FGF2 OVERRIDES KEY PRO-FIBROTIC FEATURES OF BONE MARROW STROMAL CELLS ISOLATED FROM MODIC TYPE 1 CHANGE PATIENTS

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Abstract

Extensive extracellular matrix production and increased cell-matrix adhesion by bone marrow stromal cells (BMSCs) are hallmarks of fibrotic alterations in the vertebral bone marrow known as Modic type 1 changes (MC1). MC1 are associated with non-specific chronic low-back pain. To identify treatment targets for MC1, *in vitro* studies using patient BMSCs are important to reveal pathological mechanisms. For the culture of BMSCs, fibroblast growth factor 2 (FGF2) is widely used. However, FGF2 has been shown to suppress matrix synthesis in various stromal cell populations. The aim of the present study was to investigate whether FGF2 affected the *in vitro* study of the fibrotic pathomechanisms of MC1-derived BMSCs. Transcriptomic changes and changes in cell-matrix adhesion of MC1-derived BMSCs were compared to intra-patient control BMSCs in response to FGF2. RNA sequencing and quantitative real-time polymerase chain reaction revealed that pro-fibrotic genes and pathways were not detectable in MC1-derived BMSCs when cultured in the presence of FGF2. In addition, significantly increased cell-matrix adhesion of MC1-derived BMSCs was abolished in the presence of FGF2. In conclusion, the data demonstrated that FGF2 overrides key pro-fibrotic features of MC1 BMSCs *in vitro*. Usage of FGF2-supplemented media in studies of fibrotic mechanisms should be critically evaluated as it could override normally dominant biological and biophysical cues.

Keywords: Bone marrow stromal cells, fibroblast growth factor 2, basic fibroblast growth factor, Modic changes, fibrosis, extracellular matrix, cell-matrix adhesion.

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List of Abbreviations

ACK	ammonium-chloride-potassium
ACTA2	actin alpha 2
BMSC	bone marrow stromal cell
BMI	body mass index
BSA	bovine serum albumin
CD	cluster of differentiation
CFU-F	colony-forming unit fibroblast
COL1A1	collagen type I alpha
DEG	differentially expressed gene
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ENA	European Nucleotide Archive
FDR	false discovery rate
FGF2	fibroblast growth factor 2
FGFR	fibroblast growth factor receptor
FN1	fibronectin
FRS2	FGFR substrate 2
GO	gene ontology
GRB2	growth factor receptor-bound protein 2

GSEA	gene set enrichment analysis
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT1	hypoxanthine phosphoribosyltransferase 1
IQR	interquartile range
ITGA	integrin subunit alpha
JNK	c-Jun N-terminal kinase
LBP	low-back pain
LEPR	leptin receptor
MAPK	mitogen activated protein kinase
MC1	Modic type 1 changes
MC2	Modic type 2 changes
MC3	Modic type 3 changes
MEM-α	minimum essential medium α
MFI	mean fluorescence intensity
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
MSigDB	molecular signatures database
NES	normalised enrichment score
ODI	Oswestry disability index
P/S	penicillin/streptomycin
PBS	phosphate buffered saline
PDGF-B	platelet-derived growth factor B
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma virus
RT-qPCR	real-time quantitative polymerase chain reaction
SD	standard deviation
STIR	short tau inversion recovery
T1w	T1-weighted
T2w	T2-weighted
TGF-β	transforming growth factor β
VAS	visual analogue score

Introduction

LBP is one of the most disabling conditions worldwide, with a tremendous socioeconomic burden (James *et al.*, 2018). MC are vertebral endplate signal intensity changes, visualised by T1w and T2w MRI, that highly associate with non-specific LBP (Jensen *et al.*, 2008; Mera *et al.*, 2021; Modic *et al.*, 1988; Saukkonen *et al.*, 2020). According to their appearance on MRI, three interconvertible types of MC are distinguished: MC1 (T1w: hypointense, T2w: hyperintense), MC2 (T1w and T2w: hyperintense) and MC3 (T1w and T2w: hypointense). A few histological studies have shown inflammation, fibrosis and high bone turnover in MC1, increased bone erosion and fatty replacement of normal bone marrow in MC2, and a stable sclerotic state in MC3 (Dudli *et al.*, 2016; 2022). Despite the high prevalence of MC, the pathobiology is poorly understood and an MC-specific treatment does not exist (Jensen *et al.*, 2008). Hence, targeted treatment for MC would improve the life quality of these patients and have a dramatic impact on the huge financial costs of LBP. Of the three types, MC1 have the largest association with non-specific chronic LBP (Mera *et al.*, 2021; Saukkonen *et al.*, 2020) and were, therefore, the focus of a previous pathomechanistic study (Heggli *et al.*, 2021). Fibrosis is an important hallmark of the MC1 pathomechanism and is a potential candidate when selecting MC1 serum biomarkers (Dudli *et al.*, 2016; 2017; 2020; 2022; Modic *et al.*, 1988). Fibrosis in MC1 seems to be mediated by BMSCs. CD90 is an important BMSC marker and MC1 bone marrow biopsies have more $CD90^+$ cells, which correlates with fibrotic changes (Dudli *et al.*, 2022). Furthermore, MC1 BMSCs have a pro-fibrotic transcriptome, secrete more ECM and have a stronger adherence to their matrix as well as a higher contractile capacity (Heggli *et al.*, 2021). When studying fibrotic MC1 BMSC features *in vitro*, it was found that FGF2 strongly suppresses these fibrotic mechanisms and changes the overall assay outcome (Heggli *et al.*, 2021). Therefore, the present study hypothesised that FGF2 overrode fibrotic MC1 BMSCs features.

In vitro, BMSCs can spontaneously differentiate and lose their stem cell characteristics. FGF2 is often used in the cell culture medium to avoid spontaneous differentiation, maintain BMSC stem cell character and promote their division (Ahn *et al.*, 2009; Blache *et al.*, 2016; Gharibi and Hughes, 2012; Martin *et al.*, 1997). FGF2 belongs to the FGF family of secreted signalling proteins and binds to and signals through FGFR. FGFRs are receptor tyrosine kinases that activate FRS2 upon FGF binding, which leads to the recruitment of GRB2. Among other effects, this leads to the activation of the Ras-Raf-MAPK signalling pathway (Ornitz and Itoh, 2015). FGF2-induced BMSC proliferation seems to be mediated by the transient activation of the MAPK JNK (Ahn *et al.*, 2009). However, FGF2 strongly suppresses ECM production by BMSCs *in vitro* (Horton *et al.*, 2020) and exhibits anti-fibrotic effects by preventing the differentiation of fibroblasts into myofibroblasts (Dolivo *et al.*, 2017a; Liguori *et al.*, 2018). The antifibrotic effect of FGF2 seems to be context- and cell-dependent: *e.g.* FGF2 antagonises fibroblast activation through a crosstalk between TGF-β and MAPK signalling or inhibits fibrosis *via* JNK-dependent activation of the antifibrotic cytokine hepatocyte growth factor (Dolivo *et al.*, 2017b). Thus, the use of FGF2 as a supplement in BMSC *in vitro* model systems of MC1 might be critical when assessing pro-fibrotic pathomechanisms. The present study investigated the effect of FGF2 on the pro-fibrotic transcriptome and cell-matrix adhesion of MC1-derived BMSCs in comparison to intra-patient control BMSCs and showed that FGF2 overrode key pro-fibrotic features of MC1 BMSCs *in vitro*. Thereby, the study showed the importance of critically evaluating and standardising the FGF2 usage *in vitro* when assessing pro-fibrotic features.

Materials and Methods

The study was approved by the local Ethics Commission (#2017-00761; approved June 05, 2017)

Table 1. Patient characteristics. MC1 and intra-patient control BMSCs were isolated from 10 patients. Bottom line indicates mean and SD or IQR. Level indicates anatomical level of bone marrow aspirates. f: female. m: male. [a]: median with iQR.

ID	Age (years)	Sex (f/m)	Height (cm)	Weight (kg)	BMI (kg/m²)	Smoker	VASback (0-10)	VASleg (0-10)	ODI (%)	Control level	MC1 level	Assay
1	70	f	161	91	35.11	yes	9	9	66	L4	L5	Bulk RNA sequencing
2	87	f	156	76.6	31.48	no	9	0	66	L2	L5	
3	73	f	155	91.5	38.09	no	NA	NA	36	L4	S1	
4	58	f	151	68.7	30.13	yes	6	NA	NA	L4	L5	RT-qPCR
5	68	m	188	166.9	47.22	no	8	6	52	S1	L5	
6	58	f	153	90	38.45	yes	8	8	53	L5	S1	
7	55	m	182	106.3	32.09	yes	6	6	30	L5	L3	Adhesion capacity
8	50	f	174	105	34.68	no	9	9	82	L4	L5	
9	55	f	172	60	20.28	yes	7	8	50	L5	L4	
10	83	f	163	69	26	yes	7	5	38	L4	L5	ECM staining
Mean ± SD	63.78 ± 11.68	80 % f	165.78 ± 13.6	90.5 (70.9, 101.6)[a]	34.17 ± 7.3	60 % yes	7.75 ± 1.28	6.57 ± 3.15	54.38 ± 16.87			

and performed in accordance with the Declaration of Helsinki. If not stated otherwise, chemicals were purchased from Sigma-Aldrich.

Patients and bone marrow aspirate collection

10 patients undergoing spinal lumbar fusion surgery at Balgrist University Hospital from April 2018 to July 2020 were included in the study (Table 1). Informed consent was obtained from each patient. Study subjects were pre-operatively identified based on T1w, T2w and STIR lumbar MRI.

Inclusion criteria were (i) lumbar spinal fusion at a level with predominant MC1 large enough that the pedicle screw came to lie within the MC1 lesion and (ii) an additional pedicle screw that was placed in a vertebral body not affected by any type of MC, serving as an intra-patient control. Exclusion criteria were infectious diseases, malignancies, juvenile scoliosis, autoantibodies, prior instrumented back surgery and listhesis > grade 2. Bone marrow aspirates were collected using Jamshidi needles through the pedicle screw trajectory shortly before the screws were placed. An experienced radiologist confirmed MC1.

BMSC isolation and cell culture

MC1 and intra-patient control bone marrow aspirates were centrifuged in K2-EDTA collection tubes for 15 min at 700 ×*g* and 4 °C. Bone marrow plasma and fat were removed and red blood cells were lysed using ACK lysis buffer (0.15 mol/L NH_4Cl, 0.01 mol/L $KHCO_3$, 0.0001 mol/L Na_2EDTA, pH = 7.2-7.4). Isolation of BMSCs was conducted through plastic adherence (Dominici *et al.*, 2006; Hoch and Leach, 2015). Mononuclear cells were resuspended in BMSC growth medium consisting of MEM-α without nucleosides (Gibco), 50 U/mL P/S (Gibco), 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Gibco), 10 % heat-inactivated foetal calf serum and 2.5 ng/mL FGF2 (PeproTech), seeded in T75 flasks and incubated at 37 °C and 5 % CO_2. All assays were performed using BMSCs from passage 3-5. For all experiments, MC1 and intra-patient control BMSCs were used at the same passage. Table 1 shows which assays were performed for each patient.

Bulk RNA sequencing

MC1 and control BMSCs were grown to 90-100 % confluency in BMSC growth medium. Then, BMSCs were incubated for 24 h with either BMSC growth medium (FGF2+) or BMSC growth medium without FGF2 (FGF2−). BMSCs were harvested and RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Ribosomal RNA was depleted (Ribo-Zero Gold kit, Illumina) and a sequencing library was prepared (TruSeq® Stranded total RNA preparation kit, Illumina) with an input of 150 ng total RNA. Paired-end sequencing of libraries with 151 cycles and > 40 million reads per sample was performed, quality was assessed (Fast QC), adaptor sequences at the 3' end were removed and 4 bases at each end were trimmed (Trimmomatic v0.36). Reads with > 30 nt were mapped to the reference genome hg38 (STAR; 2.6.0c) and counted using FeatureCounts. Statistical analysis was performed using EdgeR (v3.22.1) to i) investigate the effect of FGF2, comparing control (FGF2+) *vs.* control (FGF2−) and MC1 (FGF2+) *vs.* MC1 (FGF2−); ii) compare MC1 to intra-patient control cultivated with or without FGF2. For i), genes were considered to be differentially expressed if the *p*-value was < 0.01 and $\log_2$ fold change was > ± 0.5; for ii), genes were considered to be differentially expressed if the *p*-value was < 0.05 and $\log_2$ fold change was > ± 0.5. Due to the strong FGF2 effect, a more stringent cut off was used in i) than in ii). Both GO overrepresentation analysis and GO enrichment analysis were performed using the identified DEGs. Overrepresentation analysis was run using «clusterProfiler», GO enrichment analysis was performed using Metacore (version 19.4) and

Table 2. Primer sequences used for RT-qPCR.

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)
HPRT1	AGAATGTCTTGATTGTGGAAGA	ACCTTGACCATCTTTGGATTA
ACTA2	GACAATGGCTCTGGGCTCTGTAA	ATGCCATGTTCTATCGGGTACTT
COL1A1	CCGATGGATTCCAGTTCGAG	GGTAGGTGATGTTCTGGGAG
FN1	TACACTGGGAACACTTACCG	CCAATCTTGTAGGACTGACC
ITGA8	AAAAGCAGACGGAAGTGGCT	AGCAGCAACTGAGTATCCAAGG

DAVID (version 6.8) databases. GSEA was carried out using the GSEA software v4.1.0 (UC San Diego and Broad Institute) (Subramanian *et al.*, 2005), signal to noise as gene ranking metric and 1,000 random permutations of the gene set with the complete dataset. GSEA was performed with all hallmark gene sets from the Molecular Signatures Database (MSigDB) v7.1 (Subramanian *et al.*, 2005) and for a specific fibrosis gene set (Wohlfahrt *et al.*, 2019) and an ECM assembly gene set obtained from MSigDB. Gene sets were considered to be enriched with an FDR q-value < 0.1. Enrichment map visualisation was carried out in the GSEA software v4.1.0 using GSEA result of c2.cp.reactome with $p < 0.01$, FDR q-value < 0.1 and network was visualised in Cytoscape version 3.8.0 using an overlap coefficient of 0.5. RNA sequencing data are available at the ENA at EMBL-EBI under accession number PRJEB39993 and PRJEB46346 (Web ref. 1 and Web ref. 2).

RT-qPCR

RNA isolation, cDNA synthesis and RT-qPCR were performed according to manufacturer's protocol. Briefly, RNA was isolated using the RNeasy mini kit (Qiagen). 100 ng of isolated RNA was reverse transcribed to cDNA using the SensiFAST cDNA Synthesis Kit (Labgene, Châtel-Saint-Denis, Switzerland). 2.5 % of total cDNA was added to the SensiFAST™ SYBR® No-ROX Kit and RT-qPCR was performed for 40 cycles of 5 s 95 °C, 20 s 60 °C, 10 s 72 °C followed by a melting curve analysis using a Mic Real-Time PCR system (Labgene). Melting curve analysis was performed to ensure amplification of single products and samples were measured in technical duplicates. Gene expression was quantified using the ΔΔCq method, with *HPRT1* as reference gene. ΔΔCq values were tested against the null hypothesis (μ0 = 0) with one sample *t*-tests. Primer sequences are displayed in Table 2.

Flow cytometry

ITGA8 protein expression was measured by flow cytometry. MC1 and control BMSCs were incubated for 24 h with or without FGF2 and immunolabelled with ITGA8 antibody (R&D, anti-human Integrin α8 APC conjugated, FAB6194A) for 45 min at room temperature, washed with PBS containing 1 % FCS and analysed using a BD LSRFortessa™ Flow Cytometer. Data were analysed using FlowJo (version 10.7.1). The non-normal distributed difference of MC1 and control MFI with FGF2 (FGF2+) was tested against the null hypothesis (μ0 = 0) using the Wilcoxon test, the normal distributed MFI difference of MC1 and control without FGF2 (FGF2−) was tested against the null hypothesis (μ0 = 0) using a one sample *t*-test.

Adhesion capacity

The effect of FGF2 on MC1 and control BMSC adhesion capacity to a fibronectin-coated surface was assessed. 96-well plates were coated with 16 μg/mL fibronectin overnight at 4 °C. Non-specific binding was prevented by incubating the wells with 1 % heat-inactivated BSA for 1 h at 37 °C. MC1 and control BMSCs were expanded and cultivated as described above. Prior to quantifying adhesion, medium was changed for 24 h to growth medium with (FGF2+) or without (FGF2−) FGF2. BMSCs were detached and 2,500 cells/well were seeded in 6 replicates. At both the 15 min and 4 h time points, cell suspension was discarded and wells were washed with PBS to remove remaining non-adherent cells. Adherent cells were fixed using 4 % neutral buffered formalin, stained with Hoechst 33342 (ThermoFisher Scientific) and imaged at four predefined spots per well using a Nikon Eclipse Ti2 upright brightfield microscope. Cells were counted manually using ImageJ and the cell count at 15 min was normalised to the respective 4 h count. Percentages of attached cells were compared by paired *t*-tests.

Immunofluorescent staining of extracellular type I collagen

500 MC1 and control BMSCs were seeded in growth medium with or without FGF2. Both media contained 100 μmol/L ascorbic acid to ensure extracellular collagen deposition. After a 72 h culture, cells were incubated for 4 h at 37 °C with the Anti-Collagen I primary antibody (BosterBio, Pleasanton, CA, USA, #PA2140-2) (1:150 dilution). Medium was removed, cells were fixed with 4 % neutral buffered formalin and incubated with Hoechst 33342 (ThermoFisher Scientific) and an Alexa Fluor 546-conjugated secondary antibody (1:100 dilution in PBS containing 3 % BSA) for 1 h at room temperature. Wells were imaged using a Nikon A1R+ HD confocal microscope and the same brightness and contrast was applied to the images using Image J.

Statistics

All statistical analyses were performed using GraphPad prism version 9.1.2. Normal distribution

of data was tested using the Shapiro Wilk test. In case of normal distribution, parametric tests were run, in case of non-normal distribution, non-parametric tests were applied. An outcome was considered significant if $p < 0.05$ ($p < 0.01$ for RNA sequencing).

Results

No morphological difference between MC1 and intra-patient control BMSCs *in vitro*

To study the effect of FGF2 on fibrotic assay outcomes, BMSCs were isolated from patients undergoing spinal fusion surgery from a MC1 lesion and from an adjacent vertebral body without MC1, serving as intra-patient control BMSCs (Fig. 1**a**). In-depth population and stemness characterisations of MC1 and intra-patient control BMSCs have been previously performed (Heggli *et al.*, 2021). Briefly, > 93 % of both MC1 and intra-patient control BMSCs express the consensus MSC surface markers CD73, CD90, CD105 but not CD14, CD19, CD34, CD45, show tri-lineage differentiation potential (with reduced adipogenic differentiation of MC1 BMSCs) and form CFU-F on plastic dishes (Dominici *et al.*, 2006; Heggli *et al.*, 2021). Assessment of BMSC subpopulation markers revealed overrepresentation of a leptin receptor high ($LEPR^{high}$)-expressing BMSC population in MC1 BMSCs (Heggli *et al.*, 2021). Size, structure, form and shape of isolated and expanded MC1 and intra-patient control BMSCs did qualitatively not differ *in vitro* (Fig. 1**b**).

FGF2 upregulated mitogenic pathways in MC1 and control BMSCs

To assess the effect of FGF2, the transcriptome of MC1 and control BMSCs cultured in standard growth medium containing 2.5 ng/mL FGF2 or no FGF2 were compared. 3,734 DEGs were identified in control and 4,062 DEGs in MC1 BMSCs comparing FGF2+ to FGF2− (Fig. 2**a**). Overrepresentation analysis of the DEGs revealed, in both MC1 and control BMSCs, GOs associated with cell proliferation among the top three upregulated GOs. Comparing FGF2+ to FGF2−, in both MC1 and control, the top two GOs were "cell division" and "regulation of G2/M transition of mitotic cell cycle" (Fig. 2**b**). Enrichment visualisation of significantly enriched gene sets of the Reactome pathway database (820 gene sets), as identified by GSEA, further revealed upregulated pathways linked to cell division (Fig. 2**c**). This transcriptomic comparison demonstrated the strong mitogenic effect of FGF2 on both MC1 and control BMSCs. It further showed that there was no difference between MC1 and intra-patient control BMSCs as they similarly upregulated mitogenic pathways in response to FGF2.

The effect of FGF2 on the transcriptome was considerably stronger than transcriptional changes between MC1 and intra-patient control BMSCs

Transcriptional differences between MC1 and intra-patient control BMSCs cultivated with or without FGF2 for 24 h were compared. 391 and 449 DEGs were detected between MC1 and intra-patient control when cultivated with or without FGF2, respectively (Fig. 3**a**). Only 46 of the DEGs overlapped between the two conditions (Fig. 3**b**). GSEA of "hallmarks" gene sets revealed significantly enriched processes in MC1 BMSCs associated with inflammation when cultivated with FGF2, such as interferon alpha (FDR $q = 0.000$), interferon gamma (FDR $q = 0.000$) and inflammatory response (FDR $q = 0.017$). In comparison, the significantly enriched "hallmarks" in MC1 BMSCs when cultivated without FGF2 revealed the pro-fibrotic signalling processes "Hedgehog signalling" (FDR $q = 0.017$), "Notch signalling" (FDR $q = 0.009$) and "Epithelial mesenchymal transition" (FDR $q = 0.037$) (Fig. 3**c**). The only overlapping enriched gene set was "Hedgehog signalling"

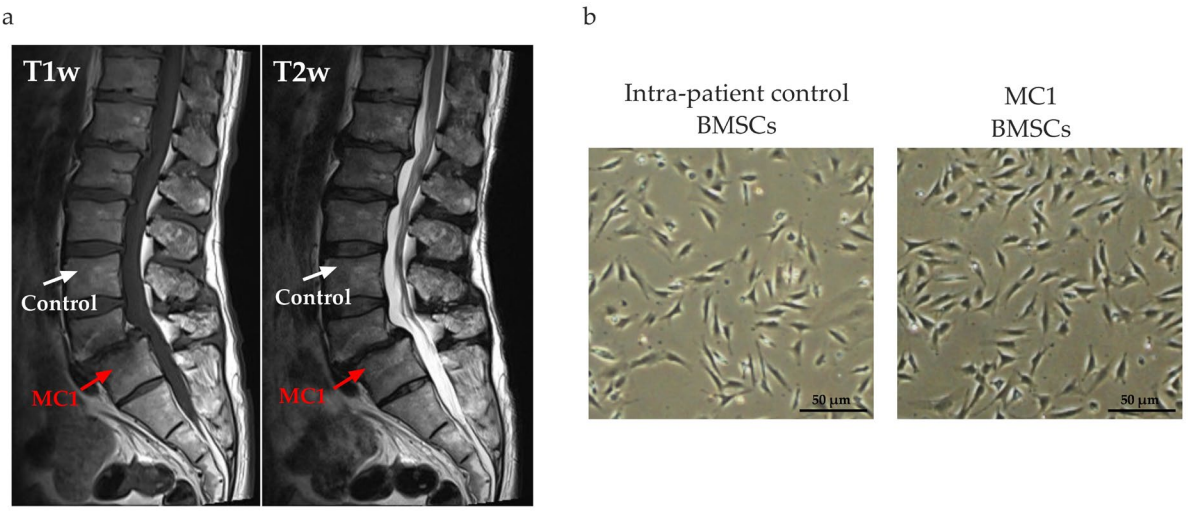

Fig. 1. BMSCs isolated from patients with MC1. (**a**) MC1 on T1w (left) and T2w (right) MRI. Red arrows indicate MC1 lesion, white arrows indicate intra-patient control. (**b**) Isolated and expanded BMSCs from control (left) and MC1 (right) visualised by phase contrast microscopy.

(cultivated with FGF2, FDR q = 0.032). However, the enrichment score was much lower with FGF2 and it was ranked only at the 7th position of the top enriched gene sets (Fig. 3**c**). Due to the known matrix-suppressing effect of FGF2, GSEA was performed with two fibrosis-specific gene sets (Subramanian *et al.*, 2005; Wohlfahrt *et al.*, 2019). Both fibrotic gene sets were significantly enriched in MC1 BMSCs when FGF2 was removed for 24 h, whereas no enrichment was found when cultivated with FGF2 (Fig. 3**d**). The top three GOs (Metacore) of "Cellular components" comparing MC1 to control without FGF2 were all associated with ECM production. The same three GO terms were not found in the top three upregulated GO terms in the presence of FGF2 (not shown) and their p-values were substantially higher than without FGF2 (Fig. 3**e**). These findings indicated that FGF2 use *in vitro* resulted in different transcriptional outcomes. It marginalised or even covered the pathophysiological pro-fibrotic transcriptome of MC1.

FGF2 overrode the pro-fibrotic phenotype of MC1 BMSCs *in vitro*

To confirm the transcriptomic analysis, the question of whether the use of FGF2 affected the expression of prototypic pro-fibrotic genes between MC1 and intra-patient control was tested. When cultivated in the presence of FGF2, *ACTA2* ($\log_2$ fold change

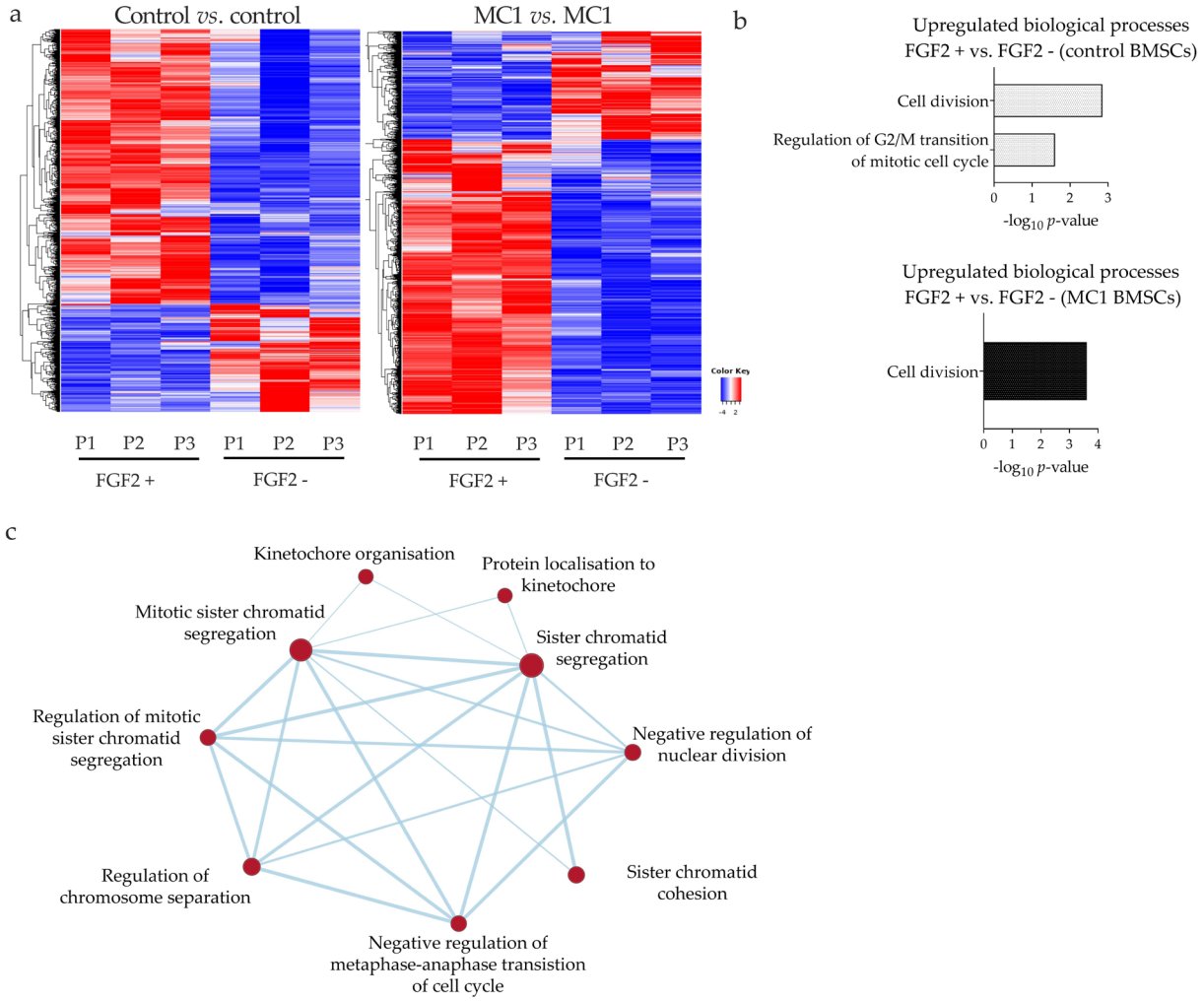

Fig. 2. FGF2 promoted transcriptomic changes associated with cell division in both MC1 and control BMSCs. Transcriptomic changes in MC1 and control BMSCs cultivated for 24 h in growth medium containing FGF2 were compared to growth medium without FGF2. (**a**) Heatmap of the DEGs showing FGF2 effect in control (left) and MC1 BMSCs (right). Differential expression analysis was conducted using EdgeR. DEGs were defined as $p < 0.01$ and log2 fold change > 0.5. (**b**) Top 3 enriched upregulated biological processes identified by overrepresentation analysis comparing FGF2+ to FGF2− in control (top) and MC1 (bottom). Overrepresentation analysis was run using clusterProfiler and the identified DEGs. (**c**) Visualisation of significantly enriched pathways of MC1 BMSCs in response to FGF2 identified by GSEA of Reactome pathway gene sets. GSEA was carried out using the GSEA software v4.1.0 (UC San Diego and Broad Institute) (Subramanian *et al.*, 2005), signal to noise as gene ranking metric and 1,000 random permutations of the gene set with the complete dataset. Gene sets were considered to be enriched with FDR q-value < 0.1. Enrichment map visualisation was carried out in the GSEA software v4.1.0 using GSEA result of c2.cp.reactome with $p < 0.01$, FDR q-value < 0.1 and network was visualised in Cytoscape version 3.8. using an overlap coefficient of 0.5.

MC1-control = 0.98 ± 0.70, p = 0.14), *COL1A1* (log_2 fold change MC1-control = 0.75 ± 0.95, p = 0.46) and *FN1* (log_2 fold change MC1-control = 1.66 ± 1.33, p = 0.16) expression was similar in MC1 and control BMSCs. In contrast, when cultivated without FGF2, expression of *COL1A1* (log_2 fold change MC1-control = 1.52 ± 0.02, p = 0.006), *FN1* (log_2 fold change MC1-control = 2.36 ± 0.72, p = 0.029) and *ACTA2* (log_2 fold change MC1-control = 2.09 ± 1.01, p = 0.07) was upregulated (Fig. 4**a**). Staining of extracellular type I collagen further demonstrated the overriding effect of FGF2 on the pro-fibrotic phenotype of MC1 BMSCs (Fig. 4**b**).

Increased cell-matrix adhesion is an important pro-fibrotic mechanism (Hinz *et al.*, 2003; 2006). Transcriptomic analysis revealed that fibronectin

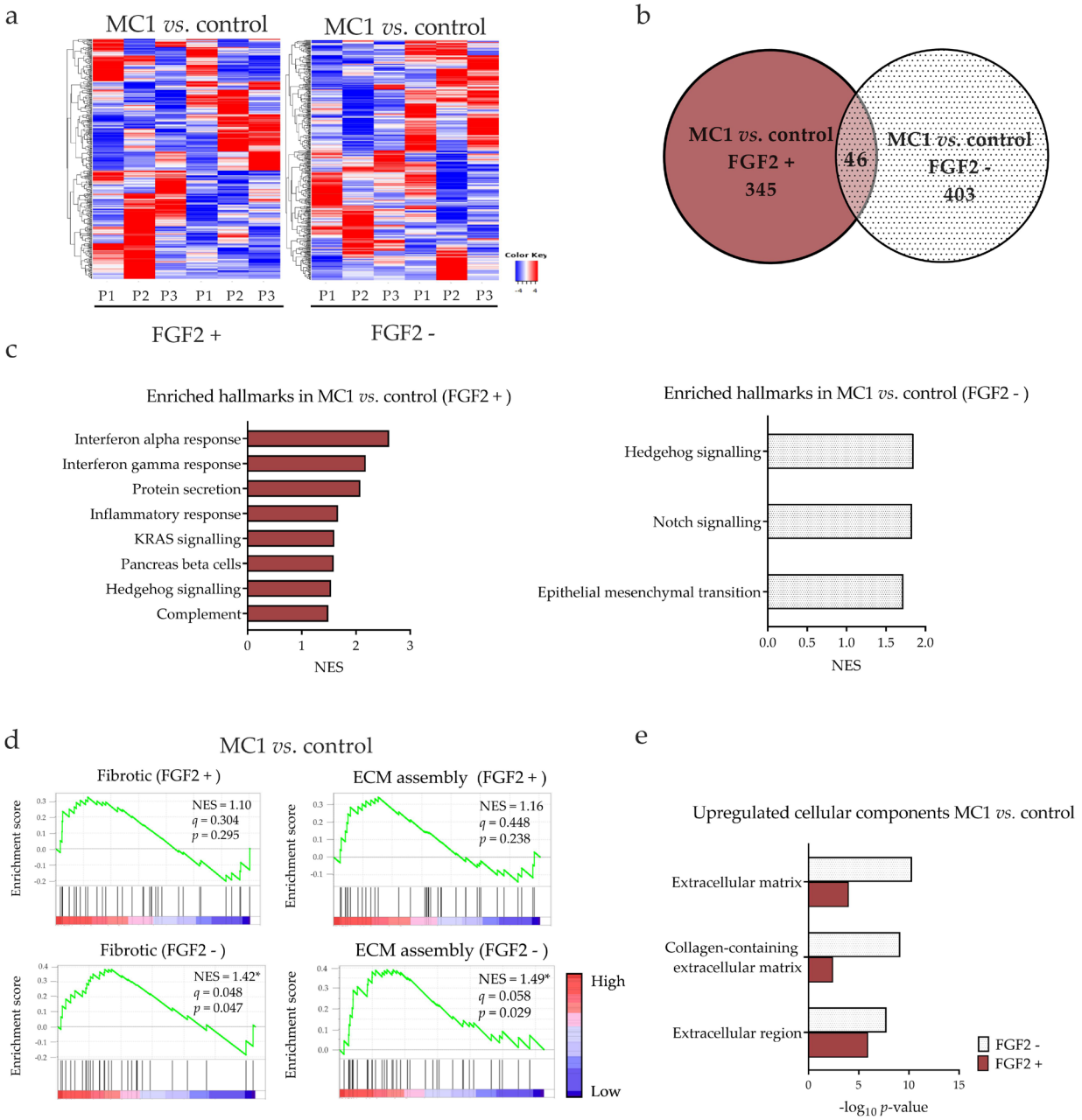

Fig. 3. FGF2 changed the outcome when comparing the transcriptome of MC1 to intra-patient control BMSCs. (**a**) Heatmap of the DEGs (p < 0.05, log2 fold change > 0.5) between MC1 and control BMSCs cultivated with (391 DEGs, left) and without (449 DEGs, right) FGF2. Differential expression analysis was conducted using EdgeR. (**b**) Venn diagram of overlapping DEGs between MC1 and control BMSCs with and without FGF2 in cell culture medium. (**c**) GSEA of "hallmarks" revealed different significantly enriched processes between MC1 and intra-patient control with and without FGF2. (**d**) Fibrotic gene sets were solely significantly enriched in MC1 BMSCs when cultivated without FGF2. GSEA was carried out using the GSEA software v4.1.0 (UC San Diego and Broad Institute) (Subramanian *et al.*, 2005), signal to noise as gene ranking metric and 1,000 random permutations of the gene set with the complete dataset. Gene sets were considered to be enriched with FDR q-value < 0.1. (**e**) Top 3 upregulated localisations when cultivated in the presence or absence of FGF2. GO enrichment analysis was performed using Metacore (version 19.4) and the DEGs.

binding was upregulated in MC1 BMSCs in FGF2− but not in FGF2+ (Fig. 4**c**). Therefore, cell-matrix adhesion to fibronectin-coated surface was measured performing a functional *in vitro* adhesion assay. In the presence of FGF2, adhesion capacity did not significantly differ between MC1 and control BMSCs (MC1-control: + 7.05 % ± 5.59 %, p = 0.18). In contrast, adhesion was significantly increased in MC1 BMSCs in the absence of FGF2 (MC1-control: + 15.96 % ± 1.06 %, p = 0.002) (Fig. 4**d**).

ITGA8, among other integrin subunits, contributes to adhesion of cells to fibronectin (Barczyk *et al.*, 2010). Whether differential expression of *ITGA8* was affected by FGF2 was tested. Increased *ITGA8* expression was found in MC1 BMSCs FGF2− (log_2 fold change MC1-control = 4.62 ± 1.36, p = 0.03) but not in FGF2+ (log_2 fold change MC1-control = 1.75 ± 2.15, p = 0.30) (Fig. 4**e**, left). This was supported by flow cytometric analysis of ITGA8 protein level, where a trend towards increased ITGA8 protein expression was observed in FGF2− (MFI MC1-control = 78.67 ± 52.38, p = 0.12) compared to FGF2+ [MFI MC1-control = 55.00, IQR (29.50, 55.50), p = 0.25]. These findings showed that *in vitro* transcriptomic and functional outcomes between MC1 and control BMSCs depended on the use of FGF2.

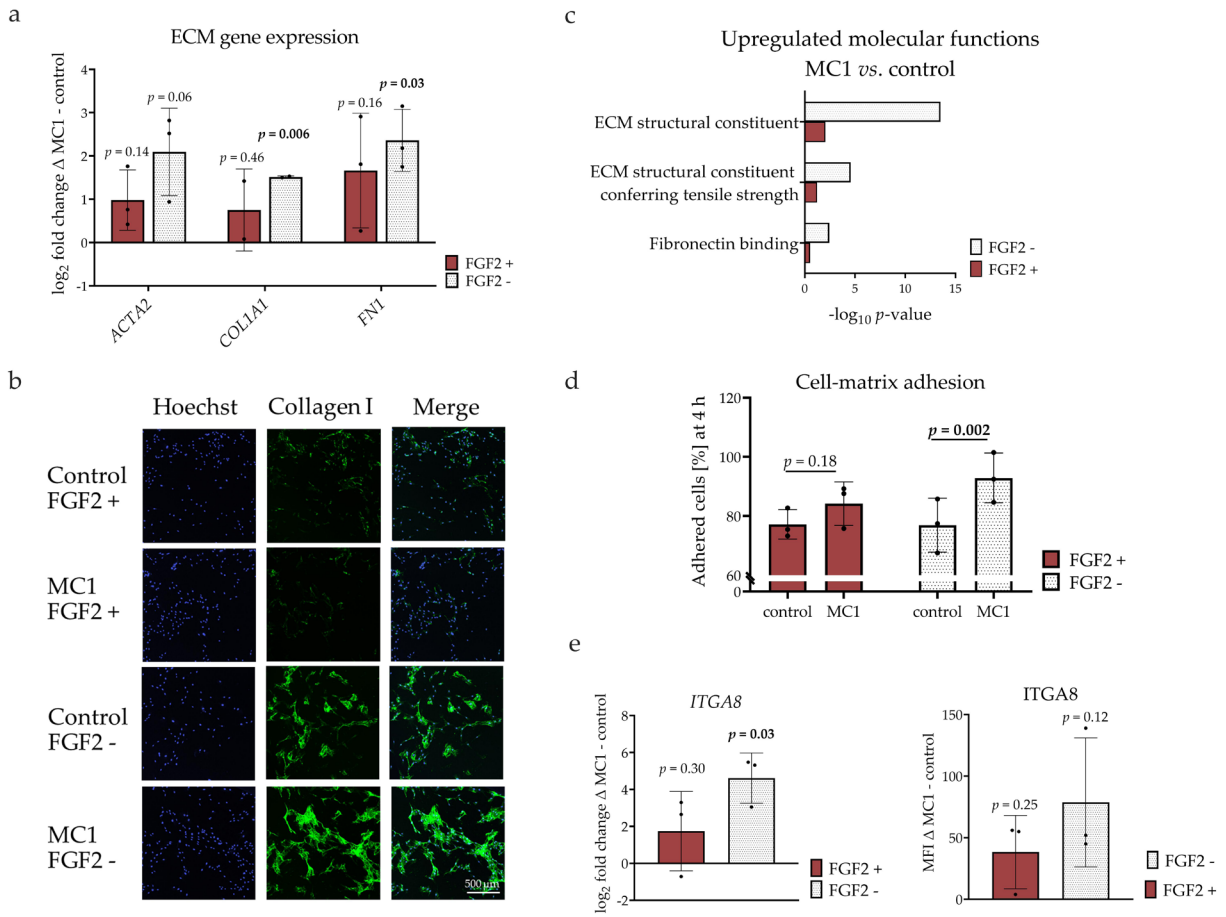

Fig. 4. Increased ECM production and adhesion capacity of MC1 BMSCs were suppressed by FGF2. (**a**) RT-qPCR of pro-fibrotic gene expression. *ACTA2*, *COL1A1* and *FN1* expression in MC1 BMSCs was increased only in the absence of FGF2. ΔΔCq values were tested against the null hypothesis (μ0 = 0) using the one sample *t*-tests. (**b**) Staining of extracellular type I collagen FGF2 almost completely suppressed type I collagen synthesis in both MC1 and control BMSCs (upper two rows). Increased collagen I synthesis in MC1 BMSCs was only visible when BMSCs were cultivated in the absence of FGF2 (lower two rows) (2× magnification). (**c**) Upregulated molecular functions comparing MC1 to control. Significant enrichment of ECM-related functions and fibronectin binding only occurred under FGF2− conditions. GO enrichment analysis was performed using Metacore (version 19.4) and the DEGs. (**d**) Functional *in vitro* adhesion assay confirmed that the difference in adhesion capacity to fibronectin-coated surface between MC1 and control increased upon removal of FGF2. Percentages of attached cells were compared using paired *t*-tests. (**e**) Gene expression of fibronectin-specific *ITGA8* subunit was increased in MC1 BMSCs in the absence but not in the presence of FGF2 (left). Similarly, a trend towards increased ITGA8 protein expression was observed without but not with FGF2 (right). ΔΔCq values were tested against the null hypothesis (μ0 = 0) using one sample *t*-tests. The non-normal distributed difference of MC1 and control MFI with FGF2 (FGF2+) was tested against null hypothesis (μ0 = 0) using the Wilcoxon test, the normal distributed MFI difference of MC1 and control without FGF2 (FGF2−) was tested against null hypothesis (μ0 = 0) using a one sample *t*-test.

Discussion

The anti-fibrotic and ECM-suppressing effects of FGF2 have recently been described for healthy BMSCs (Horton *et al.*, 2020). The present study, using clinical MC1 BMSCs, showed that this effect could lead to misleading results from pathomechanistic studies. As little as 2.5 ng/mL FGF2 overrode key pro-fibrotic features of MC1 BMSCs and led to no significant differences compared to intra-patient control BMSCs (Fig. 3). While significantly increased matrix synthesis and matrix adhesions were observed in MC1 BMSCs compared to intra-patient control BMSCs in the absence of FGF2, no significant differences were found in the presence of FGF2 (Fig. 4,5). It is conceivable that FGF2 has similar effects when studying stromal cells under other fibrotic conditions. Therefore, the *in vitro* usage of FGF2 in experiments that study the fibrotic effects of stromal cells should be carefully evaluated.

FGF2 as media supplement strongly suppresses ECM and pro-fibrotic gene transcription of BMSCs in 3D *in vitro* cell culture (Horton *et al.*, 2020). The anti-fibrotic effect of FGF2 is not restricted to BMSCs. It has also been shown for other cell types, *i.e.* dermal fibroblasts, where FGF2 reduces scarring processes (Dolivo *et al.*, 2017b; Eto *et al.*, 2012). The anti-fibrotic effects of FGF2 are critical when studying MC1. MC1 BMSCs have a pro-fibrotic phenotype, which is characterised by a pro-fibrotic transcriptome and increased cell-matrix adhesion (Heggli *et al.*, 2021). The present study showed that the anti-fibrotic effect of FGF2 overrode the pro-fibrotic phenotype of MC1 BMSCs. This potentially could lead to a different interpretation of MC1 BMSC characteristics that poorly reflects the pathophysiological *in vivo* phenotype. First, GSEA analysis in the presence of FGF2 revealed upregulated inflammatory but not fibrotic mechanisms in MC1 BMSCs. Consequently, the presence of FGF2 would not only have covered the fibrotic transcriptome of MC1 BMSCs but could also have redirected the study focus towards investigating inflammatory instead of fibrotic BMSC mechanisms. Second, even if fibrotic features such as ECM gene expression, cell-matrix adhesion and integrin expression of MC1 BMCS were analysed, the pro-fibrotic phenotype would not have been revealed (Awan *et al.*, 2018; Grella *et al.*, 2016; Herrera *et al.*, 2018; Thannickal *et al.*, 2003). Hence, it would have not been possible to conclude that MC1 BMSCs with FGF2 have a pro-fibrotic phenotype, which is not in agreement with the current understanding of MC1 pathophysiology (Dudli *et al.*, 2020; Dudli *et al.*, 2022; Heggli *et al.*, 2021; Modic *et al.*, 1988).

The present study showed that FGF2 overrode key pro-fibrotic features of MC1 BMSCs. However, other growth factors, such as PDGF-B or the epidermal growth factor, are also used to promote cell proliferation in MSC/BMSC culture (Gharibi and Hughes, 2012). PDGF-B *e.g.* shares similar signalling pathways to FGF2 for promoting proliferation (Kang *et al.*, 2005). Due to those similar shared signalling pathways, an interference with the fibrotic mechanisms could be imaginable. It needs to be elucidated in further studies whether these supplemented growth factors in BMSC/MSC medium also override key fibrotic pathomechanisms.

FGF2 is often used to expand MSCs and BMSCs *in vitro* because it promotes expansion and maintains stemness (Ahn *et al.*, 2009; Blache *et al.*, 2016; Gharibi and Hughes, 2012; Martin *et al.*, 1997). The present study showed that removal of FGF2 from culture should be considered when transitioning from the cell expansion phase to pathomechanistic studies,

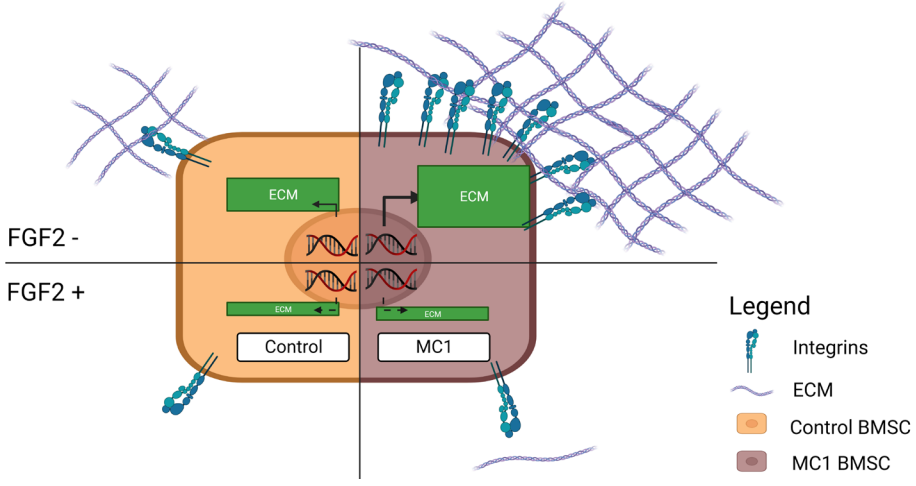

Fig. 5. Schematic illustration of how FGF2 overrides the pro-fibrotic MC1 BMSC phenotype. ECM production and cell-matrix adhesion are increased in MC1 BMSCs (upper left and right quadrant) (Heggli *et al.*, 2021). This likely reflects the pathophysiological *in vivo* phenotype. In the presence of FGF2, ECM synthesis and cell-matrix adhesion are strongly suppressed in both MC1 and control BMSCs (lower left and right quadrant). Consequently, the significant differences in pro-fibrotic features observed in the absence of FGF2 (FGF2−) cannot be observed in the presence of FGF2 (FGF2+) Hence, FGF2 overrides key pro-fibrotic features of MC1 BMSCs.

in particular if fibrotic mechanisms are being investigated. Further studies are needed to unravel the fibrotic-suppressing mechanism of FGF2 on BMSCs.

Limitations

The present study did not assess the fibrotic overriding effect of FGF2 on the complete MC1 BMSC phenotype (which also includes *e.g.* increased cell contractility, pro-collagen type 1 alpha 1 synthesis, *etc.*). The study focused on fibrotic features (gene expression, cell-matrix adhesion) that have been previously shown to be important when studying the fibrotic-overriding effect of FGF2 in MC1 (Heggli *et al.*, 2021). Another limitation was that the study did not assess whether cultivation > passage 5 with FGF2 permanently overrode the pro-fibrotic MC1 BMSCs features. It is well known that *in vitro* expansion progressively changes the BMSC phenotype (Yang *et al.*, 2018). Typically, when investigating pathomechanisms of BMSCs/fibroblasts, cells in low passage numbers from 3-8 are used (Frank-Bertoncelj *et al.*, 2017; Sareen *et al.*, 2018). In the present study [and also in a previous characterisation study (Heggli *et al.*, 2021)], only BMSCs from passage 3-5 were used. Therefore, the present study allowed to draw conclusions about the effect of FGF2 on fibrotic MC1 BMSC features up to passage 5. It can be assumed that long-term culture changes the fibrotic MC1 BMSC phenotype, which likely influences FGF2 effects. However, it might be challenging to distinguish at later passages whether this is a result of an irreversible FGF2 effect or if the pro-fibrotic features changed as a result of long-term culture/passaging. Finally, FGF2 can activate five of the seven FGFR variants (FGFR1 IIIb, FGFR1 IIIc, FGFR2 IIIc, FGFR3 IIIc and FGFR-4 but not FGFR2 IIIb and FGFR3 IIIb). The anti-fibrotic effect of FGF2 is versatile, cell- and context-dependent and includes promotion of apoptosis in myofibroblast *in vivo*, inhibition of TGF-β signalling in fibroblasts and myofibroblasts and suppression of pro-fibrotic gene expression with involvement of multiple signalling pathways (Dolivo *et al.*, 2017b). The present study did not investigate the anti-fibrotic mechanism of FGF2 on MC1 BMSCs or the expression of the five FGFR variants in MC1 and intra-patient control BMSCs in the presence or absence of FGF2. Therefore, it was not possible to draw any conclusion about the underlying anti-fibrotic mechanism of FGF2 in BMSCs. However, the present study allowed to conclude that pro-fibrotic gene expression and functional cell-matrix adhesion were overridden by FGF2 in MC1 BMSCs.

Conclusion

The present study showed that the addition of FGF2 to BMSC culture medium covered disease-specific pro-fibrotic features. Although BMSCs are intensively studied, a consensus about the *in vitro* usage of FGF2 when performing transcriptomic analyses and functional assays has yet to be established. This demonstrates that the *in vitro* use of FGF2 in BMSC culture should be critically evaluated and questioned and its application for assays standardised.

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Discussion with Reviewers

Reviewer: Growth factors such as PDGF share similar signalling pathways with FGF2 and are used in cell expansion medium of MSCs/BMSCs. Should, for instance, addition of PDGF also be considered an issue when studying MC1 BMSC pathomechanics?
Authors: The reviewer addresses an important point, namely, that besides FGF2, other growth factors, such as *e.g.* PDGF-B or epidermal growth factor, are used *in vitro* to promote cell proliferation in MSC/BMSC culture (Gharibi and Hughes, 2012). Even though PDGF-B and FGF2 share similar signalling pathways that promote proliferation (*e.g.* MAPK signalling), they result in different cellular behaviours. For example, corneal fibroblast under PDGF-B stimulation upregulate α5β1 integrin subunits at the leading edge of the cell with a high turnover that results not only in proliferation but also in cell migration. In contrast, FGF2 stimulation leads to the downregulation of α5β1 integrin subunits and, therefore, results only in proliferation but not migration (Gallego-Muñoz *et al.*, 2017, additional reference). Furthermore, in contrast to FGF2, which interferes with TGF-β signalling leading to an anti-fibrotic effect, PDGF-B in combination with TGF-β is strongly pro-fibrotic and also considered as a target for tissue fibrosis (Bonner, 2004; Klinkhammer *et al.*, 2018, additional references). Due to similar shared signalling pathways of FGF2 and PDGF-B, an interference with fibrotic mechanisms could be imaginable. As both growth factors result in different cellular behaviours, the potential fibrotic overriding effect of PDGF-B needs to be studied. However, this clearly shows that growth factor supplementation needs to be critically evaluated when a cell phenotype that can be influenced by these growth factors is studied.

Uruj Zehra: How would the authors explain the clinical relevance of their findings?
Authors: The study is important as it shows that use of FGF2 in cell culture medium for assessing pro-fibrotic BMSC features can lead to wrong pathomechanistic conclusions. For example, the use of FGF2 for the *in vitro* characterisation of MC1 BMSCs (Heggli *et al.*, 2021) would have led to the conclusion that BMSCs were not relevant in the fibrotic mechanisms in MC1. With the knowledge that BMSCs are pro-fibrotic in MC1, targeted treatments for BMSCs in MC1 could be defined. In general, the study demonstrated the need to standardise FGF2 use in the research field for *in vitro* assays. This is significant when considering how many fibrotic diseases are driven by MSCs and when considering that this cell type is studied by multiple research groups.

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Editor's note: The Scientific Editor responsible for this paper was Sybille Grad.