yellow colour. (D) Human ischaemic LVAD sections stained with vimentin (green), DAPI (blue), and fibulin-6 (red). Fibulin-6 staining observed in the scar regions (bar = 50 μm).
Figure 3  Fibulin-6 expression by fibroblasts in vitro (fibulin-6 = green, α-SMA = red, DAPI = blue, for all the images). Fibroblasts isolated from neonatal mouse hearts and stimulated with TGF-β1 at indicated time points were subjected to RNA or protein extraction for RT-PCR or western blotting or stained for fibulin-6 or α-SMA expression. (A) RT-PCR expression analysis of α-SMA in the TGF-β1-stimulated and -non-stimulated cells. (B) RT-PCR expression analysis of fibulin-6 in TGF-β1-stimulated and -non-stimulated CFs. (C and D) Fibulin-6 deposition into the matrix by cultured neonatal CF in vitro. The lower panel is the magnified image of area marked with rectangle (bar = 50 μm). (E) CFs stimulated with TGF-β1 have regions with and without fibulin-6 deposition. The left and lower panel images are the magnified image of area marked with rectangle. (F) Differentiation of CFs into myofibroblasts with the appearance of α-SMA stress fibres after TGF-β1 stimulation with less fibulin-6 deposition. (G) Regions with fibulin-6 in TGF-β1-stimulated cells with non-differentiated fibroblasts (bar = 50 μm). (H) α-SMA stress fibre formation in scr siRNA-transfected CF with and without TGF-β1 stimulation. A diminished expression of α-SMA in fibulin-6 KD CF after TGF-β1 stimulation (bar = 50 μm). (I) RT-PCR expression analysis of α-SMA in scr siRNA and fibulin-6 siRNA-transfected CFs with and without TGF-β1 stimulation (n = 3, P < 0.05). (J) Immunoblot and densitometric analyses for α-SMA in scr siRNA and fibulin-6 siRNA-transfected CFs with and without TGF-β1 stimulation (n = 3, P < 0.05).
expression is also observable in endocardial endothelial cells in sham mouse heart. Fibulin-6 expression increases at the transcriptional level in the infarcted but not the remote myocardium during the early and late phase of infarct healing. RNA in situ hybridization revealed the expression of fibulin-6 along the endocardial endothelial cell layer and in the peri-infarct zone but not in sham tissue. Its spatial expression pattern also changes drastically in the course of infarct healing in mice, while the vascular expression remains unchanged. Even at the translational level fibulin-6 expression is not different from sham at the early stages (until Day 3 post-MI), but as the remodelling advances from the inflammatory phase to scar formation, remarkable amounts of the protein are deposited in the peri-infarct zone, specifically progressing from the endocardium into the myocardium associated with CFs but not myofibroblasts. Fibulin-6 is deposited in regions experiencing high mechanical strain, i.e. the junction between the remote and infarct zone of myocardium apparently oriented along force trajectories.

For maintenance of ventricular function and during remodelling, CFs play a critical role. Cardiac injury provokes proliferation of resident CF as much as the proliferation and differentiation of myofibroblasts. These proliferating CFs grant ECM homeostasis by regulating the turnover of ECM proteins and confer ECM remodelling by producing various essential ECM proteins (fibrillar collagen types I and III; collagen types IV, V, and VI; fibronectin, laminin, elastin, fibrillin, proteoglycans, and glycoproteins) required during MR. Thus, where cardiomyocytes are responsible for the ‘real weight lifting’ and contractility, CF build the thrust bearing abutment to adjust the matrix architecture.

**Figure 4** Matrix or CF-derived fibulin-6 restores TGF-β1 stimulation response. Neonatal mouse CFs were subjected to a 3T3 protocol. Resulting cells were used to study the effect of fibulin-6 on stress fibre formation upon TGF-β1 stimulation, which is deposited either in matrix or released from cells. Matrix (cardiogel) was produced by neonatal CF treated with either scr siRNA or fibulin-6 KD by prolonged confluent culture and subsequent lysis. 3T3 cells reversely transfected with scrambled or fibulin-6 siRNA were plated on that matrix and stress fibre formation was assessed by staining fibres with Alexa 488 phalloidin. Representative images are of three independent experiments. Stress fibre formation of 3T3 cells plated on WT cardiogel (A) with scr siRNA transfection and no stimulation, (B) scr siRNA transfection with TGF-β1 stimulation, (C) fibulin-6 siRNA KD and no stimulation, and (D) fibulin-6 siRNA KD with TGF-β1 stimulation. 3T3 cells were also plated on fibulin-6-deficient cardiogel and stimulated with TGF-β1 (E) after scr siRNA transfection and (F) fibulin-6 siRNA KD (bar = 50 μm).
after infarction has occurred and when tissue remodelling is required. Various members of the fibulin family have been reported to be secreted and expressed by fibroblasts in culture or in vivo. Fibulin-6 has also been shown to be expressed at mRNA level in human skin fibroblasts. We observed deposition of fibulin-6 specifically around vimentin-positive cells in the infarct zone of mice. Often a colocalization signal was also obtained, which could be because of the deposition of fibulin-6 beneath the cells, or closely surrounding the cells in the tissue. This indicated that CFs could be the major source for secreting the protein. Endocardial or VSMC may also contribute. However, myofibroblasts (α-SMA-positive cells) did never directly co-localize and were not found in close proximity with the fibulin-6 signal. In human ischaemic cardiomyopathy, we demonstrate similar expression patterns of fibulin-6 as in mice. The samples from human heart are small and originate from the left ventricular apex. We were thus not able to discern the infarct/remote junction as in the animals to examine the interface between large infarcted areas and the scar insertion regions. Still, fibulin-6 expression in the human samples was also observed at the border zone between scarry stretches and viable cardiomyocytes. Likewise, the vascular expression of fibulin-6 was very much comparable to mouse myocardium. In addition, we have shown that neonatal primary CFs secret and deposit fibulin-6 as a part of the ECM synthesized in culture. When CFs were activated by TGF-β1, expression of α-SMA was increased, while fibulin-6 expression dropped significantly both at the transcript and the protein level around α-SMA-positive cells. However, regions with dense deposition of fibulin-6 were also found

Figure 5 Fibulin-6 is required for the migration of 3T3 CF. An automated scratch assay was carried out by plating 3T3 cells on a 96-well plate and growing them to confluency. A scratch was inflicted after mitomycin treatment before measurement was started. The area of the scratch normalized to its circumferance was measured on fluorescence images obtained from the DiR-stained cells at 6 and 24 h after infliction of the scratch. Cells that had migrated into the scratch were harvested for RNA extraction. (A) Relative mRNA expression of fibulin-6 in migrated vs. non-migrated cells. (B) Representative fluorescence micrograph of a section of a 96-well plate after a scratch assay with fibulin-6 KD 3T3 cells compared with scr siRNA-transfected 3T3 cells and (C) with TGF-β1-stimulated 3T3 cells, at 6 h and 24 h post-scratch with their corresponding migration index.
in cultures stimulated with TGF-β1. In these regions, CF remained largely α-SMA negative, which means that TGF-β1 did not induce differentiation of the entire population of fibroblasts in confluent cultures, likely because of the heterogenous expression of functional TGF-β receptors in cell culture. Additionally, fibulin-6 that had been previously deposited before TGF-β1 induction remained in the matrix and was

Figure 6 Role of fibulin-6 for ex vivo migration of fibroblasts from explants of remote and ischaemic myocardium. WT mice were subjected to 14 days MI and the endocardial layer of the heart was harvested and separated into small explants. Explants were cultured and maintained with DMEM/10% FCS for 72 h. Explants were treated with scr siRNA or fibulin-6 siRNA in the meanwhile. Micrographs of the CTG-stained explants after 72 h were taken under fluorescence microscope and the number of cells as well as the average distance migrated from the closed edge of the explants were measured morphometrically. (A) Fibroblast outgrowth from remote and ischaemic tissue explants (bar = 100 μm). (B) Analysis showing the total number of cells migrating out of the explant (n = 29 explants from three animals, P < 0.0001) and (C) the average maximum distance covered by the cells from the closest possible point of origin (explant border) to the farthest point migrated (n = 29 explants from three animals) in ischaemic explants compared with remote. (D) Fibulin-6 KD in ischaemic explants compared with scr siRNA (bar = 100 μm). (E) Number of cells that migrated out of the explant (n = 46 explants from three animals). (F) Average distance covered by the migrated cells away from the explant (n = 46 explants from three animals).
Fibulin-6 expression in migrating cardiac fibroblasts during remodelling

... effective as evidenced in our rescue experiments, which demonstrate that fibulin-6 that is either produced by CF or available in preformed matrix can steer fibroblast differentiation. However, in the complete absence of fibulin-6 in cells and matrix, a-SMA expression in fibroblasts upon TGF-β stimulation was highly impaired, suggesting that fibulin-6 probably regulates stress fibre formation during differentiation. Thus, although fibulin-6 expression is down-regulated in CF when exposed to TGF-β, it can still induce the TGF-β-mediated effects probably because of the long half life of the protein and its spatial presence in the matrix. This is well in line with the staining pattern seen in mouse and human infarcted myocardium in vivo, suggesting that CFs represent the origin of fibulin-6 rather than myofibroblasts.

In addition to ECM homeostasis, CFs have miscellaneous other functions such as migration, myofibroblast differentiation, proliferation, secretion of growth factors etc. during MR.38 These activities of CF are in turn controlled and supported by their microenvironment, that is the surrounding matrix components, interstitial fluid composition, cell–cell contacts, and substrate supply to the location.39 – 41 With respect to their surrounding matrix, CFs partly create their own microenvironment.41,42 Thus, ECM provides both signal and substrate for fibroblast function during MR. One such vital function is the migration of fibroblasts in wound healing and MR.41,42 The role of other fibulins in migration has been documented in many studies.15,43 – 46 Explants from the infarct region of WT mice, isolated on Day 14 after MI, yield enormous outgrowth of fibroblasts when compared with the remote region. Fibulin-6 siRNA KD in ischaemic explants significantly diminished the outgrowth of cells. We go on to show that fibulin-6 in the matrix or produced by the cells itself can profoundly alter migratory behaviour of the cell, constituting a parallel motif to autocrine and paracrine effects of the endocrine system that could be termed ‘automatricellular’ or ‘paramatricellular’ effect. However, the origin of the emerging fibroblast remains unclear since it may be derived from endothelial precursor cells,47 monocytes, resident fibroblasts, or mesenchymal progenitor cells.48 It is possible that the emerging fibroblasts may be derived from endothelial-to-mesenchymal transition. The fibroblasts could, however, also be derived from the subendocardial and/or paravascular mesenchymal stem cell niche.

Scratch closure in a wound-healing assay by fibulin-6 KD 3T3 cells was delayed in vitro, suggesting impaired migration of the cells with reduced fibulin-6 levels. Based on these findings, we examined the effect of fibulin-6 on TGF-β1-induced migration. It had been reported that TGF-β1 induces the expression of a-SMA and facilitates the migration of cells.35,49,50 The migration of 3T3 CF after TGF-β1 stimulation in fibulin-6 KD cells was slowed, compared with control cells probably due to impaired a-SMA expression. 3T3 cells that were harvested after having migrated expressed more fibulin-6 compared with adherent cells. We therefore conclude that fibulin-6 is instrumental in CF migration, which is a pivotal event during MR.

However, our study has certain limitations. Owing to a lack of more specific fibroblast markers, vimentin (intermediate filament abundant found in fibroblasts) has been chosen as a marker for fibroblasts, which also stains other cells of mesenchymal origin. Moreover, fibroblasts were identified according to cytomorphological differences with other cell types apart from vimentin staining. The direct or the indirect interaction partners of fibulin-6 that might be responsible for its impact on fibroblast migration and differentiation have not been addressed here. This question has to be solved in future studies. Definitive proof of the role of fibulin-6 in myocardial wound healing is subject to following studies using mice deficient for fibulin-6 in the MI model. However, the lethal phenotype of the global knockout established in Bruce Vogels laboratory may necessitate a complex conditional knockout strategy, which is very likely to also display a wide variety of connective tissue phenotypes and may thus not be such a straightforward approach. These experiments are in preparation. We have attempted to circumvent this issue by using ex vivo explant tissue culture in conjunction with siRNA silencing and validated our findings in human heart failure samples.

In conclusion, we have demonstrated the differential expression, localization, and distribution of fibulin-6 in ischaemic myocardium upon MI undergoing remodelling in mouse and human. We suggest that fibroblasts are the primary cell type to secrete fibulin-6 during remodelling following MI. However, TGF-β signalling is influenced by fibulin-6 leading to the expression of a-SMA, thus regulating the migration of CFs. From our in vitro and ex vivo explant data, we prove that fibulin-6 is importantly involved in CF migration. Thus, we propose that fibulin-6 is a pivotal ECM protein regulating crucial processes during MR in close interplay and complementation with TGF-β signalling.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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