Role of fibroblast growth factor receptors (FGFR) and FGFR like-1 (FGFRL1) in mesenchymal stromal cell differentiation to osteoblasts and adipocytes

Kähkönen TE, Ivaska KK, Jian M, Büki KG, Väänänen HK, Härkönen PL

University of Turku, Institute of Biomedicine, Turku, Finland

Corresponding author:

Tiina Kähkönen
University of Turku, Finland
Institute of Biomedicine
Turku, Finland
tesilv@utu.fi
+358443285136

Abstract

Fibroblast growth factors (FGF) and their receptors (FGFRs) regulate many developmental processes including differentiation of mesenchymal stromal cells (MSC). We developed two MSC lines capable of differentiating to osteoblasts and adipocytes and studied the role of FGFRs in this process. We identified FGFR2 and fibroblast growth factor receptor like-1 (FGFRL1) as possible actors in MSC differentiation with gene microarray and qRT-PCR. FGFR2 and FGFRL1 mRNA expression strongly increased during MSC differentiation to osteoblasts. FGF2 treatment, resulting in downregulation of FGFR2, or silencing FGFR2 expression with siRNAs inhibited osteoblast differentiation. During adipocyte differentiation expression of FGFR1 and FGFRL1 increased and was down-regulated by FGF2. FGFR1 knockdown inhibited adipocyte differentiation. Silencing FGFR2 and FGFR1 in MSCs was associated with decreased FGFRL1 expression in osteoblasts and adipocytes, respectively. Our results suggest that FGFR1 and FGFR2 regulate FGFRL1 expression. FGFRL1 may mediate or modulate FGFR regulation of MSC differentiation together with FGFR2 in osteoblastic and FGFR1 in adipocytic lineage.

Keywords

Mesenchymal stromal cell, osteoblast, adipocyte, fibroblast growth factor, fibroblast growth factor receptor, fibroblast growth factor receptor like-1

1. Introduction

Bone marrow contains many cell types including mesenchymal stromal cells (MSCs). The MSCs are a rare population, counting only 0,001% of bone marrow nucleated cells (1). These cells can be isolated and enriched by plastic adherence in culture and identified on the basis of surface marker expression. MSCs are CD73 and CD105 positive and lack the expression of hematopoietic markers such as CD14, CD34 and CD45 (2, 3). They can also be defined by their multilineage differentiation capacity. In living organism, and also *in vitro*, MSCs can differentiate into many cell types including osteoblasts and adipocytes (1, 4). The cells retain their capacity of proliferating and differentiating to a certain extent and therefore they can be used in culture for several passages. However, primary cells display a high variation between species and individuals (1-3) and the reproducibility of the results has often been poor. Therefore, there is a need for MSC cell line models capable of unlimited proliferation and multi lineage differentiation.

The fibroblast growth factor (FGF) family consists of 23 members which can be divided into 6 subfamilies. They bind to FGF-receptors (FGFRs) 1-4 (5, 6) with different binding affinities but only FGF1 and FGF2 are able to bind and activate all the receptors (5, 6). Activation of FGFRs leads to phosphorylation and action of several molecules on the downstream signaling pathways including ERK/MAPK, PI3K/AKT and PLCy.

The FGFs and FGFRs are known to be important for many developmental processes (5, 6) and they also have a role in MSC differentiation (7). Mutations in the FGFR genes can lead to skeletal defects such as craniosynostosis and chondrodysplasias (4, 7, 8). Particularly FGFR2 has been found to be an important driver of osteoblast differentiation (4, 7). FGFs and FGFRs are also expressed in human white and brown adipose tissue (9, 10). Silencing FGFR1 expression as well as the use of dominant-negative form of FGFR1 resulted in the inhibition of adipocyte differentiation *in vitro*, suggesting the importance of FGFR1 in the process (11).

In addition to the four classical FGFRs there is an additional receptor, FGFR like-1 (FGFRL1, also known as FGFR5) (6, 12). FGFRL1 gene was discovered in a cartilage specific cDNA library in 2000 (8, 12) and thereafter it has been found in many mammalian tissue types including kidney, liver, skeletal muscle, heart and lung (8). It is also expressed in skeleton and especially in the growth plates of long bones (8) and targeted inactivation of FGFRL1 gene in mice led to an array of phenotypes

including disturbed skeletal development (13). Patients with craniosynostosis have been found to carry FGFRL1 mutations (8) and in meta-analyses of genome-wide association studies FGFRL1 through critical microRNA target site polymorphisms for bone mineral density proved to be important for bone formation (14). FGFRL1 is located on the cell membrane, able to bind several FGFs of which FGF2, FGF3 and FGF8 bind it with high to intermediate affinity (6, 8, 12). FGFRL1 differs from the classical FGFRs as it has only a truncated intracellular domain which is unable to cause transphosphorylation of the tyrosine residues and activate most downstream signaling pathways (6, 8). For this reason it was first thought to be a nonfunctional member of the FGFR family. However, FGFRL1 has been shown to have a negative effect on proliferation (8, 12) but the data on differentiation is controversial and calls for new studies to explore this issue further. The mechanisms of FGFRL1 are not known but it has been suggested to function as a decoy receptor for various FGFs and/or modulator of secondary intracellular signaling transducers such as SHP-1 and -2 (6, 8, 15). Interestingly, in a recent study SHP-1 was reported to be a positive regulator of osteoblastogenesis (16).

The aim of this study was to examine the role of FGFRs in the differentiation of osteoblasts and adipocytes from MSCs, their progenitor cells. For this purpose we created two immortalized MSC-lines capable for unlimited proliferation and multilineage differentiation. With this model we focused on FGFRs, and especially on a novel member of the FGFR-family, FGFRL1, the role of which in MSC differentiation is currently unknown.

2. Materials and methods

2.1 Development of immortalized MSC lines

The animal experimentation was approved by the local review committee of Central Animal Laboratory, University of Turku (Turku, Finland). Bone marrow cells were isolated from long bones of C57Bl male mice (age 8-20 days, N=3-10) and MSCs were enriched by plastic adherence for 48h. Adherent cell population was expanded for 4-5 days in alphaMEM (Gibco) supplemented with 15% fetal bovine serum (Gibco), 1mM GlutaMAX (Gibco) and penicillin-streptomycin (Gibco). To create immortalized cell lines, MSCs were transfected by electroporation with a pRITA plasmid linearized with ScaI containing SV40 large T antigen (SV40TAg) under the control of tet-on promoter (17) using Human MSC Nucleofection Kit (Lonza) and Amaxa (Nucleofector II, Lonza) according to manufacturer's instructions. The tet-on promoter drives the expression of SV40TAg (immortalization) and neomycin (selection). Immortalization was achieved with promoter activation

by 12,5µg/ml doxycycline (Thermo Fisher Scientific), and stably transfected clones were selected based on antibiotic resistance using 0,4mg/ml G418 (Lonza). Reversal of immortalization was evaluated by SV40TAg expression and cell proliferation (alamarBlue, Invitrogen) in cells grown with or without doxycycline. Cell surface marker expression was analyzed with immunohistochemial staining for CD44, Sca1 and CD45 using Mouse MSC marker panel 93759 (Abcam).

2.2 Differentiation of immortalized MSCs to osteoblasts and adipocytes and treatments

Cells were grown on culture dishes in normal medium (alphaMEM, 10% iFBS, GlutaMAX, Hepes (Gibco) and PS) with 12,5 µg/ml doxycycline and 0,4 mg/ml G418 in humidified incubator at 37°C and 5% CO_2.

For differentiation the MSC cells were seeded to 6-well plates in normal medium. After attachment (24h) the media was replaced with the differentiation medium, which for osteoblasts was supplemented with 15% iFBS, 10mM Na-β-glyserophosphate (Fluka) and 70µg/ml ascorbic acid phosphate. For adipocyte differentiation the medium was supplemented with 10µg/ml insulin, 0,5mM xantine, 0,1mM indomethacin and 10^{-6}M dexamethasone (all from Sigma-Aldrich). During the differentiation cultures half of the medium was replaced with fresh medium every 3-4 days.

For short treatment 25ng/ml FGF2 (R&D systems), 100nM FGFR inhibitor PD173074 (a gift from Pfizer) or their combination was added 24h prior to the sample collection and the vehicle (DMSO) was used as a control. For long treatments FGF2 and/or PD173074 were included in the medium throughout the culture time and when replacing half of the medium with fresh, also new FGF2/PD173074 was added every 3-4 days. The inhibitor PD173074 was administered to cultures 30 min prior to addition of FGF2.

2.3 Microarray

The MSCs were grown in osteoblastic or adipogenic differentiation medium for 7 days in T25 tissue culture flasks in three replicates. RNA was isolated using an RNeasy Kit (Qiagen) according to the manufacturer's instructions and RNA was subjected to microarray analysis using a Mouse Genome 2.0 Array (Affymetrix). The induction of expression of osteoblast and adipocyte marker genes was compared to that in undifferentiated controls and up/down regulation was defined as higher than 2-fold change in expression together with statistical significance of $p<0,05$.

2.4 qRT-PCR

RNA was isolated using RNeasy kit (Qiagen) with DNAse treatment (Qiagen). 0,5µg of RNA was used as a starting material for cDNA and Oligo-dT mRNA-primers (BioLabs) with Maxima RT

enzyme (Thermo Fisher Scientific) was used. For quantitative RT-PCR Dynamo HS SYBR green (Thermo Fisher Scientific) was used to detect the expression of osteoblast and adipocyte marker genes and FGFRs with gene-specific primers (Supplement 1) using CFX96/384 qRT-PCR machine (Biorad). The data was analyzed by ΔΔCT-method and mRNA expression was normalized to cyclophilin D expression and presented in relative to undifferentiated and/or untreated samples.

2.5 Western blot

The cells were harvested to 5x sample buffer (0,5M Tris-HCl, glycerol, 10%SDS and 0,01% bromophenolblue) and denaturated with 0,5µl of β-mercaptoethanol (Fluka) by heating in 95°C for 5min. Samples were run on 12% SDS-PAGE gels and transferred to nitrocellulose membrane (Millipore). The membranes were blocked with 8% fat-free milk solution prior to incubation with a primary antibody. Primary antibodies were anti-FGFR1 (Abcam, ab10646), anti-FGFR2 (Abcam, ab10648), anti-FGFRL1 (Biorbyt orb101861 and RD Systems AF1899), anti-pFRS2 (Cell signaling, #3864), total-FRS2 (Abcam, ab10425) anti-pERK1/2 (Cell signaling, #9101S), anti-ERK1/2 (Cell signaling, #9102) and anti-tubulin (Abcam, ab4074). Immune complexes were detected with fluorescent secondary antibodies (donkey anti-rabbit lgG cw800, #925-32213, Li-Cor) with Li-Cor (Li-Cor).

2.6 Cytochemical stainings

The cells were fixed with 4% paraformaldehyde (PFA) for 15min and washed with 1 x phosphate buffered saline (PBS). Alkaline phosphatase (ALP) activity was detected with an Alkaline Phosphatase Kit 86R (Sigma-Aldrich) according to manufacturer's instructions with volumes adjusted to the microtiter plates. Prior to Oil-red-O staining, cells were washed with 60% isopropanol and air-dried. Oil-Red-O solution (Sigma- Aldrich) was added to the cells for 10 min and washed with PBS. Images of representative areas were taken with Axiovert 200M (Zeiss).

2.7 Transfection of shFGFR constructs

The expression of FGFRs was silenced by transfecting the cells with specific shFGFR constructs. Transfections were done with electroporation as described in paragraph 2.1. For transfections, 200 000 cells were transfected using 2µg of shFGFR for FGFRL1 or its control (NT) (Santa Cruz Technologies). For FGFR1 and FGFR2 silencing two different constructs (FGFR1: B and D, FGFR2: I and A) were used to improve silencing efficiency and were compared to their control (LZ) (18). Cell pools surviving the transfection were selected with 0,3µl/ml puromycin (Gibco) and subjected to differentiation experiments.

2.8 Statistical analysis

Statistical analysis was done by GraphPad Prism software using one-way ANOVA with Bonferroni correction for multiple comparisons. Each experiment was repeated 2-4 times and the number of parallel samples was 3 to 6. Data (mean±SD) of representative experiments are shown. Statistical significance is presented as * $p<0,05$, ** $p<0,01$ and *** $p<0,001$.

3. Results

3.1 Establishment of MSC-like cell lines

Development of MSC-like cell lines gave rise to 27 clones. Immortalized clones were studied for the integration of an immortalization construct to genomic DNA and expression of MSC cell surface markers. Two of the cell lines, MSC6 and MSC22, were chosen for further studies on the mechanisms of differentiation capacity.

The expression of the immortalization construct SV40T-antigen integration to genomic DNA was verified by PCR and immunostaining (Fig. 1A). Both clones were positive for the MSC markers CD44, Sca-1/Ly6A/E and negative for CD45 (Fig. 1A). Promoter activation by doxycycline induced the expression of SV40T (Fig. 1A) and increased proliferation measured by the alamarBlue cell proliferation assay (Fig. 1A). In the differentiation experiments doxycycline was omitted from the medium to ensure efficient differentiation.

Differentiation of MSCs towards osteoblastic and adipocytic phenotypes was characterized by the expression of mRNA and cytochemical or immunocytochemical stainings of selected marker genes. The mRNA levels of type 1 collagen (COL1) increased after 4 days in osteoblast differentiation and decreased after that (Fig. 1B). On day 16 of osteoblastic differentiation cultures, the expression of ALP mRNA increased up to 60 fold when compared to MSCs (Fig. 1B), and cytochemical staining for ALP activity was elevated (Fig. 1B). The mRNA levels of osteocalcin (OC), a late marker for osteoblast differentiation, increased to 4 fold after 16 days of osteoblast differentiation and OC expression on protein level was also confirmed by immunocytochemistry (Fig. 1B). The expression patterns of the genes studied were in line with the reported expression profiles for these genes. Based on the expression of the markers, the differentiation process was divided into three phases: pre-osteoblast, early-osteoblast and osteoblast (after 5, 9 and 13 days in culture, respectively) and these will be used later in the text.

During adipocyte differentiation intracellular lipid droplets started to accumulate at day 4 of culture. After 7 days the cells exhibited adipocyte-like morphology as visualized by phase-contrast microcopy and Oil Red O –staining (Fig. 1C). On day 7 the relative increase of fatty acid binding protein-4 (FABP4) mRNA levels was nearly 100 fold (Fig. 1C) when compared to MSCs. Expression of the major adipocyte transcription factor peroxisome proliferation factor gamma (PPARy) mRNA increased 3 fold (Fig. 1C) compared to MSCs. Based on the expression of adipocyte differentiation markers and Oil-Red-O –staining, the cells will be referred to as pre-adipocytes and adipocytes (phenotypes reached in on days 4 and 7 of differentiation cultures) in the text.

Both MSC6 and MSC22 cell clones were initially characterized for a differentiation capacity towards both osteoblastic and adipocytic phenotypes. Both of the cell lines do differentiate efficiently to both lineages under similar culture conditions. However, based on the levels of ALP mRNA and the intensity of staining, MSC6 cells differentiated to osteoblastic lineage slightly more efficiently than MSC22 cells (data not shown) and therefore, they were selected for further studies on osteoblastic differentiation. In contrast, MSC22 cells differentiated slightly better to adipocytes and therefore, they were chosen to model this differentiation process.

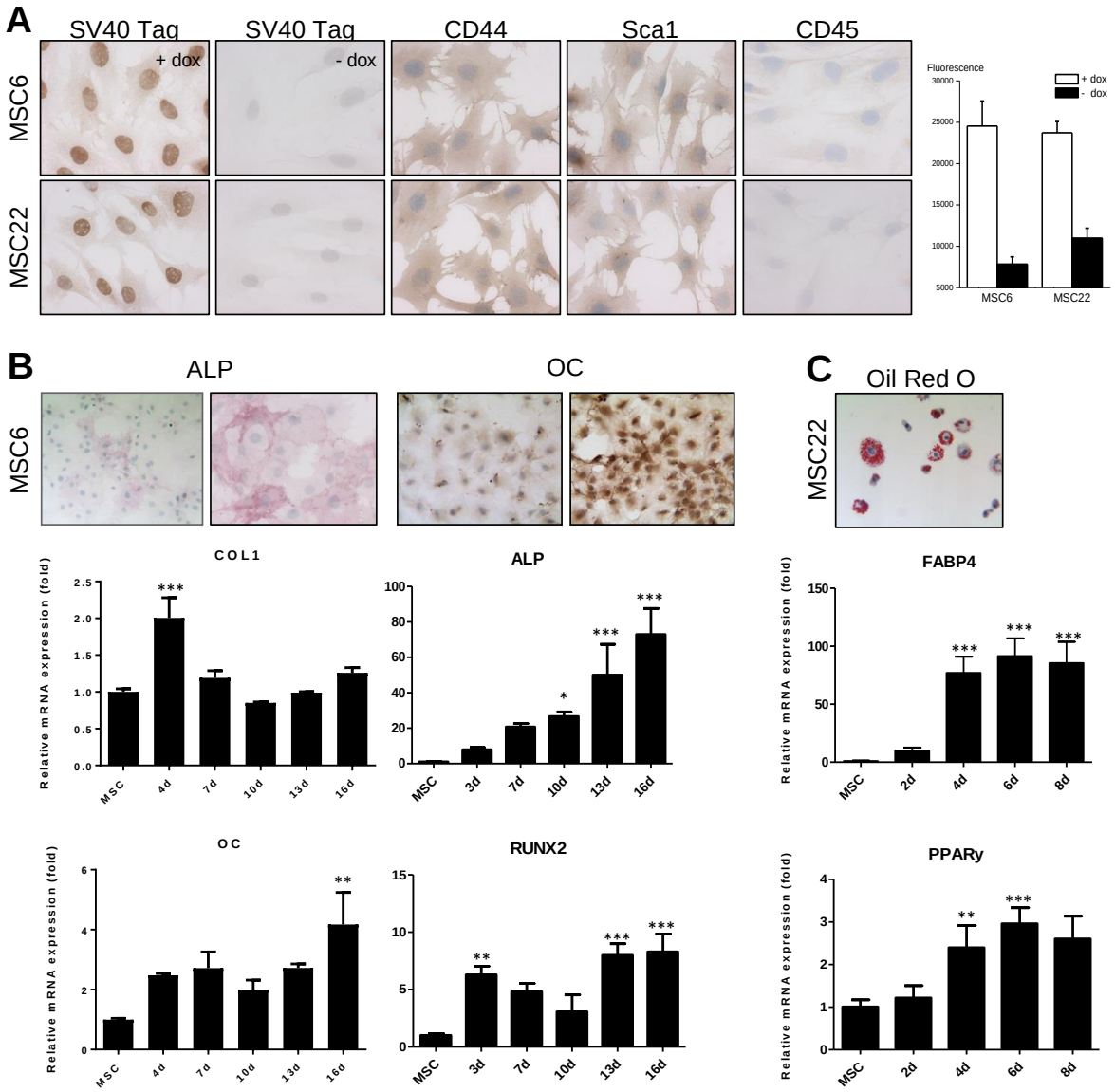

A
SV40 Tag
+ dox
SV40 Tag
- dox
CD44
Sca1
CD45
MSC6
MSC22
Fluorescence
+ dox
- dox
MSC6
MSC22
B
ALP
OC
MSC6
C
Oil Red O
MSC22
COL1
ALP
FABP4
OC
RUNX2
PPARγ
Relative mRNA expression (fold)
MSC
4d
7d
10d
13d
16d
3d
2d
6d
8d

Figure 1: Characterization of the MSC cell lines. (A) MSC6 and MSC22 were immunostained for mesenchymal (CD44 and Sca1) and hematopoietic (CD45) stem cell markers and representative images (20x magnification) are presented. Treatment of the cells with doxycycline (±dox) activates SV40Tag expression in the cells and increases cell proliferation determined with the alamarBlue-assay. The columns show a relative increase in fluorescence intensity (mean±SD, n=3) on day 6 in culture in comparison with undifferentiated MSC cells. (B) MSC6 cells were differentiated to osteoblasts for 16 days and characterized by cytochemical staining for ALP activity (left, 4x magnification; right, 20x magnification) and immunostaining for osteocalcin protein (left, negative control, 10x magnification; right, positive staining, 10x magnification) and expression of COL1, ALP, OC and RUNX2 mRNA (mean±SD, n=3)(lower panel), undifferentiated MSC6 cells were used as a control. (C) MSC22 cells were differentiated to adipocytes for 7 days and characterized by Oil-Red-O staining (upper panel) and expression of FABP4 and PPARγ mRNA (mean±SD, n=5)(lower panel), undifferentiated MSC22 cells were used as a control. Statistical significances are shown as *p<0,05, **p<0,005 and ***p<0,001.

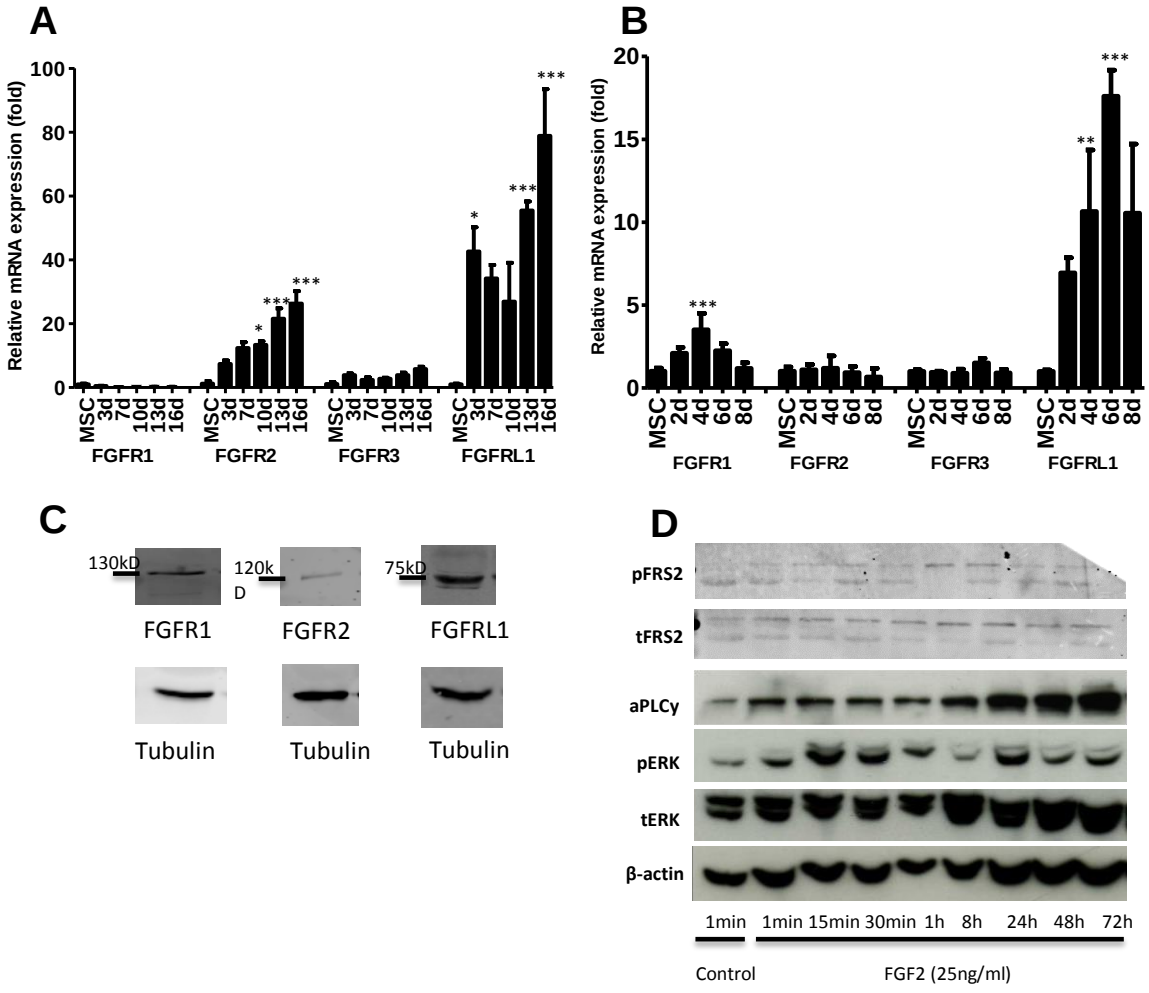
3.2 Expression profile of FGFRs during MSC differentiation

The mRNAs of undifferentiated MSCs and MSC6 and MSC22 cells and cells differentiated to osteoblasts and adipocytes were subjected to a gene microarray analysis. Upregulation of genes related to osteoblast and adipocyte differentiation was seen in MSC6 and MSC22 cells (Supplement 2) and the data was screened for the mRNAs of various FGFs, FGFRs and FGF-related signaling proteins. The expression of FGFR2 mRNA was found to change significantly during osteoblast differentiation detected with two independent probes (p=0,04 and p=0,01) (Supplement 2). The expression of FGFRL1 mRNA was significantly upregulated during both osteoblast (p=0,005) and adipocyte (p=0,001) differentiation (Supplement 2).

Verification of the results by qRT-PCR showed that the mRNAs for FGFR1-3 and FGFRL1 were expressed both in MSCs and mature osteoblasts and adipocytes (Fig. 2A, 2B). The FGFR4 mRNA was barely detectable in MSCs and in mature osteoblasts and adipocytes and therefore it was not included in further analysis (data not shown). During osteoblast differentiation the relative levels of FGFR2 and FGFRL1 mRNA increased 20 fold and over 80 fold, respectively (Fig. 2A), when compared to undifferentiated MSCs. The relative expression of FGFR1 mRNA decreased during osteoblast differentiation while that of FGFR3 mRNA remained unchanged (Fig. 2A).

During adipocytic differentiation cultures the relative level of FGFR1 mRNA increased up to day 4 (3,5 fold) after which it decreased almost to the control level (Fig. 2B). The expression of FGFR2 and FGFR3 mRNA was rather low and no changes were seen (Fig. 2B). The relative levels of FGFRL1 mRNA increased during the differentiation cultures being highest on day 6 (17 fold) (Fig. 2B). The expression of FGFR1, FGFR2 and FGFRL1 proteins was demonstrated in MSCs by western blots (Fig. 2C) and also detected during the differentiation (Supplement 3). The general pattern of FGFR2 and FGFR1 protein followed that of mRNA levels (Fig. 2A and 2B, Supplement 3). FGFRL1 protein level also increased during differentiation but as big relative changes as in mRNA was not observed (Fig. 2A and 2B, Supplement 3). The activation of FGFRs and the responsiveness of MSCs

to FGFs was studied by treating the cells with FGF2, known to activate all FGFRs, from 1min to 72h. The FRS2 and PLCy pathway was shown to be activated after a 1 min exposure to FGF2 and to stay active for at least 72h (Fig. 2D). The responsiveness of both cell lines (MSC6 and MSC22) were noted to be similar and the data on MSC6 cells is presented. The MAPK-ERK-pathway was activated at 15min after treatment but after 1h the signal started to decrease (Fig. 2D).


A
Relative mRNA expression (fold)
100
80
60
40
20
0
MSC 3d 7d 10d 13d 16d
FGFR1
FGFR2
FGFR3
FGFRL1
B
Relative mRNA expression (fold)
20
15
10
5
0
MSC 2d 4d 6d 8d
FGFR1
FGFR2
FGFR3
FGFRL1
C
130kD
120kD
75kD
FGFR1
FGFR2
FGFRL1
Tubulin
Tubulin
Tubulin
D
pFRS2
tFRS2
aPLCy
pERK
tERK
β-actin
1min 1min 15min 30min 1h 8h 24h 48h 72h
Control
FGF2 (25ng/ml)

240

Figure 2: FGFR expression during MSC differentiation. (A) MSC6 cells were differentiated to osteoblasts and FGFR1-3 and FGFRL1 mRNA levels were determined by qRT-PCR on days 3, 7, 10, 13 and 16 of cultures, undifferentiated MSC6 cells were used as a control. The columns represent relative mRNA levels (mean±SD, n=3) in comparison to undifferentiated MSCs (B) MSC22 cells were differentiated to adipocytes and the levels of FGFR1-3 and FGFRL1 mRNA were analyzed on day 2, 4, 6 and 8 of cultures, undifferentiated MSC22 cells were used as a control. The columns represent mRNA levels in comparison to undifferentiated MSC cells (mean±SD, n=5). The mRNA values are normalized to those of cyclophilin B and presented relative to the mRNA level of each individual receptor in MSCs (set as 1). Statistical significances are presented as *p<0,05, **p<0,005 and ***p<0,001. (C) The expression of FGFR1 (130kDa), FGFR2 (120kDa) and FGFRL1 (75kDa) in undifferentiated MSCs was demonstrated by western blots. Tubulin was used as a loading control and is presented individually for each separate western blot runs. (D) MSC6 cells were treated with 25ng/ml of FGF2 for 1min, 15min, 30min, 1h, 8h, 24h, 48h and 72h, or control (DMSO for 1min) and the protein samples were run on SDS-PAGE gels. The activation of downstream signaling pathways of FGFRs was studied with specific antibodies for pFRS2 (upper band), aPLCy, and phospho-ERK, here total FRS2, total ERK and β-actin were used as a loading control.

3.4 The effect of a short and long FGF2 treatment on osteoblast differentiation

A short 24-hour treatment with FGF2 decreased the expression of the mRNAs for osteoblast marker genes. Downregulation of ALP mRNA levels in osteoblasts was up to 80% (Fig. 3A). Similar effects were also seen after a long (continuous) treatment in osteoblasts where the decrease was almost 90% (Fig. 3B) compared to control-treated cells. Similar trend was also seen in pre- and early-osteoblasts as well as in expression of other osteoblast marker genes studied (COL1, OC, RUNX2, data not shown).

Next we asked whether the inhibitory effect of FGF2 could be abolished by blocking the FGF2-mediated signaling. Simultaneous treatment with FGF2 and the FGFR inhibitor 100nM PD173074 blocked FRS2 phosphorylation (Supplement 4) and 100nM PD173074 was used in the later studies. During osteoblast differentiation, the addition of PD173074 together with FGF2 as a short and long treatment maintained the ALP mRNA levels at a control level in osteoblasts (Fig. 3A and 3B, respectively). Treatment with the FGFR inhibitor alone did not have any effect on differentiation (Fig. 3A, 3B).

Treatment of differentiating cells with FGF2 altered the expression of FGFRs. The levels of FGFR2 mRNA decreased by short and long treatments (Fig. 3A and 3B) by about 50% compared to control treated osteoblasts. Short treatment had no effect on the FGFRL1 mRNA level (data not shown), but during a long treatment it was decreased at all stages on differentiation (Fig. 3B). Interestingly, a short treatment increased FGFR1 mRNA levels at all stages of differentiation about 2 fold (Fig. 3A) but such an effect was not seen during a long treatment (data not shown). Similar results were also observed with short FGF8 treatment in a preliminary experiment with decreased differentiation and changes in receptor expression (data not shown).

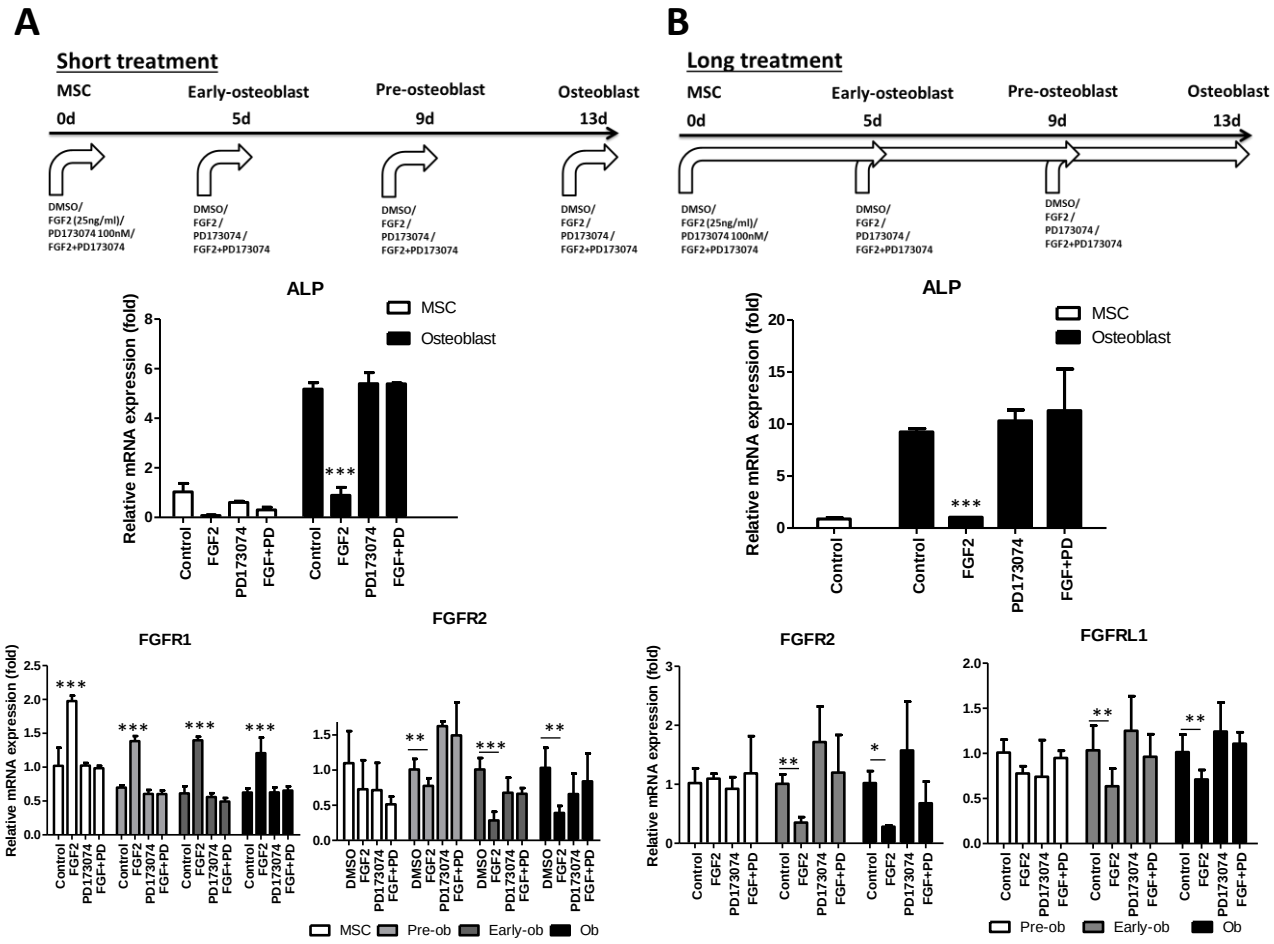


Figure 3: The effect of short and long FGF2 treatments on osteoblast differentiation. (A) The effects of a short 24-hour incubation with FGF2 (25ng/ml) with or without PD173074 (100mM), on the levels of ALP, FGFR1 and FGFR2 mRNAs was studied with qRT-PCR. The columns represent means±SD, (n=5) corrected to cyclophilin B mRNAs and related to the mRNA levels in control-treated samples at each timepoint. (B) The effects of long (continuous) treatment of cultures with 25 ng/ml FGF2, 100mM PD173074 or a combination of both on the levels of ALP, FGFR1 and FGFRL1 mRNAs was studied with qRT-PCR. Columns present means±SD, (n=5) corrected to cyclophilin B mRNAs and related to the corresponding mRNA levels in non-treated MSCs. Abbreviations: pre-ob=pre-osteoblast, early-ob=early-osteoblast, ob=osteoblast.

3.5 The effect of FGFR2 and FGFRL1 downregulation on osteoblast differentiation

To study the role of FGFRs in osteoblast differentiation we transfected MSCs with shRNA-constructs to silence the expression of FGFR2 and FGFRL1 separately and simultaneously. In MSCs we did not see downregulation of the receptor mRNA levels, possibly due to low initial expression levels (ct-values over 30). However, during MSC differentiation to osteoblasts the levels of both FGFR2 and FGFRL1 mRNA increased (Fig. 2), which enabled to study the effect on silencing in mature osteoblasts. In shFGFR2 cells differentiated to osteoblasts, FGFR2 mRNA level was decreased to 25% when compared to the control and a similar change was observed in in double-silenced shFGFR2+shFGFRL1 cells (Fig. 4A). Despite of several attempts, shFGFRL1 silencing was not

294 successful and no significant decrease of FGFRL1 mRNA was obtained (Fig. 4A). Interestingly
295 however, the level of FGFRL1 mRNA was decreased by 90% in in double-silenced
296 shFGFR2+shFGFRL1 cells (Fig. 4A). Based on these experiments we concluded that FGFR2 may
297 regulate expression of FGFRL1 which could, in the absence of silencing of FGFRL1 in shFGFRL1
298 cells, explain decreased FGFRL1 mRNA levels in shFGFR2+shFGFRL1 cells. This conclusion was
299 supported by further experiments and determination of FGFRL1 mRNA in shFGFR2 cells
300 (Supplement 5).

301 Silencing of FGFR2 was associated with a small but statistically non-significant decrease in the level
302 of ALP mRNA in osteoblasts but a marked decrease in the cytochemical staining of ALP activity
303 (Fig. 4B, Supplement 5). In shFGFR2+shFGFRL1 cells differentiated to osteoblasts, the expression
304 of ALP mRNA was decreased by 93% in comparison with the control (Fig. 4B). A slight decrease in
305 FGFRL1 mRNA levels was associated with upregulation of ALP and RUNX2 mRNA levels
306 (Supplement 5).

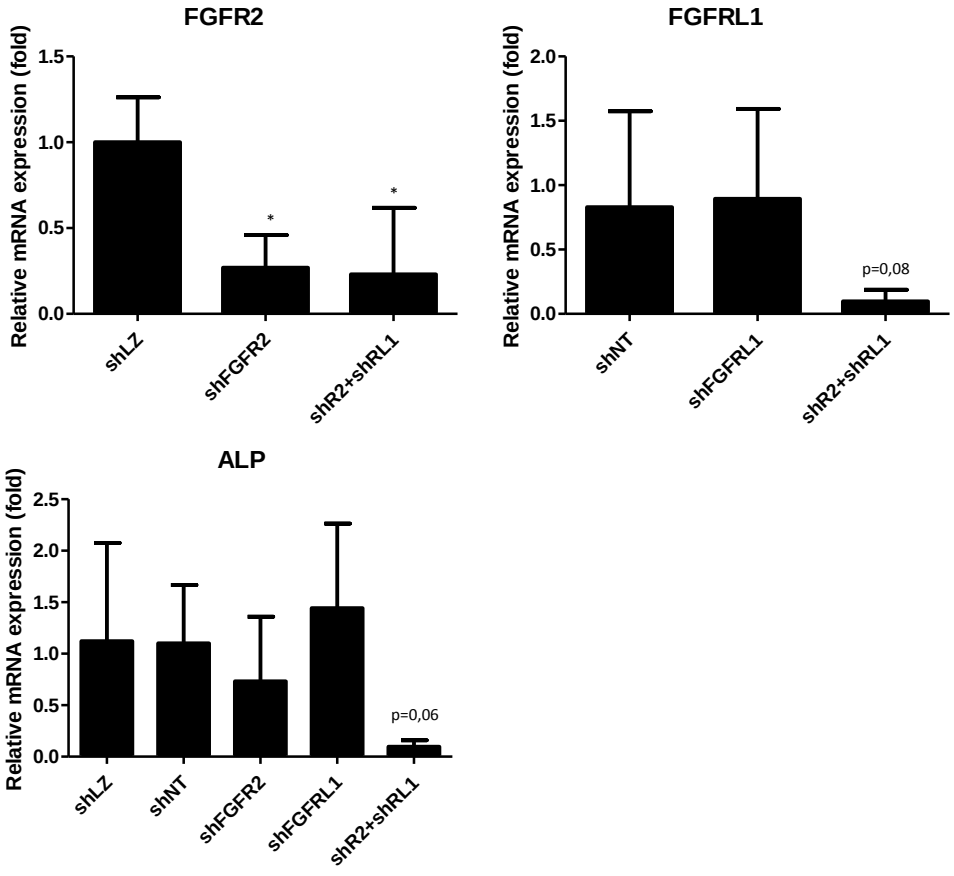


Figure 4: Effect of FGFR2 and FGFRL1 downregulation on osteoblast differentiation. (A) MSCs were transfected by nucleofection using shRNA-constructs and differentiated to osteoblasts. The FGFR mRNA expression is reported relative to transfection control (for shFGFR2 cells shLZ and shFGFRL1 shNT, respectively) where the columns present mean±SD, n=5. (B) Differentiation was studied measuring the levels of ALP mRNA in comparison of that in controls (set as 1) (columns, mean±SD, n=5).

3.6 The effect of a short and long FGF2 treatment on adipocyte differentiation

Treatment of pre-adipocytes and adipocytes with FGF2 for 24h decreased the expression of PPARγ mRNA by about 50% (Fig. 5A). The level of FABP4 mRNA was 85% in pre-adipocytes and in adipocytes of that in controls (data not shown). During a long (continuous) FGF2 treatment, the level of FABP4 mRNA in pre-adipocytes was 60% and in adipocytes 35% of that in control-treated cells (Fig. 5B). We also detected a decrease in the PPARγ mRNA levels but it was not as prominent as that of FABP4 (data not shown). Addition of PD173074 to FGF2 in the cultures prevented the FGF2-induced decrease of PPARγ (Fig. 5A) and FABP4 expression (Fig. 5B). The PD173074 treatment alone did not have any effect on differentiation of the cells (Fig. 5A, 5B).

Exposure of MSCs to FGF2 altered FGFR expression during adipocyte differentiation. During a short treatment FGFR1 mRNA increased 2 fold compared to control-treated MSCs (Fig. 5A) whereas during differentiation FGFR1 mRNA levels decreased to almost 40% in pre-adipocytes and adipocytes compared to those in controls (Fig. 5A). The decrease of FGFRL1 mRNA expression was more prominent in pre-adipocytes (about 50%) (Fig. 5A). A long treatment altered FGFR1 mRNA levels only slightly and the decrease in adipocytes was about 30% (Fig 5B). FGFRL1 mRNA level in pre-adipocytes and adipocytes was almost 30% and 20% of that in controls, respectively (Fig. 5B).

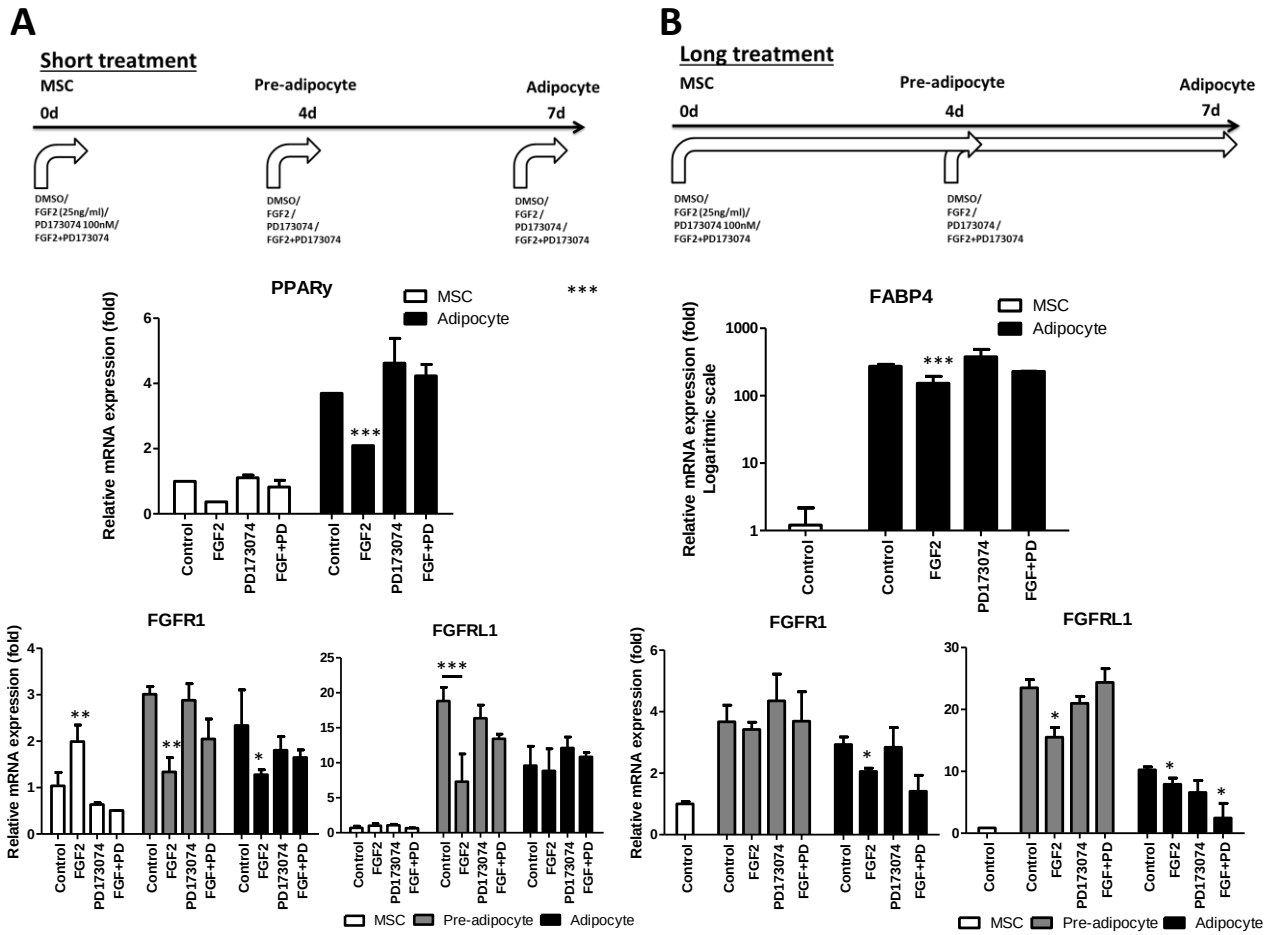


Figure 5: The effect of a short and long FGF2 treatment on adipocyte differentiation. (A) The MSCs were incubated in the presence of absence of FGF2 (25ngml), PD173074 (100mM), a combination of both or control for 24h at different stages of adipocytic differentiation cultures and studied for PPARγ, FGFR1 and FGFRL1mRNA levels by qRT-PCR. Columns represent means±SD (n=5), normalized to cyclophilin B mRNA expression and related to control-treated MSCs. (B) The effects of a long (continuous) treatment of the cultures with FGF2 (25ngml), PD173074 (100mM), a combination or control were studied with qRT-PCR for FABP4 (Log-scale), FGFR1 and FGFRL1 mRNAs. Columns represent mean±SD (n=5) normalized to cyclophilin B mRNA and related to the corresponding mRNA levels in untreated MSC cultures.

3.7 The effect of FGFR1 and FGFRL1 silencing on adipocyte differentiation

To study the possible role of FGFR1 and FGFRL1 in adipocyte differentiation we transfected MSC lines with shFGFR1 and/or shFGFRL1 shRNA constructs. A significant decrease of 80% in the expression of FGFR1 mRNA was obtained in shFGFR1 cells and the silencing effect was comparable in double-silenced cells (shFGFR1+shFGFRL1) when differentiated to adipocytes (Fig. 6A, Supplement 6). Knockdown of FGFRL1 was not successful in these cells as there was no significant difference between control and shFGFRL1 cells. However, a 60% decrease in FGFRL1 mRNA levels was observed in double-silenced compared to their controls (Fig. 6B, see also Supplement 6). Silencing of FGFR1 changed the expression of adipocyte marker genes. There was a significant decrease in the expression of FABP4 which was decreased down to 75% in FGFR1 silenced cells (Fig. 6B, Supplement 6). The knockdown of FGFR1 and FGFRL1 simultaneously did not, however, affect the level of FABP4 mRNA (Fig. 6B). FGFR1 silencing also decreased FGFRL1 mRNA levels (Supplement 6). Surprisingly, the knockdown of FGFR1 and FGFRL1 simultaneously did not affect the level of FABP4 mRNA (Fig. 6B).

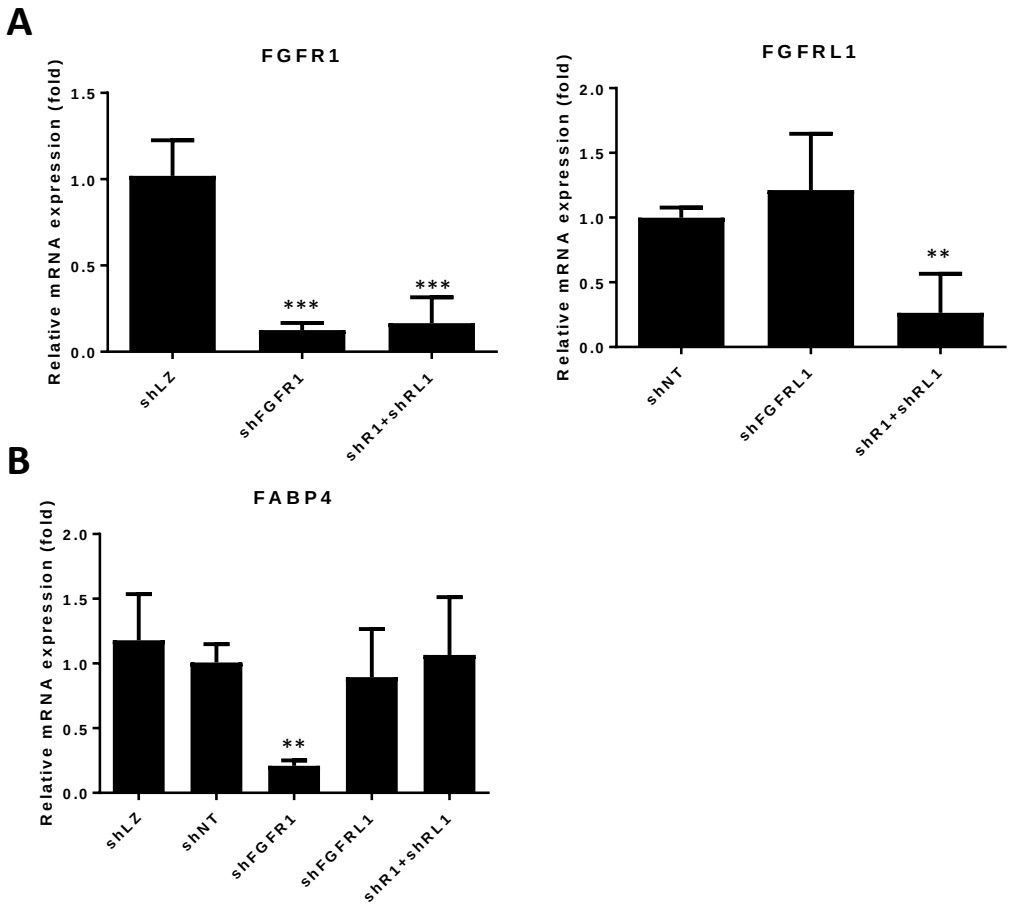


Figure 6: The effect of FGFR1 and FGFRL1 downregulation in adipocyte differentiation. (A) MSC cells were transfected with FGFR1 shRNA and/or FGFRL1 shRNA constructs, and differentiated to adipocytes and expression of the mRNAs for FGFR1 and FGFRL1 mRNA was studied by qRT-PCR. The mRNA levels were related to transfection controls (for shFGFR1 shLZ and for shFGFRL1 shNT, respectively). (B) Adipocytic differentiation was demonstrated by expression of FABP4 mRNA (mean±SD, n=5).

4. Discussion

4.1 FGFRs in MSC differentiation

In this study we report the development of two immortalized mouse mesenchymal stromal cell lines which under controlled growing conditions can be differentiated to osteoblasts and adipocytes. Using these cell lines we have studied the role of FGFRs in the differentiation towards osteogenic and adipogenic lineages and found that, besides FGFRs, FGFRL1 is but also a possible actor in the differentiation of these cells.

FGFs and FGFRs have been shown to regulate differentiation of many cell types (5-7). Here we show that FGFR1, -2 and -3 and FGFRL1 are expressed in MSCs and their expression is altered upon differentiation. The expression of various FGFRs in MSCs has been reported previously (4, 7, 10, 19) but to the best of our knowledge, this is the first analysis of the changes in all of the FGFRs, including FGFRL1, during differentiation of MSCs to osteoblasts and adipocytes, and studying the changes in FGFR expression with respect to FGFRL1.

4.2 FGFR2 in osteoblast differentiation

The expression of FGFR2 was observed in MSC and was significantly increased upon osteoblast differentiation. Therefore it can be hypothesized that FGFR2 plays a role in osteoblast differentiation. When the differentiation was inhibited by a short or long FGF2 treatment, the expression of FGFR2 was decreased, which may be associated with the observed blockade in differentiation. To verify the role of FGFR2 in the osteoblast differentiation, we silenced the expression in MSCs using a shRNA approach. This led to decreased expression of FGFR2 and inhibition of differentiation in mature osteoblasts. Corresponding findings on the role of FGFR2 in osteoblast differentiation have also been showed by others by using constantly-active FGFR2 (4). Elevated FGFR2 expression and activity was found to increase osteoblast differentiation via stimulated ERK- pathway signaling. FGFR2 is thought to act as a positive regulator of long bone growth (20) and accordingly, FGFR2 knock-out mice have skeletal dwarfism and decreased bone mineral density (7, 21). In our study, an increase of FGFR2 levels during differentiation was associated with decrease of the levels of FGFR1 mRNA. A short FGF2 treatment, resulting in the inhibition of differentiation, increased FGFR1 mRNA levels at all stages of differentiation. FGFR1 could function as a fast-acting negative regulator of

differentiation. Our findings are in line with the studies of White and co-workers (22) who suggested that FGFR1 is a negative regulator of long bone growth. Taken together, our results suggest that FGFR2 is an important positive regulator of osteoblastogenesis whereas FGFR1 may act as a fast-acting negative regulator during the differentiation process.

4.3 FGFR1 in adipocyte differentiation

During adipocyte differentiation the expression of FGFR1 increased while the expression of FGFR2 and FGFR3 remained unchanged. Inhibition of differentiation by a short and long FGF2 treatment was associated with a decrease in the expression of FGFR1 which was more marked after a short than a long treatment. Silencing of FGFR1 in MSCs and differentiating them to adipocytes significantly decreased the expression of adipocyte marker genes. FGFR1 may thus act as a fast-acting positive regulator of adipocyte differentiation which would be opposite to its effects on osteoblast differentiation. Our results are in line with earlier studies on the role of FGFR1 in adipocyte differentiation using adipose-tissue derived cell line models (10, 19). Silencing of FGFR1 by siRNA was shown to reduce the activation of FGFR-mediated signaling pathway and PPARy levels and decrease differentiation (10).

4.4 FGFRL1 alterations are associated with MSC differentiation

We identified FGFRL1 as another FGF signaling modulating actor possibly involved in MSC differentiation to osteoblasts and adipocytes. FGFRL1 was expressed in MSCs and its expression greatly increased during differentiation towards mature osteoblasts and adipocytes. When differentiation was inhibited by FGF2, the expression of FGFRL1 was downregulated. Interestingly, only a long FGF2 treatment decreased the levels of FGFRL1 mRNA suggesting that its modulatory effects are time-dependent. The mechanism of action of FGFRL1 is not well known. It has been suggested to act as a ligand trap, disabling the binding of FGFs to other receptors, or by recruiting protein tyrosine phosphatases such as SHP-1 to alter the intracellular signaling (6, 8, 12). SHP-1 known to interact with the intracellular domain of FGFRL1 is also known to promote bone formation (Tang et al., 2017). Other indirect interactions with FGFRs are also likely to occur. We observed that silencing of FGFR1 in adipocytic and FGFR2 in osteoblastic lineage was associated with decrease in FGFRL1 expression. This suggests that the regulation of FGFLR1 expression is caused or mediated by FGFR1 and FGFR2. It was notable that FGF2 treatment caused parallel effects on FGFR2 and FGFRL1 in osteoblastic and on FGFR1 and FGFRL1 in adipocyte lineage, which also supports although not proves mutual dependence of the changes.

FGFRL1 has been suggested to act as positive or negative regulator of differentiation depending on the context (8, 12). Our results suggest that FGFRL1 may act as a positive regulator of MSC differentiation depending on the lineage in association with FGFR1 or FGFR2. It may also function as a modulator of FGFR1 and FGFR2. Silencing of FGFR2 also decreased FGFRL1 which was associated with inhibition of osteoblast differentiation. FGFRL1 could thus act as a positive regulator of osteoblast differentiation together with FGFR2. Correspondingly in adipocytes, silencing of FGFR1 was associated with a concomitant decrease of FGFRL1 which suggests that FGFRL1 mediates or supports the effects of FGFR1 on adipocytic differentiation. A co-operative action of these receptors has previously been observed in xenopus embryos (8). Overexpression of a truncated form of FGFR1 or injection of FGFRL1 mRNA led to defects in trunk, tail and notochord and that the effects could be reversed by co-injection of FGFRL1 mRNA into FGFR1 overexpressing animals (8). In our study, unfortunately, silencing of FGFRL1 in MSCs was not successful or the cell pools lost their silencing after a number of passages. To obtain better understanding of FGFRL1 actions in MSCs better transfection and silencing efficiency should be obtained.

4.5 The effect of FGF2 treatment on differentiation

FGF2 is a potent member of the FGF-family which is able to activate all FGFRs. In our experiments a short and long FGF2 treatment inhibited osteoblast and adipocyte differentiation. FGF2 has been reported to have both stimulatory and inhibitory effects on osteoblast differentiation depending on the differentiation stage (23, 24). The stimulatory effect is mainly seen in the proliferative phase and inhibitory effect during later stages of differentiation. FGF2 transgenic mice with non-targeted overexpression have a dwarf phenotype caused by the premature closure of the growth plates while FGF2 deficient mice have a normal skeleton (7). In the absence of FGF2 the balance in the bone microenvironment may be maintained by several other growth factor pathways activated during MSC differentiation (25). In addition to FGF signaling, PDGF and TGF-β growth factor families have been observed to be important for MSC differentiation to several lineages (25). This may also explain our observations that FGFR inhibitor alone had no effect on MSC differentiation.

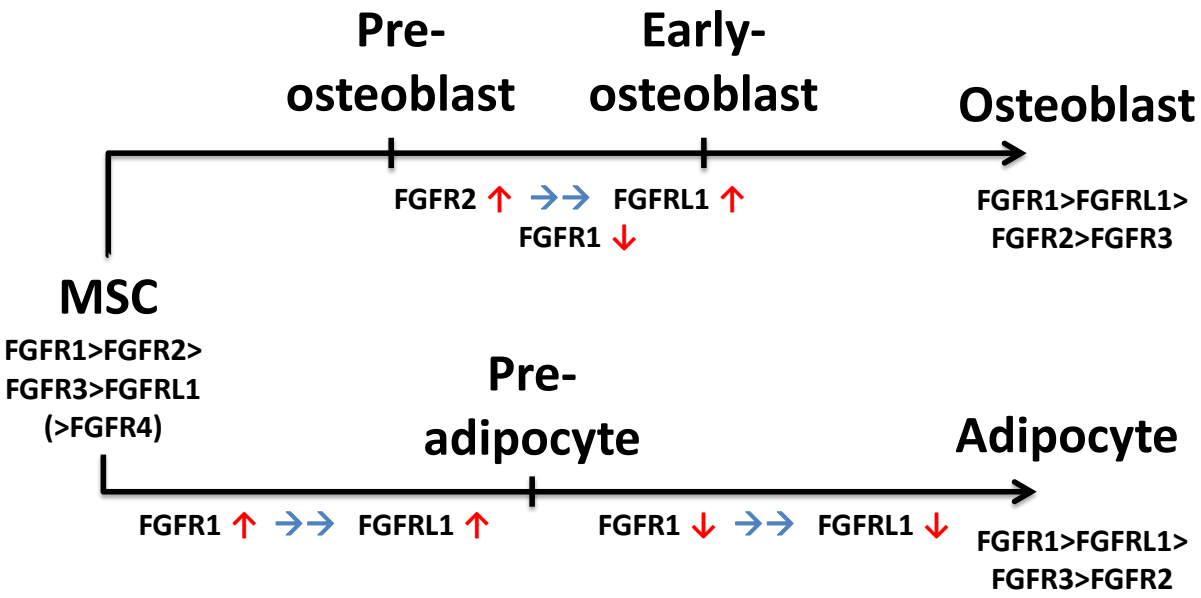
In adipocytes, previous reports have focused on studying the stimulatory effects on adipocyte differentiation obtained by priming MSCs with FGF-1 (9, 26). In contrast, no effect was seen when the cells were treated with FGF1 during differentiation (9). FGF1, similar to FGF2, is able to activate all classical FGFRs but there are differences in the receptor binding profile and affinity toward different FGFR isoforms (5, 6) which could explain some differences in the findings. Taken together,

the effects of FGFs on adipocyte differentiation appear to be dependent on the FGF isoform and differentiation stage.

4.6 Conclusions

We developed two immortalized mesenchymal stromal cell lines which can be used to model osteoblast and adipocyte differentiation. Osteoblast differentiation during cultures was demonstrated with osteoblast marker genes and ALP staining. Adipocyte differentiation was characterized on the basis of the morphology of the cells and expression of marker genes. These cell lines are valid models for in vitro studies on osteogenic and adipogenic differentiation of MSCs.

Our study suggests that FGFRL1 is involved in FGFR2- and FGFR1-mediated differentiation of MSCs to osteoblasts and adipocytes, respectively (Fig. 7). Expression of FGFRL1 is strongly increased during the differentiation process and it seems to follow the changes in FGFR1 and FGFR2. Furthermore, FGF2 treatment caused similar responses in FGFRL1 as in FGFR2 and in FGFR1 during osteoblast and adipocyte differentiation, respectively. Our results suggest that FGFR1 and FGFR2 regulate expression of FGFRL1 which in turn may support or modulate FGFR-driven signaling in MSCs. The study highlights a novel role for FGFRL1 on MSC differentiation to osteoblasts and adipocytes.



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465 **Figure 3: Summary of the findings.** In MSCs, FGFR1, 2, 3 and FGFRL1 are expressed. During
466 differentiation to osteoblasts the pattern of FGFRs changes as expression of FGFR2 and FGFRL1 is
467 elevated whereas that of FGFR1 is decreased. During adipocyte differentiation the expression of
468 FGFR1 is increased at the pre-adipocyte stage and then decreased. The expression of FGFRL1
469 continued to increase upon differentiation to mature adipocytes but seemed to decrease at very late
470 stage. The summary represents suggested regulation of FGFRL1 by FGFR2 and FGFR1 in osteoblast
471 and adipocyte lineage, respectively.

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