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Original Article

Cardiac FGF23: new insights into the role and function of FGF23 after acute myocardial infarction☆,☆☆

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ABSTRACT

Objective: We aimed to elucidate the local role of FGF23 after myocardial infarction in a mouse model induced by left anterior descending artery (LAD) ligation.
Approach and results: (C57BL/6 N) mice underwent MI via LAD ligation and were sacrificed at different time-points post MI. The expression and influence of FGF23 on fibroblast and macrophages was also analyzed using isolated murine cells.
We identified enhanced cardiac FGF23 mRNA expression in a time-dependent manner with an early increase, already on the first day after MI. FGF23 protein expression was abundantly detected in the infarcted area during the inflammatory phase. While described to be primarily produced in bone or macrophages, we identified cardiac fibroblasts as the only source of local FGF23 production after MI. Inflammatory mediators, such as IL-1β, IL-6 and TNF-α, were able to induce FGF23 expression in these cardiac fibroblasts. Interestingly, we were not able to detect FGF23 at later time points after MI in mature scar tissue or remote myocardium, most likely due to TGF-β1, which we have shown to inhibit the expression of FGF23. We identified FGFR1c to be the most abundant receptor for FGF23 in infarcted myocardium and cardiac macrophages and fibroblasts. FGF23 increased migration of cardiac fibroblast, as well as expression of Collagen 1, Periostin, Fibronectin and MMP8. FGF23 also increased expression of TGF-β1 in M2 polarized macrophages.
Conclusion: In conclusion, cardiac fibroblasts in the infarcted myocardium produce and express FGF23 as well as its respective receptors in a time-dependent manner, thus potentially influencing resident cell migration. The transitory local expression of FGF23 after MI points towards a complex role of FGF23 in myocardial ischemia and warrants further exploration, considering its role in ventricular remodeling.

1. Introduction

Despite a magnitude of diagnostic and therapeutic progress, acute and chronic ischemic heart failure, particularly in combination with renal failure, is still associated with a high morbidity and mortality [1]. Recent observational studies in humans suggest that fibroblast growth factor 23 (FGF23) is linked to the pathophysiology of heart failure [2], increased mortality [3–5] and pathological cardiac hypertrophy [6–9].

FGF23 is mainly produced in osteocytes and osteoblasts, preventing mineralization and sustaining stem cell differentiation [10]. FGF23 also acts directly on kidney tubular structures regulating phosphate homeostasis [11–13] via increases in renal phosphate excretion and parathyroid hormone activity [12,13]. FGF23 is considered to be a

Abbreviations: FGF23, Fibroblast growth factor 23; LAD, Left anterior descending artery; FGFR, Fibroblast growth factor receptor; ACTA2, Smooth muscle alpha actin; FCS, Fetal calf serum; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TGF, Transforming growth factor; TNF, Tumor necrosis factor; MI, Myocardial infarction; BMDM, Bone marrow-derived macrophage.

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valuable biomarker for phosphate metabolism disturbances in renal failure [14].

Independently of its effect on phosphate homeostasis, serum FGF23 has been established as a strong and independent factor to predict the cardiovascular risk in various cohorts [15–18]. Increased FGF23 directly induces hypertrophy in cardiomyocytes [19,20], since the blockade of the FGF23 pathway in cardiomyocytes could significantly reduce cardiomyocyte hypertrophy [21,22]. Thus, FGF23 is assumed to be important in the context of chronic kidney disease patients who have a demonstrated increased risk of a cardiovascular death [9].

Recent data have shown an association between FGF23 and the de-novo development of heart failure [16,23,24] as well as with subsequent hospitalizations due to heart failure [25] after an acute myocardial infarction. Furthermore, a significant rise in serum FGF23 is also documented in cardiogenic shock patients after MI, with a strong negative correlation between the clinical outcome and mortality of patients [5]. These data suggest that FGF23 may have additional unknown roles, independent of what is believed to date. Therefore, this study aims to further elucidate the basic mechanisms of the effects and origin of FGF23. Very recent data show that FGF23 production is not necessarily limited to the production in bone cells. A local production in different organs is also currently being discussed [26–28], but the extent and effect of extraskeletal FGF23, and specifically cardiac production, is still under investigation.

Considering these data, we hypothesized that FGF23 could also have an as yet to be defined role in myocardial ischemia and healing after MI, which we aim to investigate in this study.

2. Methods

2.1. Myocardial Infarction

Male C57BL/6 N mice (Charles River, Germany) 8–10 weeks old were intubated under general anesthesia (100 mg/kg ketamine, 10 mg/kg xylazine, i.p.) and analgesia (0.1 mg/kg buprenorphine). The mice were ventilated using a rodent respirator with positive pressure and oxygen. A left-sided thoracotomy was performed, and MI was induced by permanent ligation of the proximal left anterior descending artery (LAD) [29]. The ribs, muscle and skin incisions were closed with separate sutures. The analgesia was continued 5 days after infarction using 0.1 mg/kg buprenorphine every 8 h. All mice were housed under standardized conditions in the Animal Facility of the University Hospital Aachen (Germany). All animal experiments and study protocols were approved by local authorities, complying with European and German animal protection laws (AZ: 84–02.04.2013.A185).

2.2. Histology and immunohistochemistry

FGF23 (antibodies-online, ABIN714461; working concentration: 10 μg/ml) expression was evaluated at different time points after MI induction. Mice were anesthetized (100 mg/kg ketamine, 10 mg/kg xylazine, i.p.) and the hearts were excised, fixed in formalin and embedded in paraffin. Two sections per heart were stained to analyze the infarcted area for FGF23. Co-staining with alpha actin (SMA, DAKO, M 0851 clone 1A4; working concentration: 0.35 μg/ml) or monocyte marker (Mac3, BD Pharmingen, 550,292; working concentration: 1 μg/ml) was performed. Positive-stained cells or double positive-stained cells were counted in three different fields per section and expressed as cells per power field (200-fold magnification).

2.3. Isolation and cultivation of cardiac fibroblasts and bone marrow-derived macrophages (BMDM)

Male C57BL/6 N mice (Charles River, Germany) 8–10 weeks old were sacrificed by ketamin/xylazine overdose. For macrophage isolation, tibia and femur bones were isolated under sterile conditions and all muscle tissue was removed. The bone marrow was flushed out of the bone with sterile PBS. The resultant cell suspension was resuspended in RPMI 1640 medium with L-glutamine, 50 mM HEPES, 10% FCS, 1% penicillin/streptomycin and 15% L929-conditioned media (LCM). After 8 days the mature BMDM were utilized in the experiments. For cardiac fibroblasts, hearts were excised, cut into small pieces and enzyme digested using collagenase/dispase (Sigma Aldrich). The resultant cell suspension was resuspended in DMEM medium with 1% penicillin/streptomycin and 10% FCS to cultivate the cardiac fibroblasts [30].

2.4. Polarization and stimulation of bone marrow-derived macrophages (BMDM)

The mature BMDM were stimulated for 24 h to polarize in either M1 or M2. M0 were incubated in medium without stimulation. M1 were incubated with 20 ng/ml IFN-γ (Pepro Tech) and 10 ng/ml LPS (Sigma Aldrich). M2 were incubated with 20 ng/ml IL-4 (Pepro Tech). Polarized BMDMs were incubated for 24 h with 50 ng/ml FGF23 (RD Systems). Finally, the cells were lysated for RNA extraction as described below.

2.5. In vitro stimulation of cardiac fibroblasts

Isolated murine cardiac fibroblasts were seeded into 6-well plates (200,000 cells/well) in DMEM medium with 1% penicillin/streptomycin and 10% FCS and cultivated until 85% confluence. Twenty-four hours before starting the experiments, the cells were incubated in low FCS medium (DMEM with 1% penicillin/streptomycin and 1% FCS). Afterwards, the cells were incubated for 24 h with 10 ng/ml IL-1β (Pepro Tech), 10 ng/ml IL-6 (Pepro Tech), 25 ng/ml TNF-α (Pepro Tech), 10 ng/ml TGF-β (Cell Signaling) or 50 ng/ml FGF23 (RD Systems). Finally, the cells were lysated for RNA extraction as described below.

2.6. Flow cytometry analysis

Cells were fixed and stained with anti-alpha-actin (clone A4, DAKO, Germany), followed by secondary anti-FITC antibody and quantified using FACSCanto flow cytometer and FACSDiva Software (BD Bioscience).

2.7. RNA isolation, cDNA transcription and real-time PCR (RT-PCR)

Total RNA was extracted from cultured cells and mouse heart tissues (Qiagen Kit) and transcribed (Qiagen Kit). Quantitative real-time PCR was performed using PowerUp SYBR Green Master Mix (Thermofischer) and ViiA™ 7 Real-Time PCR System (Applied Biosystems) targeting the genes of FGF23, FGFR1c, FGFR3c, FGFR4, MMP8, periostin, fibronectin, collagen 1, collagen 3, Il 6, Il 1ß, TGF β1, Arginase 1 and β-actin (primer sequences are listed in Table 1).

2.8. Migration assay

Migration was performed in low-serum medium (DMEM with 1% FCS and 1% penicillin/streptomycin), using transwell with an 8 μm pore size from Sigma. A total of 50,000 cells were seeded into transwells and allowed to transmigrate for 24 h in low-serum medium. Transmigrated cells were counted in five different fields per condition under the microscope.

2.9. Serum

Serum was collected from the mice before euthanasia under general anesthesia (100 mg/kg ketamine, 10 mg/kg xylazine, i.p.) using cardiac punction at different time points after MI (1 d, 4 d, 7 d, 14 d, 28 d).

Table 1
List of the primer sequences.

Gene	Primer Sequence
β-actin	Forward: 5′-AGG CAT GTA CGT AGC CAT CC-3′
	Reverse: 5′-CTG TCA GCT GTG GTG GTG AA-3′
FGF23	Forward: 5′-TGA CTC GAA GGT TCC TTT GTA TG-3′
	Reverse: 5′-ATG CTT CTG CGA CAA GTA GAC-3′
FGFR1c	Forward: 5′-GAC TGC TGG AGT TAA TAC C-3′
	Reverse: 5′-CTT CCA GGG CTT CCA GA-3′
FGFR3c	Forward: 5′-GAG GAG ACC CTG GAA AAG CG-3′
	Reverse: 5′-GAG AAC GCC TCT GTG GAG AC-3′
FGFR4	Forward: 5′-ACC AAC ACT GGA GCC TGG T-3′
	Reverse: 5′-AGG AGA TAG CTG TAG CGA ATG C-3′
MMP8	Forward: 5′-GAA GGC AGG AGA GGT TGT-3′
	Reverse: 5′-TGG AGG AAG ATG AGT AAT GGA A-3′
Periostin	Forward: 5′-AAG GAA AAG GGT CAT ACA CGT ACT TC-3′
	Reverse: 5′-CCT CTG CGA ATG TCA GAA TCC-3′
Fibronectin	Forward: 5′-CGA GGT GAC AGA GAC CAC AA-3′
	Reverse: 5′-CTG GAG TCA AGC CAG ACA CA-3′
Collagen 1	Forward: 5′-ACT ACT GGA GAA GTT GGC AAG C-3′
	Reverse: 5′-GTA CCA CGT TCT CCT CTT GGA C-3′
Collagen 3	Forward: 5′-TCT GAG CTG CTT CTT CCT CTC T-3′
	Reverse: 5′- GAAGAAACCAGGTTCCACTTTG-3′
Il 6	Forward: 5′-GCT ACC AAA CTG GAT ATA ATC AGG A-3′
	Reverse: 5′-CCA GGT AGC TAT GGT ACT CCA GAA-3′
Il 1β	Forward: 5′-CAG CTG AAA GCT CTC CAC CTC-3′
	Reverse: 5′-CTT TCC TTT GAG GCC CAA GGC-3′
TGF β1	Forward: 5′-AGT GTG GAG CAA CAT GTG GAA C-3′
	Reverse: 5′-TTC AGC CAC TGC CGT ACA AC-3′
Arginase 1	Forward: 5′-GAA CCC AAC TCT TGG GAA GAC-3′
	Reverse: 5′-GGA GAA GGC GTT TGC TTA GTT-3′

2.10. Mass spectrometry protein analysis

To analyze the FGF23 serum level, serum samples were collected at different time points after MI. Samples were washed/equilibrated with ammonium bicarbonate in acetonitrile and digested with 0.02 μg trypsin at 37 °C for 24 h. The resulting peptides were desalted and concentrated using the ZipTipC18 (Millipore, USA) technology. The eluates of the ZipTipC18 were spotted directly onto the MALDI target (Bruker-Daltonic, Germany) using a-cyano-4-hydroxycinnamic acid as matrix. The subsequent analyses were carried out using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) using the MALDI-Lift fragment option (MALDI-TOF/TOF-MS). Calibrated and annotated spectra were subjected to a database search (Swiss-Prot, Zürich, Switzerland) using Bruker Bio-Tool 3.2 and the Mascot 2.2 search engine, which compared the experimental MALDI-TOF-MS and MALDI-TOF/TOF-MS data sets with the calculated peptide masses in the sequence database for each entry. Using empirically determined factors, a statistical weight was assigned to each individual peptide match.

2.11. Statistical analysis

Data represent mean±S.E.M. Statistical analysis was performed with Prism 7 software (GraphPad). The means of two groups were compared with unpaired Student's-t test, using Welch's correction by significant variance. More than two groups were analyzed using 1-way ANOVA followed by Newman–Keuls post hoc test or 2-way ANOVA followed by Bonferroni's multiple comparison test, in the case of a more than two variable parameter, as indicated. *P*-values of<0.05 were considered significant.

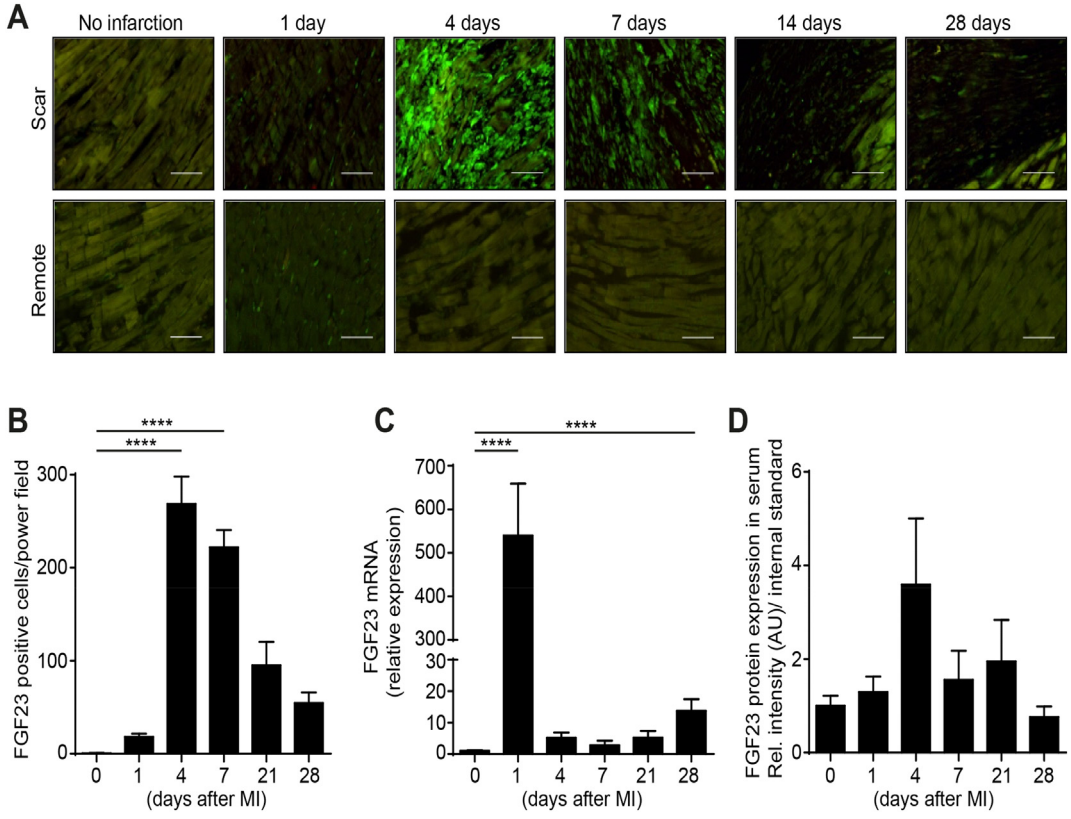

Fig. 1. *FGF23 expression after myocardial infarction in infarcted and remote areas.* **A)** Representative pictures of FGF23 expression (green) in remote area and in infarcted area of wild-type mice (C57/BL6) at different time points after MI induction using immunohistochemistry (scale bar=50 μm). **B)** Quantification of FGF23-positive cells at different time points after MI in infarcted area; *n*=3–6. **C)** RT-PCR analysis of *FGF23* mRNA in myocardial infarction area at different time points after MI. Data was normalized to mean of control mice without MI induction; *n*=6. **D)** Quantification of serum FGF23 at different time points after MI using MALDI-TOF mass spectrometry; *n*=7. Results are presented as means±S.E.M.; ****$P<.0001$.

3. Results

3.1. FGF23 localization and expression after myocardial infarction

FGF23 staining during healing after MI showed an increased protein expression of FGF23 after 4 and 7 days in the injured, infarcted area (Fig. 1A, upper panel), but not in the remote area (Fig. 1A, lower panel). No FGF23 expression was observed at later time points after MI. The quantification of FGF23-positive cells confirm an increased expression of FGF23 4 days (268.50±29.71, n=6) and 7 days (222.10±18.59, n=6) after MI vs. control (0.6667±0.1667, n=3, ****P<.0001) (Fig. 1B). Investigating the FGF23 mRNA expression at different time points after MI by RT-PCR, we found an increase in FGF23 mRNA expression only 1 day (540.2±118.8, n=8) after myocardial infarction vs. control (1.000±0.2487, n=8, ****P<.0001) (Fig. 1C). Meanwhile, quantification of serum FGF23 at different time points after MI using MALDI-TOF mass spectrometry showed a corresponding trend, but no significant changes during the healing after MI (Fig. 1D).

3.2. Cells expressing FGF23 during healing after MI

Considering the importance of macrophages [31,32] and myofibroblasts/fibroblasts [33,34] in repair after MI, we investigated FGF23 expression in cardiac macrophages and SMA-positive fibroblasts. Macrophages are strongly positive for FGF23 (Fig. 2A) during the healing phase after MI. The quantification of FGF23/Mac3 double-positive cells (Fig. 2B) showed an increase at day 4 (187.40±34.78/field n=6, ***P<.001) and day 7 (152.80±21.11/field n=6, **P<.01) after MI vs. control (0.50±0.00/field, n=3), decreasing at later time points. Further, to analyze the FGF23 synthesis in the macrophages, we isolated them from bone marrow and differentiated into M1 and M2 macrophages. As expected, M1 polarized macrophages (209.5±25.40, n=8) showed a significant higher expression of IL6 compared to the unpolarized macrophages M0 (1664±0.6131, n=8, ****P<.0001) (Fig. 2C). Interestingly, we were not able to find any mRNA expression of FGF23 in the isolated macrophages, even after their polarization (Fig. 2D). Analyzing the FGF23 receptors, we could not detect FGFR4 or FGFR3c in macrophages and polarized macrophages, rather only FGFR1c was detected (Fig. 2D). Moreover, FGFR1c was present on the undifferentiated or M2 macrophages, but not on M1 macrophages (Fig. 2E). This suggests that even if the macrophages are not able to be synthesized FGF23, they can strongly bind FGF23 via FGFR1c.

We also found FGF23 expression in cardiac fibroblasts (Fig. 3A). The quantification of FGF23/SMA double-positive cells (Fig. 3B) showed an increase at day 7 (99.20±34.49 vs. control 0.33±0.58, n=5, *P<.05) after MI. In vitro, isolated fibroblasts activated and differentiated towards α-SMA-positive myofibroblasts phenotype (Fig. 3C) during healing after MI under transforming growth factor beta (TGF-β) [35], significantly increasing alpha-actin expression in these cells (38.39±7.866 vs. 1.147±0.7434, n=3, *P<.05, Fig. 3C). Interestingly, it seemed that TGF-β is the main regulator and inhibitor of the FGF23 expression (1.00±0.3432 vs. 26.27±4.145, n=6, **P<.01, Fig. 3D), while it stops the inflammatory phase after MI [36]. Moreover, inflammatory modulators such as TNF-α, IL-6 and IL-1β induced FGF23 upregulation in fibroblasts (TNF-α: 59.74±3.719vs. 26.27±4.145, n=6, ****P<.0001, Fig. 3E; IL-1β: 743.30±77.58 vs. 26.27±4.145, n=6, ****P<.0001, Fig. 3F; IL6: 1598±47.39 vs. 54.29±10.10, n=5–6, ***P<.001, Fig. 3G), however, TGF-β1 was able to completely block this increase (Fig. 3E-G). On the contrary, the anti-inflammatory

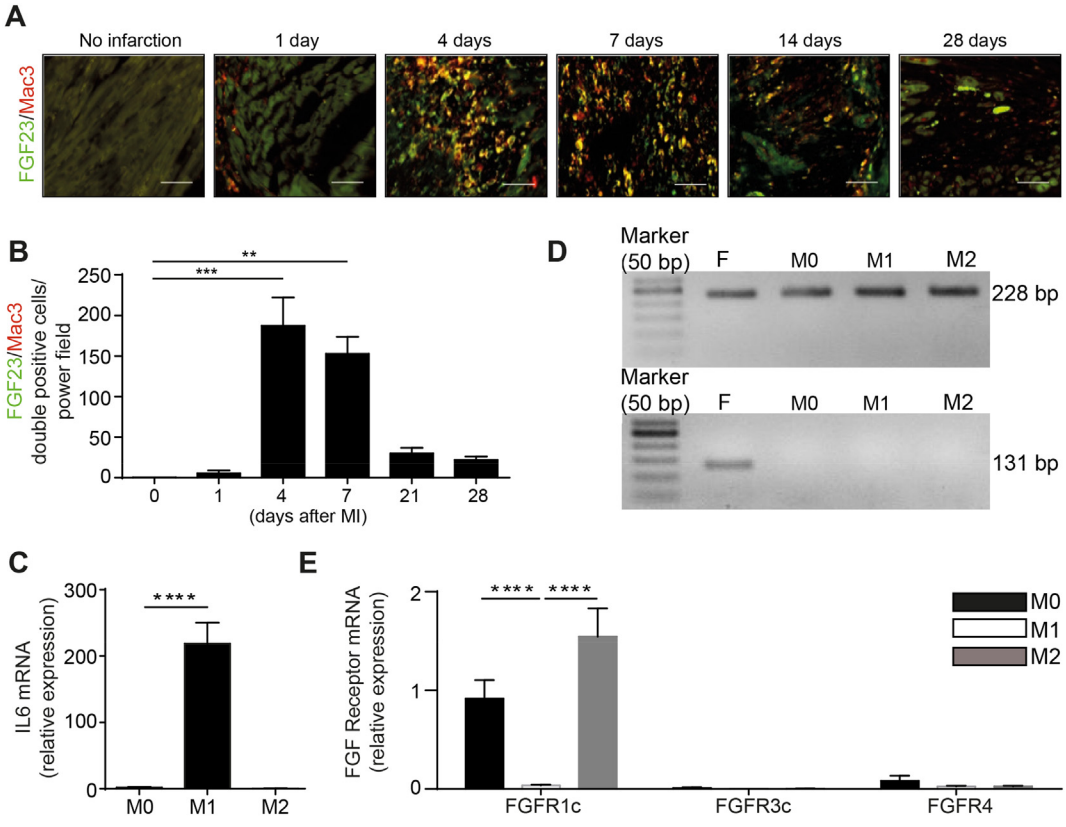

Fig. 2. *FGF23 expression in macrophage areas after myocardial infarction.* **A)** Representative pictures of FGF23 (green) and MAC3 (red) co-expression (yellow-orange, upper panel) in infarcted area of wild-type mice (C57/BL6) at different time points after MI induction using immunohistochemistry (scale bar=50 μm). **B)** Quantification of FGF23 and MAC3-positive cells at different time points after MI in infarcted area, n=3–6. **C)** IL6 mRNA expression after polarization in M1 or M2 (M0: no polarization) in BMDM (C57/BL6); n=8. **D)** Agarose gel electrophoresis of PCR transcripts (228 bp β-actin; 131 bp FGF23; F: Fibroblast; M0, M1, M2 polarized BMDM). **E)** FGF Receptor mRNA expression after polarization in M1 or M2 (M0: no polarization) in BMDM (C57/BL6); n=8. Data was normalized to mean of control. Results are presented as means±S.E.M.; *P<.05; **P<.01; ***P<.001; ****P<.0001.

cytokine interleukin 10 (IL-10) significantly inhibited FGF23 synthesis in fibroblasts (1.009±0.04970, n=6 vs. 4.228±0.2582, n=6, ****P<.0001, Fig. 3H).

Analyzing the expression of FGF23 receptors on isolated fibroblasts, we found out that FGFR1c is the most abundantly expressed receptor, with a 50-fold higher expression compared to FGFR4 (1.009 ± 0.3918, n=6, ****P<.0001) and to FGFR3c (0.3650±0. 0.1031, n=6, ****P<.0001, vs. FGFR1c mRNA expression (50.07±8.279, n=6, Fig. 3I).

Further, once we showed that the main inflammatory cytokine [37], IL-1β, increases FGF23 expression in cardiac fibroblasts, we investigated the expression of FGF23 receptors (FGFRs) after stimulation of cardiac fibroblast with IL-1β. The expression of FGFR4 did not increase after stimulation with IL-1β (0.9203±0.3930, n=6 vs. control 0.9853± 0.1104, n=6, Fig. 3J). FGFR3c expression was also not changed after IL-1β stimulation (0.7788±0.09412, n=6 vs. control 1.047±0.1484, n=6, Fig. 3K). However, there was a significant increase in FGFR1c expression after stimulation with IL-1β (2.357±0.2354, n=6 vs. control 0.9307±0.04408, n=6, **P<.01, Fig. 3L), which pointed to FGFR1c as the main receptor responsible for FGF23-fibroblast-related effects during healing after MI.

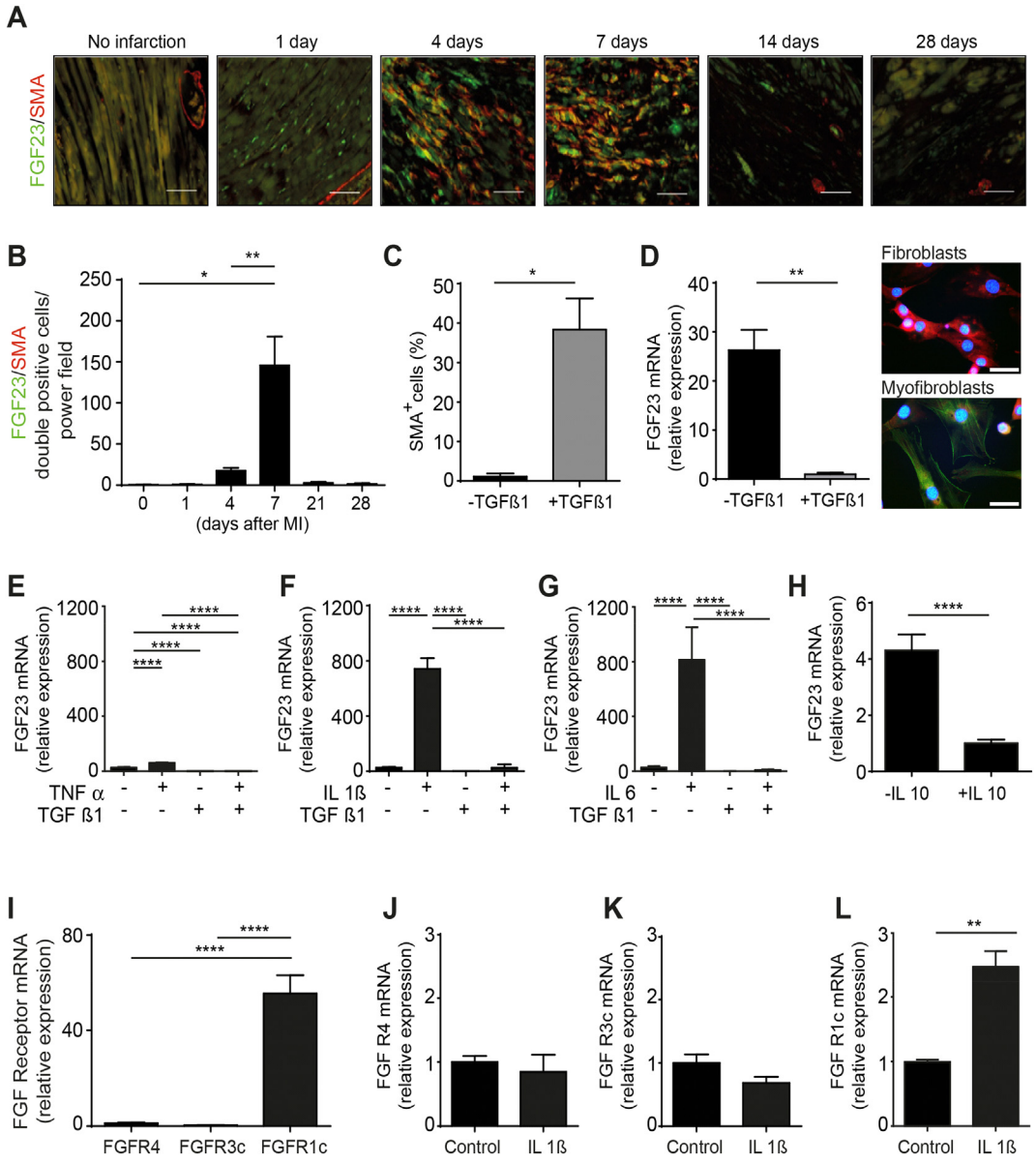

Fig. 3. *FGF23 Expression in cardiac fibroblasts after myocardial infarction.* **A)** Representative pictures of FGF23 (green) and α-SMA (red) co-expression (yellow-orange, lower panel) in infarcted area of wild-type mice (C57/BL6) at different time points after MI induction using immunohistochemistry (scale bar=50 μm). **B)** Quantification of FGF23 and α-SMA-positive cells at different time points after MI in infarcted area (200-fold magnification), n=3–6. **C)** Analysis of *α-SMA* expression in isolated mouse cardiac fibroblasts (C57/BL6) and TGF-β1-differentiated myofibroblasts; n=3. **D)** *FGF23* mRNA expression in isolated mouse cardiac fibroblasts (C57/BL6) and TGF-β1-differentiated myofibroblasts and representative pictures of FGF23 (red) and α-SMA (green) co-expression in isolated mouse cardiac fibroblasts (C57/BL6) and TGF-β1-differentiated myofibroblasts; n=6. Data was normalized to mean of TGF-β1 group. **E)** *FGF23* mRNA expression without/with TNF-α stimulation in isolated mouse cardiac fibroblasts (C57/BL6) and TGF-β1-differentiated myofibroblasts; n=6. Data was normalized to mean of control. **F)** *FGF23* mRNA expression without/with IL-1ß stimulation in isolated mouse cardiac fibroblasts (C57/BL6) and TGF-β1-differentiated myofibroblasts; n=6. Data was normalized to mean of control. **G)** *FGF23* mRNA expression without/with IL-6 stimulation in isolated mouse cardiac fibroblasts (C57/BL6) and TGF-β1-differentiated myofibroblasts. Data was normalized to mean of control; n=5–6. **H)** *FGF23* mRNA expression without/with IL-10 stimulation in isolated mouse cardiac fibroblasts (C57/BL6); n=6. Data was normalized to mean of control. **I)** *FGFR4, FGFR3c and FGFR1c* mRNA expression in isolated mouse cardiac fibroblasts; n=6. Data was normalized to mean of FGFR4. **J)** *FGFR4* mRNA expression in isolated mouse cardiac fibroblasts without/with IL-1β stimulation; n=6. Data was normalized to mean of control. **K)** *FGFR3c* mRNA expression in isolated mouse cardiac fibroblasts without/with IL-1β stimulation. Data was normalized to mean of control. **L)** *FGFR1c* mRNA expression in isolated mouse cardiac fibroblasts without/with IL-1β stimulation; n=6. Data was normalized to mean of control. Results are presented as means±S.E.M.; **P<.01; ***P<.001; ****P<.0001.

3.3. The effect of FGF23 on cardiac fibroblasts and bone marrow-derived macrophages (BMDM)

To determine the role of FGF23 after MI, we performed migration assay for isolated fibroblasts. The FGF23 (230.2±18.36, n=8) or IL1ß (259.3±32.99, n=8)-dependent migration of fibroblasts vs. control (101.9±16.78, n=8, ****P<.0001) was completely blocked by TGF-β1 (Fig. 4A). Moreover, it seems that FGF23 significantly up-regulated important markers associated with heart failure, such as MMP8 (2.804±0.4545, n=8 vs. control 1.091±0.1585, n=8, **P<.01, Fig. 4B) and periostin (1.482±0.09305, n=8 vs. control 0.9665±0.09750, n=8, **P<.01, Fig. 4C). FGF23 also plays a significant role in modulating the extracellular matrix proteins, stimulating fibronectin synthesis, the main component of provisional matrix (1.777±0.1860, n=8 vs. control 1.053±0.1381, n=8, **P<.01, Fig. 4D), as well as collagen 1 in fibroblasts (collagen 1: 1.641±0.2099, n=8 vs. control 1.033±0.04678, n=8, *P<.05, Fig. 4E) but not collagen 3 (collagen 3: 1.334±0.2919, n=8 vs. control 1.155±0.1321, n=8, n.s., Fig. 4F). Further, FGF23 seems to significantly up-regulate the TGF-β1 expression in M2 polarized BMDM (1.423±0.1425, n=8 vs. control 1.024±0.05525, n=8, *P=.0204), but not in M0 (0.7885±0.1669, n=8 vs. control 1.050±0.3221, n=8, n.s.) or M1 (0.9750±0.0848, n=8 vs. control 1.025±0.0677, n=8, n.s., Fig. 4G) polarized BMDM. On the other hand, FGF23 doesn't have any significant effect on the IL 1ß expression in M0 (1.856±0.9758, n=8 vs. control 1.737±0.5908, n=8, n.s.), M1 (1.118±0.2148, n=8 vs. control 1.172±0.2154, n=8, n.s.), or M2 polarized BMDM (1.383±0.3015, n=8 vs. control 1.181±0.2149, n=8, n.s., Fig. 4H). Additionally, FGF23 doesn't have any significant effect on the Arginase 1 expression in M0 (0.9361±0.1220, n=8 vs. control 1.091±0.1120, n=8, n.s.), M1 (0.8951±0.1119, n=8 vs. control 1.081±0.1470, n=8, n.s.), and M2 polarized BMDM (0.9808±0.1489, n=8 vs. control 1.180±0.2612, n=8, n.s., Fig. 4I).

4. Discussion

In this study, we were able to show that FGF23 and its receptor FGFR1c are up-regulated locally in the myocardium early after MI under the influence of inflammatory cytokines. Our findings challenge the view that primarily skeletal FGF23 acts on cardiac structures [6,8,38]. Most of the local production occurred in local fibroblasts (Fig. 3A). This corresponds to the early inflammatory phase after myocardial infarction, dominated by the production of various inflammatory mediators, recruitment of leukocytes, and proliferation and migration of fibroblasts [39–42]. Several inflammatory cytokines, such as IL-1ß, IL-6 and TNF-α are abundantly released, inducing up-regulation of FGF23 (Fig. 3). Our in vitro data indicates that TGF-β1 as well as IL-10 synthesis as seen in later stages (i.e. the healing phase) after MI can inhibit the transitory FGF23 up-regulation. In fact, TGF-β1 is decisive for proper healing

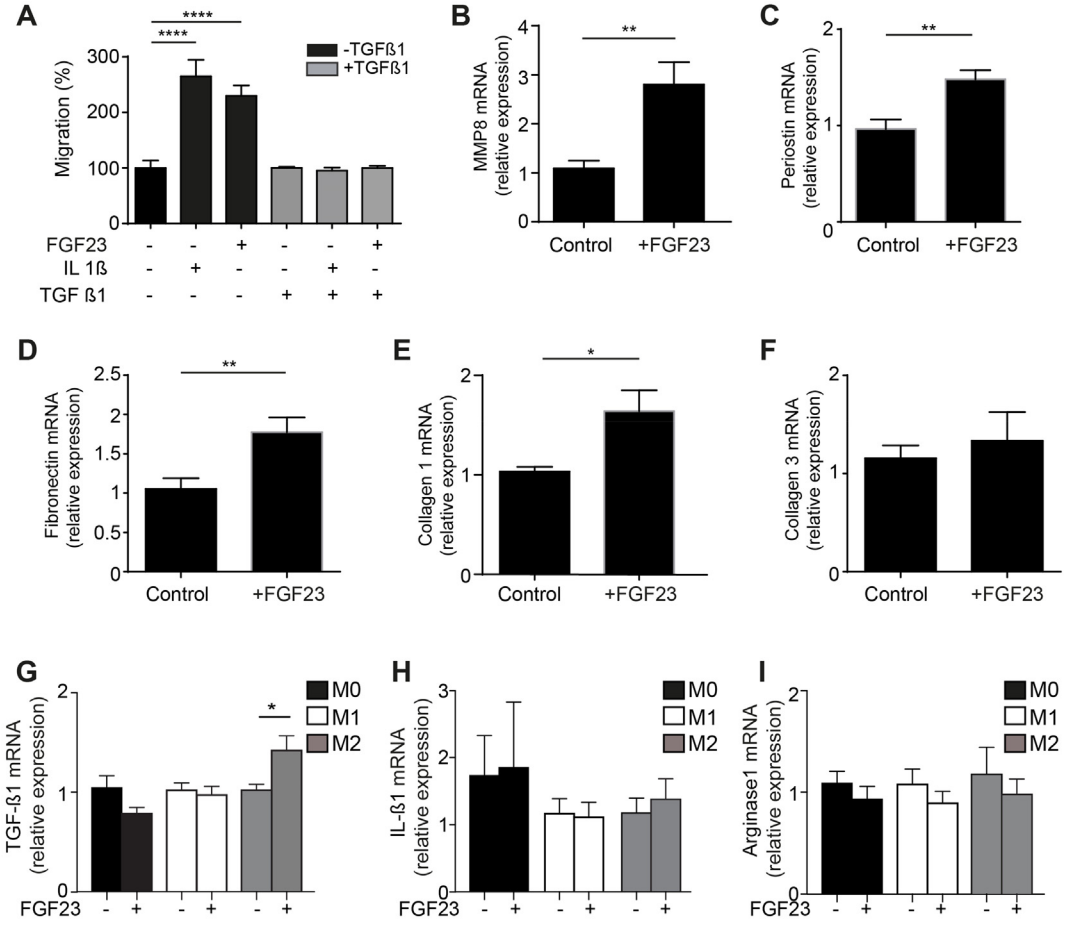

Fig. 4. *The effect of FGF23 on cardiac fibroblasts and bone marrow-derived macrophages (BMDM).* **A)** Migration of isolated mouse cardiac fibroblasts (C57/BL6, black bars) and TGF-β1-differentiated myofibroblasts (gray bars), after IL-1β and FGF23 stimulation. Data was normalized to mean of control; n=6–8. **B)** MMP8 mRNA expression without/with FGF23 stimulation in isolated mouse cardiac fibroblasts (C57/BL6); n=8. **C)** Periostin mRNA expression without/with FGF23 stimulation in isolated mouse cardiac fibroblasts (C57/BL6); n=8. **D)** Fibronectin mRNA expression without/with FGF23 stimulation in isolated mouse cardiac fibroblasts (C57/BL6); n=8. **E)** Collagen 1 mRNA expression without/with FGF23 stimulation in isolated mouse cardiac fibroblasts (C57/BL6); n=8. **F)** Collagen 3 mRNA expression without/with FGF23 stimulation in isolated mouse cardiac fibroblasts (C57/BL6); n=8. **G)** TGF β1 mRNA expression without/with FGF23 stimulation in M0 (unpolarized), M1 and M2 polarized BMDM (C57/BL6); n=8. **H)** IL-1ß mRNA expression without/with FGF23 stimulation in M0 (unpolarized), M1 and M2 polarized BMDM (C57/BL6); n=8. **I)** Arginase 1 mRNA expression without/with FGF23 stimulation in M0 (unpolarized), M1 and M2 polarized BMDM (C57/BL6); n=8. Data was normalized to mean of control. Results are presented as means±S.E.M.; *P<.05; **P<.01; ****P<.0001.

after MI, as it stops the inflammation and allows further scar maturation [40]. Since elevated FGF23 expression was negatively correlated with the clinical outcome and increased mortality of patients suffering an MI [5], we hypothesize that the inhibitory effect of TGF-β1 on FGF23 could be essential to ensure proper healing and reduce complications after MI.

Currently, it is believed that FGF23 is mainly secreted from osteocytes and osteoblasts [13] and an elevated FGF23 level in myocardial cells results from an increased uptake of circulating FGF23 [43–46]. Our results support these findings [43] (Fig. 1A), however we identified the fibroblasts as the main source of FGF23 after MI. While other studies pointed out macrophages as a potential source of FGF23 [47,48], we were not able to detect any FGF23 mRNA expression, despite obvious staining by immunofluorescence. This suggests that the macrophages in our model do not produce FGF23, but can bind it through its receptor FGFR1c. Moreover, FGF23 seems to not influence the polarization of macrophages, but to increase the TGF-β1 expression in anti-inflammatory macrophages (Fig. 4), which is essential for the proper healing and scar formation after myocardial infarction.

We were able to demonstrate for the first time that fibroblasts are the main source of FGF23 in infarcted myocardium after MI. FGF23 mRNA expression can be induced by various inflammatory mediators, such as TNF-α, IL-1β and IL-6, and are abundantly expressed immediately after MI [37] (Fig. 3D). In vitro data showed that FGF23 has an important function in stimulating the proliferation [49] but also the migration of fibroblasts (Fig. 4A), which is essential in populating the areas with massive necrosis and tissue destruction in the first phase after MI. Interestingly, despite the fact that FGFR4 is described as the most predominant receptor in the heart [7,8], we were not able to correlate this receptor with the fibroblast function. However, FGFR1c, also detected in the heart [50], seems to be abundantly expressed in fibroblasts (Fig. 3I). Additionally, its expression is increased in inflammatory conditions (Fig. 3L), as demonstrated immediately after MI.

Particularly interesting, the transient expression of FGF23 is inhibited by TGF-β1. During the healing after MI, TGF-β1 plays a multitude of decisive roles: it assures the differentiation of fibroblasts into myofibroblasts [33], but also ensures the proper transition between the inflammatory and proliferative/healing phase, by blocking the inflammatory, and sustaining the anti-inflammatory processes [40]. Once differentiated, myofibroblasts start to synthesize collagen and maturate the scar tissue. We have shown in this study for the first time that TGF-β1 is able to block the FGF23 expression in fibroblasts, even in the presence of inflammatory mediators (Fig. 3E-H), which explains the absence of FGF23 in the late course after MI. Moreover, upregulation of anti-inflammatory cytokines, such as IL-10 also reduce the synthesis of FGF23.

However, the exact role of FGF23 after MI can only be speculated. On one hand, FGF23 seems to play an important role in healing after MI, increasing TGF-β1 expression in anti-inflammatory macrophages, stimulating proliferation and migration of cardiac fibroblasts. On the other hand, FGF23 significantly up-regulates factors which have been shown to be heart failure biomarkers, such as periostin and MMP8 [52–54] (Fig. 4). Indeed, we cannot exclude that FGF23 can have a positive role to a certain extent on healing after MI, stimulating the fibroblasts to fill the empty area and produce extracellular matrix protein after cell debris clearance, thus avoiding early heart failure or even heart rupture. This could explain the differences between our results and those reported by Andrukhova et al. [43], who showed increased amounts of circulating FGF23 four weeks after MI, as well as FGF23 expression at later time points after MI, due probably to a massive infarction, mimicking the severe clinical conditions [51]. Of course, we cannot exclude the possibility of increased FGF23 serum level at later time points when the hypertrophy of the remote regions becomes significant [55] or in case of heart failure. Since FGF23 plays an important role in increasing the intracellular Ca^{2+} and contractility, leading to hypertrophy of cardiomyocytes [7,8], it is possible that local FGF23 helps to transiently compensate the loss of cardiac function through increased contractility of the surrounding viable myocardium. It is already known that prolonged elevation of FGF23 under other pathological conditions, such as chronic kidney disease, seems to promote cardiovascular disease including left ventricular hypertrophy [8,9,19]. Thus, FGF23 seems to play a crucial role in the remodeling process. In summary, our study and all recent findings suggests, that FGF23 is crucial in acute healing following myocardial infarction. However, increased and prolonged stimulation with FGF23 seems to lead to pathological conditions such as left ventricular hypertrophy. In fact, TGF-β1 seems to be the essential stimuli to avoid prolonged and increased stimulation with FGF23 after myocardial infarction.

In conclusion, our study proves for the first time that local cardiac fibroblasts produce and express FGF23. This is sustained by the inflammatory cytokines during the inflammatory phase, modulating fibroblast and macrophage function. Once terminating the inflammatory phase, TGF-β1 blocks the FGF23 expression and its effects on fibroblasts, allowing the healing to complete. Considering all these findings, we hypothesize that FGF23 may play an important role in ventricular remodeling after MI.

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