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The small leucine-rich proteoglycan fibromodulin exerts anti-fibrotic effects in cultured human cardiac fibroblasts

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ABSTRACT. Cardiac fibrosis is common and detrimental in numerous heart diseases, affecting millions of patients worldwide. Cardiac fibrosis is characterized by excessive production of extracellular matrix (ECM) constituents such as fibrillar collagens, produced by activated cardiac fibroblasts, i.e.: myofibroblasts. Therapeutic targeting of cardiac fibrosis is highly attractive; however, it remains a major medical challenge. Fibromodulin is a small leucine-rich proteoglycan localized in the ECM. Fibromodulin binds to collagen fibrils, and plays a critical role in collagen fibrillogenesis, ECM organization, wound healing and regulation of the pro-fibrotic cytokine transforming growth factor beta (TGFβ) in several organs. Fibromodulin is highly upregulated in mice and patients with heart failure, but little is known about its role in cardiac fibrosis. Our recent findings from primary cultures of cardiac fibroblasts from neonatal rat hearts suggest that fibromodulin has anti-fibrotic effects. Here we investigated the translational value of these findings by overexpressing fibromodulin in cultured human fetal and adult cardiac fibroblasts. The effects of fibromodulin overexpression on

gene expression were measured by qPCR and gene arrays, whereas protein levels were measured by Western blotting, and collagen synthesis by radioactive proline incorporation. The results support our previous findings and indicate relevance for human disease. We found that fibromodulin reduced the expression levels of the collagen cross-linking enzymes lysyl oxidase (*LOX*) and transglutaminase 2 (*TGM2*). Fibromodulin also reduced the levels of connective tissue growth factor (*CTGF*) and periostin (*POSTN*), indicating reduced TGFβ activity. Reduced levels of intercellular adhesion molecule 1 (*ICAM1)* and vascular cell adhesion molecule 1 (*VCAM1)* suggested reduced potential for immune cell adhesion, and gene arrays indicated altered integrin expression, suggesting altered ECM-cell adhesion. Expression of fibrillar collagens was unaffected. In conclusion, fibromodulin reduced TGFβ activity and down-regulated central collagen-crosslinking enzymes, in line with an anti-fibrotic effect of fibromodulin in human cardiac fibroblasts.

Key words: Extracellular matrix; Fibrosis; Cardiac remodeling; Transforming growth factor; Integrin

INTRODUCTION

Cardiac fibrosis stiffens the heart and is a hallmark feature of numerous forms of heart diseases, affecting millions of patients worldwide and with a strong impact on patient outcome (de Boer et al., 2019). Therapeutic targeting of cardiac fibrosis has become an attractive research area, with focus on identifying anti-fibrotic pathways and agents that could form the basis for important therapeutic treatment in the future (Bjørnstad et al., 2011; de Boer et al., 2019; Parichatikanond et al., 2020; Song and Zhang, 2020).

Cardiac fibrosis is defined as excessive production of extracellular matrix (ECM) by activated cardiac fibroblasts (CFBs), called myofibroblasts (Talman and Ruskoaho, 2016; Ma et al., 2018; Mohammadzadeh et al., 2019). The cardiac ECM constitutes a dynamic network of molecules, mainly fibrillar collagens (collagen I and collagen III), elastins, fibronectins, laminins, and proteoglycans (Iozzo and Schaefer, 2015; Talman and Ruskoaho, 2016; Ma et al., 2018; Christensen et al., 2019). The cardiac ECM provides structural support for the cells of the heart, and is required for cell adhesion, migration, and communication (Mohammadzadeh et al., 2019; Song and Zhang, 2020). The ECM closely interacts with cell surface receptors, e.g.: integrins and transforming growth factor beta (TGFβ) receptors (Iozzo and Schaefer, 2015). TGFβ is a well-known mediator of fibrosis and has for long been an attractive therapeutic target for treatment of cardiac fibrosis (Parichatikanond et al., 2020).

Fibromodulin (encoded by *FMOD*) is a small leucine-rich proteoglycan localized to the ECM (Iozzo and Schaefer, 2015). Proteoglycans are proteins substituted with covalently-linked glycosaminoglycan (GAG) chains, and are important constituents of the ECM (Iozzo and Schaefer, 2015; Christensen et al., 2019; Mohammadzadeh et al., 2019). Fibromodulin binds directly to collagen fibrils, the central collagen cross-linking enzyme lysyl oxidase (LOX), and TGFβ (Soo et al., 2000; Kalamajski and Oldberg, 2007;

Kalamajski et al., 2016; Tillgren et al., 2016; Neff and Bradshaw, 2021;). In lung and skin, fibromodulin regulates collagen fibrillogenesis, collagen cross-linking, ECM organization, and TGFβ activity (Rydell-Tormanen et al., 2014; Pang et al., 2020). Fibromodulin is important for skin wound healing, reducing scarring, suggesting anti-fibrotic properties (Zheng et al., 2017; Jiang et al., 2018). However, the role of fibromodulin in the cardiac ECM and fibrosis is not well understood.

We recently showed that fibromodulin is three- to ten-fold upregulated in hearts of mice and patients with heart failure (Andenæs et al., 2018). We found that CFBs express fibromodulin, and that its expression is increased in response to pro-inflammatory mediators (Andenæs et al., 2018). In primary cultures of CFBs from neonatal rats, we found that fibromodulin affected ECM remodeling and had anti-fibrotic effects in the form of reducing expression of *LOX* and another important collagen cross-linking enzyme, transglutaminase 2 (*TGM2*) (Andenæs et al., 2018). Here we aimed to increase our knowledge about the role of fibromodulin as an anti-fibrotic molecule and examined the translational potential of our previous findings from cultured rat CFBs in CFBs from human fetal and adult hearts.

MATERIAL AND METHODS

Cultured human cardiac fibroblasts

CFBs isolated from ventricles of a human adult heart (C-12375, PromoCell) and a human fetal heart (306-05F, Sigma-Aldrich), haCFbs and hfCFBs, respectively, were used. Of note, hfCFBs are known to produce a more mature ECM in culture than adult CFBs including structural fibrils and the TGFβ system (Kapur et al., 2007; Peng et al., 2010; Rypdal et al., 2021), while CFBs from the adult human heart are more relevant to heart failure patients. Both haCFBs and hfCFBs followed the same protocol for defreezing, seeding, passaging, stimulation and transduction, but with use of different reagents clarified below. In general, cells were kept in a 37˚C, 5% CO_2 humidified incubator, in uncoated culture flasks with 20-25 ml serum-containing medium. For experiments, cells were seeded at cell passage number six and seven, onto uncoated six-well plates at a density of $1x10^5$ cells/ml for 24 hours, allowing cells to attach. HaCFBs were defreezed, seeded and passaged according to the PromoCell's protocol and by using PromoCell's products (C-41210, C-23025, C-29912). HfCFBs were defreezed, seeded and passaged according to the Sigma-Aldrich Human Cardiac Fibroblasts Culture protocol, using Sigma-Aldrich products (115-500, 316-500).

Adenoviral transduction

Viral transduction of haCFBs and hfCFBs was performed in the same way. On day 0, cells were seeded onto uncoated six-well plates at a density of $1x10^5$ cells/ml with 2 ml of serum-containing medium. On day 1 the serum-containing medium was removed, and 1 ml of fresh serum-containing medium was added. One hour later, adenovirus serotype 5 encoding human fibromodulin (AdFMOD, #ADV-209185, Vector Biolabs, Malvern, PA) or empty vector control (AdVeh, #1300, Vector Biolabs) diluted in serum-containing medium (a total of 100 µl) was carefully added to each well, at a titer of $5x10^7$ PFU/ml. Twenty-four hours later, on day 2, the medium was removed, cells were washed in sterile PBS (at 37°C)

and 2 ml of serum-free medium was added carefully to each well. On day 3, 48 hours after viral transduction, medium, RNA, or protein lysate were harvested from the cells. Non-treated cells were also used as controls, following the same protocol, but without adding virus or empty vector. Experiments were conducted in three or four separate cell culture experiments, i.e. n = 3-4 biological replicates.

LDH assay

Medium was harvested from the cells by pipetting it into a clean 2 ml Eppendorf tube and placed directly on ice. Harvested medium was cleared by centrifugation at 5000 g for 15 minutes at 4˚C and the supernatant stored at -80˚C. Cell medium from hfCFBs was tested for the presence of lactate dehydrogenase (LDH), as a measure of cell death to account for cytotoxic effects of the adenovirus, using the Cytotoxicity Detection Kit (LDH) (Cat# 11644793001, Roche Diagnostics, Oslo, Norway). Absorbance was measured at 490 nm on the Hidex Sense Microplate Reader (LabLogic Systems, Sheffield, UK). Addition of Triton X-100 to cells was used as positive control for cell death.

Radioactive proline incorporation

A radioactive [^{3}H] proline incorporation protein synthesis assay was performed to estimate collagen synthesis in haCFBs. In brief, haCFBs were transduced with AdFMOD or AdVeh for 24 hours prior to culturing in serum-free medium containing vitamin C (ascorbic acid) 50µM/ml and 1 µCi L-[2,3-^{3}H]-Proline (Cat# NET323001MC, PerkinElmer, Inc, MA) for 48 hours. At harvest, cells were washed with cold PBS and lysed in 1M NaOH. Lysates were diluted in OptiPhase HiSafe 3 liquid scintillation cocktail (Cat# 1200.437, PerkinElmer) and incorporation of radiolabeled proline was quantified as counts per minute using the Wallac Winspectral 1414 liquid scintillation counter (PerkinElmer). Samples were measured in duplicates and serum-treated cells were used as positive control. Experiments were conducted in three separate cell culture passages at different time points (i.e. three biological replicates), with n = 3-6 technical replicates.

RNA extraction and gene expression analyses

Cells were harvested for RNA analysis by removing medium, adding 250µl of Buffer RLT (Qiagen Nordic, Oslo, Norway) with 1% β-mercaptoethanol (Sigma-Aldrich, Missouri, United States) per well. Cell scraper and pipette were used to collect the samples into 1.5 ml Eppendorf tubes. One well was collected for each sample, immediately put on ice and frozen at -80°C as soon as possible. RNA was extracted using the RNeasy Mini Kit (Qiagen Nordic, Oslo, Norway). RNA concentration was measured using the Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States). RNA quality was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was made using iScript (BioRad Laboratories, Inc., Hercules, CA), according to manufacturer's protocol. For qPCR, the following pre-designed TaqMan Real-Time PCR assays (Applied Biosystems, Foster City, CA) were used to determine gene expression: *ACTA2* (Hs00426835_g1), *COL1A2* (Hs01028956_m1), *COL3A1* (Hs00943809_m1), *CTGF* (Hs00170014_m1), *FMOD* (Hs05632658_s1), *ICAM1* (Hs00164932_m1), *LOX* (Hs00942483_m1), *PCNA* (Hs00427214_g1), *POSTN*

(Hs01566750_m1), *RPL4* (Hs03044646_g1), *TGM2* (Hs01096681_m1) and *VCAM1* (Hs01003372_m1). Results were detected in a QuantStudio 3 Real-Time PCR System, and data analyzed using QuantStudio Design & Analysis Software (Thermo Fisher Scientific). RPL4 was used as reference gene for normalization of qPCR expression. For gene array analysis, a total of nine samples (3 x 3 biological replicates) were pooled for each group (AdVeh or AdFMOD) and run on two gene arrays (TaqMan Array Human TGFB Pathway and TaqMan Array Human Extracellular Matrix & Adhesion Molecules, Thermo Fisher Scientific, Massachusetts, United States). A total of 176 genes, plus 16 endogenous control genes, were analyzed, comparing gene expression in AdFMOD vs. AdVeh samples. GAPDH was used as reference gene in these experiments. Results from these experiments are provided as supplemental information (Table SI and SII). String analysis was performed in string-db.org (version 11.0) by plotting gene names into the "multiple protein"-analysis.

Protein extraction and immunoblotting

Medium was harvested from the cells 48 hours after viral transduction. Cell lysate was harvested after removing the medium, using 200µL/well of 1X PBS-based lysis buffer containing 1% Triton X-100 (Sigma-Aldrich, Missouri, United States), 0.1% Tween-20 (Bio-Rad, CA, United States), protease inhibitors (Complete EDTA-free tablets, Roche Diagnostics, Oslo, Norway) and phosphatase inhibitors (PhosStop, Roche Diagnostics), cell scrape and pipette. Cell lysates were spun at 20 000 g for 10 minutes at 4˚C and the supernatant stored at -80˚C. Protein concentrations were measured using Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). SDS-PAGE and blotting were performed according to the Criterion BIO-RAD protocol. Membranes were blocked in non-fat dry milk (PanReac AppliChem, Barcelona, Spain) or casein (Roche Diagnostics, Oslo, Norway) prior to incubation with the following primary antibodies; fibromodulin (#NBP1_31657 (1:750), Novus Biotech), pAKT (#9271 (1:1000), Cell signaling), AKT (#9272 (1:1000), Cell signaling) or vinculin (#V9131 (1:1 000 000), Sigma-Aldrich) and appropriate HRP-conjugated secondary antibodies. Vinculin was used for loading control. Membranes were developed using Amersham ECL Prime Western Blotting Detection System (GE Healthcare, UK) in the Las-4000 (Fujifilm, Tokyo, Japan). Images of immunoblots were quantified and processed using ImageJ (NIH) and Adobe Photoshop CS6. Blots were stripped using the Western blot stripping buffer (210591, Thermo Scientific). For fibromodulin positive controls on immunoblots, human endothelial kidney (HEK)293 cells were transfected with *FMOD*-encoding plasmids, as described (Andenæs et al., 2018). For de-glycosylation, medium samples from transduced hCFBs, both AdVeh and AdFMOD, were added 1 µl of bacterial enzyme PNGase F per 20 µg sample and incubated at 37°C for 1 hour. Through this process, PNGase F de-glycosylates fibromodulin by removing N-linked oligosaccharides from the glycoprotein, leaving only the fibromodulin core protein of 42 kilodaltons (kDa).

Statistics

Data are given as group means ± standard error of means (SEM). Statistical analyses were performed using GraphPad Prism 8. The statistical tests applied were

unpaired t-test, comparing AdVeh to AdFMOD. Statistical significance was accepted for $P < 0.05$.

RESULTS

Fibromodulin was successfully overexpressed in cultured human cardiac fibroblasts

To better understand the potential anti-fibrotic role of fibromodulin in cultured human CFBs, we overexpressed fibromodulin using replication-deficient adenovirus 5 (Ad5-dE1/E3) in CFBs from adult and fetal human hearts. Cells were seeded, transduced and harvested according to a three-day protocol, where fibromodulin was overexpressed for two days (Figure 1A). We found no differences in cell death in AdFMOD vs. AdVeh-transduced cells (Figure 1B). *FMOD* was successfully increased in AdFMOD vs. AdVeh in both hfCFBs and haCFBs (Figure 1C and D, respectively). Accordingly, fibromodulin protein was increased intracellularly (Figure 1E-F), and as expected for an ECM-localized molecule, secreted into the medium in both cultures (Figure 1G-H). To confirm that the secreted fibromodulin was glycosylated, medium samples were treated with PNGase F, a bacterial enzyme that effectively removes N-linked oligosaccharides from glycoproteins. The secreted, glycosylated fibromodulin (FMODext, detected around 60kDa) was reduced to the expected fibromodulin core protein size (42kDa) after enzymatic digestion (Figure 1I). Thus, fibromodulin was successfully overexpressed, glycosylated and secreted in our human CFBs cultures.

Fibromodulin overexpression reduces expression of collagen cross-linking enzymes in human cardiac fibroblasts

First, we assessed whether fibromodulin overexpression in human CFBs affected expression of the two main structural collagens in the heart, namely collagen I (encoded by *COL1A2*) and collagen III (encoded by *COL3A1*). However, we found no differences in the expression of *COL1A2* nor *COL3A1* in hfCFBs overexpressing fibromodulin (Figure 2A). Similarly, *COL1A2* was not changed in haCFBs overexpressing fibromodulin, while we found a 16% reduction of *COL3A1* in haCFBs (Figure 2B). The radioactive proline incorporation assay used to assess collagen protein synthesis in haCFBs showed no difference in AdFMOD vs. AdVeh (Figure 2C). Thus, our results suggested that increased fibromodulin levels in human CFBs did not affect traditional fibrosis in terms of expression of the main structural collagens in the heart.

Next, we assessed whether increased fibromodulin levels in human CFBs affected expression of the collagen cross-linking enzymes lysyl oxidase (encoded by *LOX*) and transglutaminase 2 (encoded by *TGM2*). In hfCFBs, fibromodulin overexpression decreased *LOX* and *TGM2* expression by 7% and 26%, respectively (Figure 2D). In haCFBs, fibromodulin overexpression reduced *LOX* expression by 16%, while *TGM2* expression was unaffected (Figure 2E). This suggested that fibromodulin could be involved in reduction of collagen cross-linking in the heart through regulation of *LOX* expression in human CFBs.

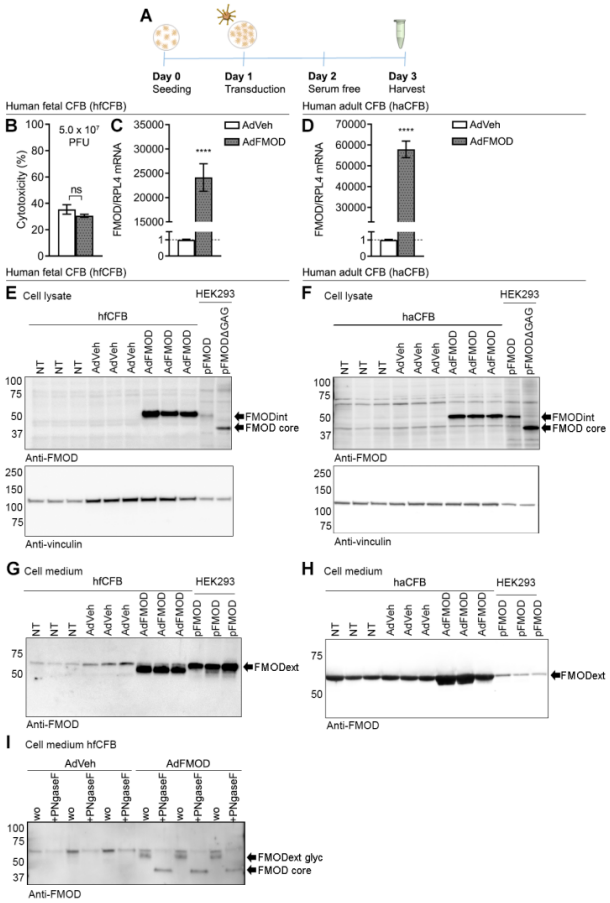

Figure 1. Fibromodulin was successfully overexpressed as a secreted glycoprotein in cultured human cardiac fibroblasts. (A) Schematic illustration of transduction protocol in cultured human fetal and adult cardiac fibroblasts (hfCFBs and haCFBs, respectively). Fibromodulin was overexpressed in hfCFBs and haCFBs using adenovirus 5 (AdFMOD) and compared to control-transduced cells with an empty adenovirus (AdVeh). (B) Percentage cytotoxicity in AdVeh vs. AdFMOD in hfCFBs (n=6). (C-D) Relative fibromodulin mRNA in hfCFBs (n=10-12, from 3 biological replicates) and haCFBs (n=12, from 3 biological replicates). RPL4 was used as reference gene. Data are shown as mean±SEM (B-D), and statistical differences were tested using an unpaired t-test, **** $P < 0.0001$; ns: not significant. (E-F) Representative immunoblots of intracellular fibromodulin (FMODint) protein in cell lysates from AdVeh and AdFMOD in hfCFBs (n=3) and haCFBs (n=3). Cell lysate from non-transduced (NT) cells was also loaded onto the gels (n=3). Vinculin was used for loading control. (G-H) Representative immunoblots of extracellular fibromodulin (FMODext) protein secreted into the cell medium in AdVeh and AdFMOD samples of hfCFBs (n=3) and haCFBs (n=3). Medium from NT cells was also loaded onto the gels (n=3).). (I) Representative immunoblots of detected extracellular fibromodulin in AdVeh and AdFMOD (the same samples as in Figure 1G, n=3), untreated (wo = without PNgaseF) or treated with the enzyme PNgaseF (+PNgaseF), removing N-linked oligosaccharides from glycoproteins, in cell medium from hfCFBs. As positive controls for fibromodulin protein immunoblot bands, human endothelial kidney (HEK)293 cells were transfected with a human FMOD-encoding plasmid (pFMOD) or a human fibromodulin mutant (pFMODΔGAG), where glycosaminoglycan (GAG) attachment sites were mutated (N127A, N166A, N201A, N291A, N341A) as described in (Andenæs et al., 2018), n=3. FMODΔGAG expression yielded a core protein of the expected size, i.e. 42 kDa, while the intracellular fibromodulin (FMODint) was slightly bigger (~50 kDa). Extracellular fibromodulin (FMODext, ~60 kDa) was detected in the HEK293 medium.

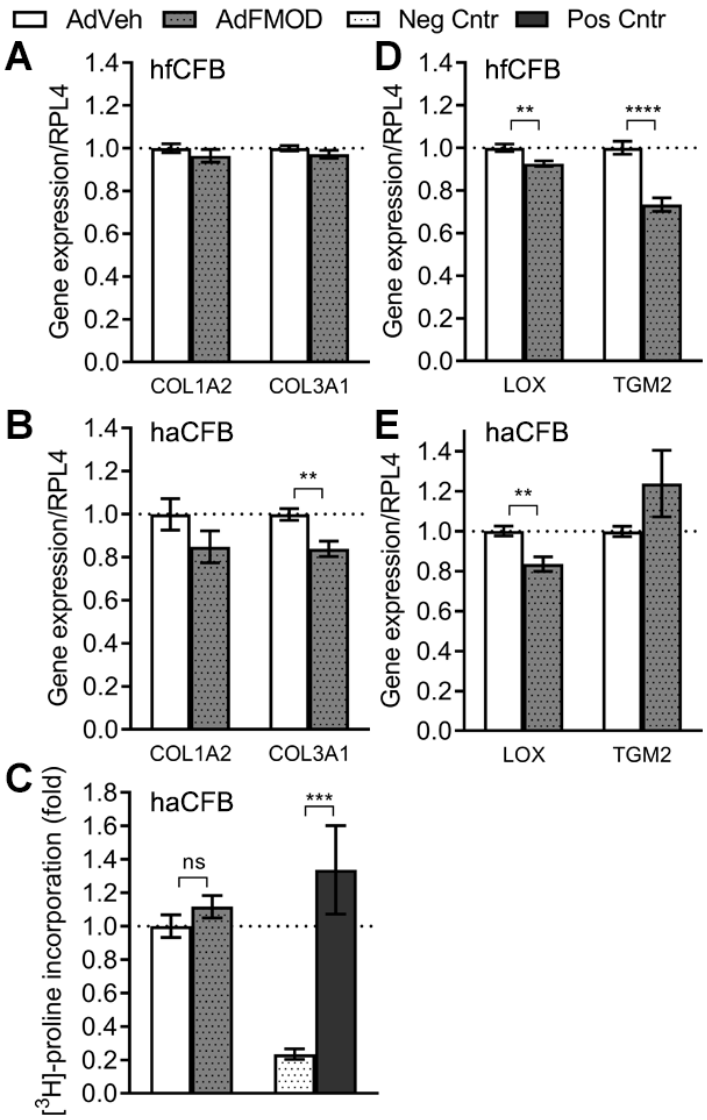

Figure 2. Fibromodulin reduces the expression of collagen cross-linking proteins. Fetal (A and D) and adult (B, C and E) human cardiac fibroblasts (hfCFBs and haCFBs, respectively) transduced with adenovirus encoding fibromodulin (AdFMOD) or empty control vector (AdVeh). (A-B) Relative gene expression of collagen 1 alpha 2 (COLA1A2) and collagen 3 alpha 1 (COL3A1), relative to housekeeping gene Ribosomal protein L4 (RPL4) in AdVeh and AdFMOD in hfCFBs (A, n=9 from 3 biological replicates) and haCFBs (B, n=11-12 from 3 biological replicates). (C) Radioactive proline incorporation was used to estimate collagen protein synthesis in AdVeh and AdFMOD in haCFBs (n=18-35 from 3 biological replicates, ns: not significant). Serum-treated haCFBs were used as positive controls (Pos Cntr) and serum free-treated haCFBs as negative controls (Neg Cntr) (n=8 from 3 biological replicates). (D-E) Relative gene expression of lysyl oxidase (LOX) and transglutaminase 2 (TGM2), relative to housekeeping gene RPL4 in AdVeh and AdFMOD in hfCFBs (A, n=9 from 3 biological replicates) and haCFBs (B, n=11-12 from 3 biological replicates). Data are shown as mean±SEM. Statistical differences were tested using unpaired t- test of AdVeh vs AdFMOD, ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

Fibromodulin overexpression affects expression of TGFβ-associated genes and reduces TGFβ activity in human cardiac fibroblasts

To examine whether fibromodulin affected TGFβ activity in human CFBs, targeted qPCR and a gene expression array with TGFβ-related genes was run in hfCFBs transduced with AdFMOD vs. AdVeh. Expression of connective tissue growth factor (encoded by *CTGF*) and periostin (encoded by *POSTN*) was measured to assess TGFβ activity, as both are direct downstream target genes of TGFβ signaling in the heart (Lorts et al., 2012; Parichatikanond et al., 2020). *CTGF* was reduced by 28% in fibromodulin overexpressing hfCFBs (Figure 3A), while it was unaffected by fibromodulin overexpression in haCFBs (Figure 3B). Importantly, fibromodulin overexpression reduced the expression of *POSTN* in both hfCFBs and haCFBs (Figure 3A-B). TGFβ-related genes whose expression was differentially regulated by fibromodulin overexpression by +/- 25% are presented in Figure 3C. We found fibromodulin overexpression to affect the expression of central TGFβ related genes (Figure 3C), e.g. reduced the expression of the pro-fibrotic interleukin-6 (IL-6) by 58%, increased the expression of different growth differentiation factors (GDF) including *GDF5* (45% increase), *GDF7* (34% increase), and *GDF15* (49% increase), whereas *GDF9* was reduced with 25%. Also, we found fibromodulin to affect direct mediators of TGFβ signaling; *SMAD1* and *SMAD2* (encoding SMAD Family Member 1 and 2, respectively). Fibromodulin overexpression reduced *SMAD2* expression by 32%, whereas *SMAD1* was increased by fibromodulin with 39% (Figure 3C). Finally, we found that the phosphoinositide 3-kinase (PI3K)/Akt (protein kinase B) pathway, a non-SMAD signaling pathway regulated by TGFβ, was inhibited by fibromodulin overexpression, through reduced protein levels of phosphorylated AKT in hfCFBs (Figure 3D). Together, these results indicated that fibromodulin reduced TGFβ activity in human CFBs.

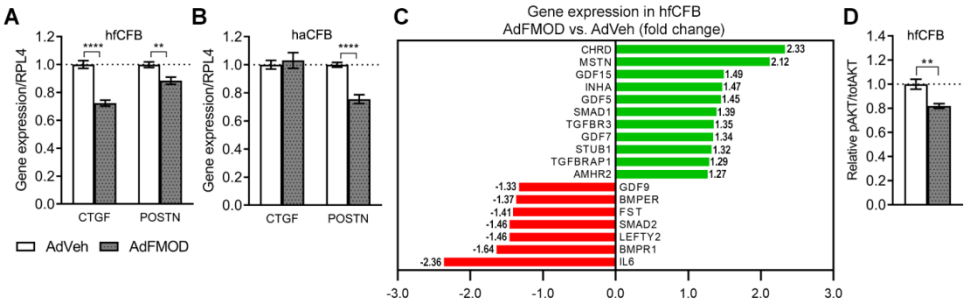

Figure 3. Fibromodulin reduced TGFβ activity and affected expression of TGFβ-associated genes in human cardiac fibroblasts. Human fetal and adult cardiac fibroblasts (hfCFBs and haCFBs, respectively) were transduced with adenovirus encoding FMOD (AdFMOD) or empty control vector (AdVeh). (A-B) Relative gene expression of CTGF and POSTN in (A) hfCFBs and (B) haCFBs, showing AdVeh vs. AdFMOD, n=9-12 from 3 biological replicates for all groups. Ribosomal protein L4 (RPL4) was used as reference gene. (C) Summary of results from gene expression array analysis of 92 TGFβ-associated genes performed on hfCFBs. Differentially regulated genes by fibromodulin +/- 25%, shown as fold change of mRNA expression in AdFMOD vs. AdVeh. Green bars show fold change of genes upregulated by fibromodulin, whereas red bars show fold change of downregulated genes by fibromodulin overexpression. (D) Relative protein levels of pAKT/totAKT in hfCFBs showing AdVeh vs AdFMOD, n=3-6 from 2 biological replicates. Data are shown as mean±SEM, and statistical differences were tested using unpaired t- test of AdVeh vs AdFMOD, ** $P < 0.01$; **** $P < 0.001$.

Fibromodulin overexpression affects the expression of ECM-associated genes including ECM-cell adhesion and integrin-laminin association molecules in human cardiac fibroblasts

To examine whether fibromodulin affected the expression of central ECM-related genes we performed a gene expression array in hfCFBs transduced with AdFMOD vs. AdVeh. ECM-related genes differentially regulated by fibromodulin overexpression +/- 25% are presented in Figure 4A. We found fibromodulin overexpression to affect the expression of central ECM-related genes. Fibromodulin increased the expression of *ITGA6* (encoding integrin alpha 6) by 88%, and the laminin encoding genes *LAMA3* and *LAMB3* by 45% and 91%, respectively. Furthermore, fibromodulin decreased the expression of the integrin encoding genes *ITGA7* (encoding integrin alpha 7) by 43%, *ITGB4* (encoding integrin beta 4) by 72%, and *ITGB2* (encoding integrin beta 2) by 75% (Figure 4A). To further investigate the relationship between all the differentially expressed genes we performed a STRING-analysis (freely available from https://string-db.org/). These analyses indicated that fibromodulin may have an effect on the integrin-laminin association and integrin signal transduction, thus affecting cell-ECM adhesion (Figure 4B). The gene array analysis indicated that fibromodulin decreased the expression of the central immune cell adhesion molecules as *ICAM1* and *VCAM1* (encoding intercellular adhesion molecule-1 and vascular cell adhesion molecule 1, respectively). This was confirmed by qPCR in both hfCFBs and haCFBs (Figure 4C and D, respectively). In hfCFBs *ICAM1* was reduced 30% and *VCAM1* was reduced 41% by fibromodulin (Figure 4C). In haCFBs *ICAM1* and *VCAM1* were reduced 32% and 26%, respectively (Figure 4D). These results indicated that fibromodulin may reduce leukocyte infiltration.

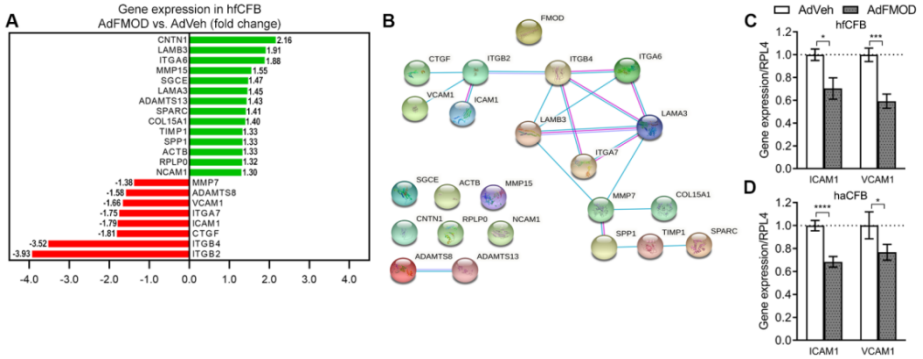

Figure 4. Increased fibromodulin levels in human cardiac fibroblasts affects the expression of ECM-associated genes and may have an effect on immune cell infiltration, integrin-laminin association and integrin signaling. Human fetal cardiac fibroblasts (hfCFBs) were transduced with adenovirus encoding FMOD (AdFMOD) or empty control vector (AdVeh). (A) Summary of results from gene expression array analysis of 84 ECM-associated genes performed on AdFMOD vs. AdVeh. Genes regulated by fibromodulin overexpression +/- 25%, shown as fold change of mRNA expression in AdFMOD vs. AdVeh. Green bars show fold change of genes upregulated by fibromodulin, whereas red bars show fold change of decreased genes by fibromodulin overexpression. (B) STRING analysis (from the freely available STRING database *https://string-db.org/*) of genes from figure 3A. The strings illustrate known interactions; blue strings show known interactions from databases, whereas pink strings show known interactions from experiments (with high confidence 0.007, see more in methods). (C-D) Relative gene expression of ICAM1 and VCAM1 (encoding intercellular adhesion molecule-1 and vascular cell adhesion molecule 1, respectively), in AdFMOD and AdVeh from (C) hfCFBs and (D) haCFBs (n=9-12 from 3 biological replicates in all groups). Ribosomal protein L4 (RPL4) was used as reference gene. Data are shown as mean±SEM, and statistical differences were tested using unpaired t- test of AdVeh vs AdFMOD, *P < 0.05; *** P < 0.005; **** P <.001.

DISCUSSION

To investigate the translational potential of our previous experimental findings in animals, we examined how fibromodulin overexpression in cultured human CFBs affected the expression of fibrosis-, TGFβ- and ECM-related genes, aiming to increase our knowledge about the role of fibromodulin in human cardiac fibrosis. We found that fibromodulin overexpression decreased the expression of the fibrosis-associated and ECM cross-linking enzymes LOX and TGM2 (Figure 2). Furthermore, we found that fibromodulin overexpression reduced TGFβ activity and altered the expression of several TGFβ-related proteins (Figure 3). Finally, fibromodulin overexpression altered the expression of laminin, integrin, and immune cell adhesion molecules (Figure 4). Our results show a translational value of our findings from experiments in mice *in vivo* and neonatal rat cells *in vitro* (Andenæs et al., 2018). Fibromodulin binds directly to collagen fibrils (Kalamajski and Oldberg, 2007; Tillgren et al., 2016) and has anti-fibrotic effects during wound healing (Pang et al., 2020; Zheng et al., 2017; Zheng et al., 2016). Our *in vitro* findings that fibromodulin promotes anti-fibrotic properties in rat and human CFBs (Andenæs et al., 2018), are in line with this.

Increasing levels of structural collagens (mainly type I and III) and collagen cross-linking are key drivers in progression of cardiac fibrosis (Lopez et al., 2010; Bjørnstad et al., 2011; Pang et al., 2020; Parichatikanond et al., 2020). In line with our findings from fibromodulin knock-out mice and AdFMOD-transduced CFBs from neonatal rat hearts (Andenæs et al., 2018), we did not see that fibromodulin affected expression levels of structural collagens type I and III. Importantly, we here show that fibromodulin decreased the expression of the ECM and collagen cross-linking enzymes LOX and TGM2 in human CFBs. Both LOX and TGM2 bind to collagen, increase collagen cross-linking and play a role in cardiac fibrosis (Lopez et al., 2010; Wang et al., 2018; Al-U'datt et al., 2019; Erasmus et al., 2020; Neff and Bradshaw, 2021). Furthermore, Kalamajski *et al.* (2106) identified a binding between fibromodulin and LOX, and suggested a fibromodulin-modulated collagen cross-linking mechanism. Our results are in line with our previous findings in neonatal rat heart CFBs, where both enzymes were reduced by increased fibromodulin levels (Andenæs et al., 2018). Thus, with a translational perspective, we here confirm that fibromodulin reduces the levels of *LOX* and *TGM2* in human CFBs (Figure 2). LOX and TGM2 are attractive drug targets for anti-fibrotic cardiac therapies (Lopez et al., 2010; Lorts et al., 2012; Wang et al., 2018; Al-U'datt et al., 2019; Erasmus et al., 2020;), and both have been linked to increased activity of the pro-fibrotic cytokine TGFβ (Wang et al., 2018; Erasmus et al., 2020;). Understanding their regulation is of importance for future research towards improved treatment for cardiac fibrosis.

Fibromodulin has previously been shown to bind TGFβ in the ECM and inhibit its activity (Hildebrand et al., 1994; Soo et al., 2000). Here we found that fibromodulin overexpression in human CFBs reduced the expression of direct downstream targets of TGFβ activity, namely *CTGF* and *POSTN* (Figure 3). *POSTN*, *CTGF* and *TGM2* are regulated by TGFβ (Cao et al., 2012; Lorts et al., 2012; Vainio et al., 2019). Thus, our results are in line with our previous findings in neonatal rat CFBs (Andenæs et al., 2018), suggesting that fibromodulin reduces TGFβ activity. As fibromodulin expression is induced by TGFβ (Barry et al., 2001), this may represent a negative feedback loop mechanism for TGFβ. *LOX* regulation by TGFβ in CFBs requires activation of PI3K/Akt (Al-U'datt et al.,

2019), and here we found that fibromodulin decreases *LOX* expression and Akt activation (Figure 2 and 3). Furthermore, we found that fibromodulin reduces *SMAD2* expression. Together, these results indicate that fibromodulin reduced TGFβ activity through SMAD2 and/or Akt. Whether the reduction directed by fibromodulin was due to direct TGFβ-binding in the ECM or other mechanisms cannot be determined from these experiments.

POSTN, CTGF and TGM2 are central pro-fibrotic proteins in the heart (Cao et al., 2012; Lorts et al., 2012; Wang et al., 2018; Vainio et al., 2019). Our finding that fibromodulin reduces the levels of these molecules in human CFBs (Figure 2 and 3) therefore suggests that the increased levels of fibromodulin found in the failing mouse and patient heart (Andenæs et al., 2018) have anti-fibrotic effects. In line with this, studies have shown that fibromodulin promotes fetal-type scarless wound healing in skin, and that administration of fibromodulin reduces scar size in skin wounds of rodents and pigs (Zheng et al., 2016; Zheng et al., 2017; Jiang et al., 2018; Pang et al., 2020; Soo et al., 2000). Interestingly, it has been shown that adding TGFβ neutralizing antibodies early after wound induction reduces scarring in rodents (Barry et al., 2001). Taken together, this supports our finding that fibromodulin reduced TGFβ activity.

Our mRNA expression arrays suggested novel pathways regulated by fibromodulin in CFBs that should be explored in future studies. As such, fibromodulin increased a downstream pathway of TGFβ, SMAD1. We found that fibromodulin increased *GDF15* expression (Figure 3), which activates SMAD1. *GDF15* is not expressed in the healthy heart, but it is highly and rapidly increased upon injury or disease, and is believed to be cardio-protective (Xu et al. 2006; Bjørnstad et al., 2011; Wesseling et al., 2020). Furthermore, we found that fibromodulin decreased the expression of IL-6 (Figure 3), which is known to be pro-fibrotic, pro-hypertrophic, pro-inflammatory and crucial to cardiac function in the acute response after injury (Meléndez et al., 2010; Hall et al., 2021). However, when chronically increased, IL-6 leads to extensive cardiac fibrosis and myocardial hypertrophy with increased ventricular stiffness (Meléndez et al., 2010; Hall et al., 2021). Additionally, IL-6 is increased, and correlates with severity, in patients with hypertension and heart failure, and is predictive of mortality (Meléndez et al., 2010; Hall et al., 2021). Thus, fibromodulin reduced the pro-fibrotic arms of TGFβ, while increasing cardio-protective arms. Furthermore, we found that fibromodulin might affect the anti-fibrotic and cardiac-protective pathway Neuregulin-1/ERBB. Fibromodulin also increased *ITGA6* expression, whereas expression of *ITGB4* was reduced (Figure 4). *ITGA6* and *ITGB4* forms a heterodimer known as the laminin receptor, binding laminin332, which consists of LAMA3, LAMB3 and LAMC2 (Hegde and Raghavan, 2013). We found that fibromodulin increased *LAMA3* and *LAMB3* expression (Figure 4). The ITGA6:ITGB4 receptor binds NRG1, and is essential for NRG1-ERBB signaling (Lemmens et al., 2007). The NRG1-ERBB signaling pathway is known to be beneficial during heart failure and cardio-protective (De Keulenaer et al., 2019), and NRG1 has anti-fibrotic and anti-inflammatory effects in the heart (De Keulenaer et al., 2019; Lemmens et al., 2007).

We here found that fibromodulin decreased the expression of *ICAM1* and *VCAM1* (Figure 4), central immune cell adhesion molecules, in human CFBs, which is in line with our previous findings in neonatal rat CFBs overexpressing fibromodulin (Andenæs et al., 2018). Additionally, fibromodulin decreased the expression of *ITGB2* (Figure 4), which is part of the heterodimeric receptor of both ICAM1 and VCAM1. These results indicate that fibromodulin is involved in regulation of the cardiac immune response. Indeed,

fibromodulin has been linked to possible immune cell responses in previous studies of fibrotic lung parenchyma, and different types of cancers (Rydell-Tormanen et al., 2014; Pourhanifeh et al., 2019).

A significant limitation of our study is that it was performed entirely *in vitro* and focused on mRNA expression changes. Nevertheless, as previous animal studies have indicated an anti-fibrotic role of fibromodulin, this is a first step towards investigating the translational potential of our previous findings and the effects of fibromodulin in the human heart. Thus, we consider our results to be of interest to human cardiac disease and ECM research fields, as there is a great need for increased understanding of the mechanisms of cardiac fibrosis and to reveal anti-fibrotic treatment targets. We used commercially available human CFBs for our study, i.e. hfCFBs and haCFBs. While CFBs from adult hearts are more relevant to human patients, the fetal CFBs constitute an important research tool as they express a more mature ECM in culture, including the TGFβ signaling system, which is considered hard to study in cultured cells (Kapur et al., 2007; Peng et al., 2010; Rypdal et al., 2021).

In conclusion, our results indicate that fibromodulin has anti-fibrotic properties in cultured human CFBs by decreasing the expression levels of collagen cross-linking enzymes, TGFβ signaling, immune cell adhesion, and ECM-cell adhesion molecules.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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