MEDICAL SCIENCE MONITOR

Down-Regulation of Fibroblast Growth Factor 2 (FGF2) Contributes to the Premature Senescence of Mouse Embryonic Fibroblast

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Freshly isolated mouse embryonic fibroblasts (MEFs) have great proliferation capacity but quickly enter senescent state after several rounds of cell cycle, a process called premature senescence. Cellular senescence can be induced by various stresses such as telomere erosion, DNA damage, and oncogenic signaling. But the contribution of other molecules, such as growth factors, to cellular senescence is incompletely understood. This study aimed to compare the gene expression difference between non-senescent and senescent MEFs to identify the key molecule(s) involved in the spontaneous senescence of MEFs.

Material/Methods: Primary MEFs were isolated from E12.5 pregnant C57/BL6 mice. The cells were continuously cultured in Dulbecco's Modified Eagle Medium for 9 passages. SA-β-Gal staining was used as an indicator of cell senescence. The supernatant from primary MEFs (P1 medium) or Passage 6 MEFs (P6 medium) were used to culture freshly isolated MEFs to observe the effects on cell senescence state. Gene expression profiles of primary and senescent MEFs were investigated by RNA-Seq to find the key genes involved in cell senescence. Adipocyte differentiation assay was used to evaluate the stemness of MEFs cultured in FGF2-stimulated medium.

Results: The senescence of MEFs cultured in the P1 medium was alleviated when compared to the P6 medium. Downregulation of FGF2 expression was revealed by RNA-Seq and further confirmed by real-time quantitative polymerase chain reaction and western blot. FGF2-stimulated medium also had anti-senescence function and could maintain the differentiation ability of MEFs.

Conclusions: The premature senescence of MEFs was at least partially caused by FGF2 deficiency. Exogenous FGF2 could alleviate the senescent phenotype.

MeSH Keywords: Cell Aging • Cell Dedifferentiation • Fibroblast Growth Factor 2

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Background

More than half a century ago Leonard Hayflick found that *in vitro* cultured primary human embryonic fibroblast had a limited replication capacity [1]. After about 50 rounds of division, these cells entered a permanent cycle arrest state called replicative senescence. Although the senescent cells stop proliferation, their metabolism remains active. Senescent cells exhibit a characteristic increase of senescence associated-β-galactosidase (SA-β-Gal) activity together with profound alterations in chromatin organization and secreted proteins including various inflammatory molecules, chemokines, and matrix remolding proteins that were collectively called the senescence-associated secretory phenotype [2–4]. Cellular senescence was involved in many physiological and pathophysiological processes such as embryonic development [5], tissue repair [6], individual aging [7], and carcinogenesis [8].

Replicative senescence of human fibroblasts is mainly caused by the telomere shorting [9] which is accumulated with each round of replication, and subsequent activation of p53-p21 [10] and/or pRB-p16 pathways [11]. In contrast to human fibroblasts, *in vitro* cultured mouse embryonic fibroblasts (MEFs) have a much smaller proliferation capacity. They usually entered a premature senescent state after 10 to 15 rounds of division [12]. Because MEFs have long telomeres and express the enzyme telomerase [13], its premature senescence is believed to be induced by other damage signals like oxidative molecules and oncogene activation rather than telomere erosion [14].

Inspired by the senescence-associated secretory phenotype of senescent cells, we hypothesized that the deregulation of secretory molecules might also contribute to the senescence of MEFs. Here we investigated the gene expression changes between primary and senescent MEFs by RNA-Seq and identified FGF2 as an important player in premature senescence.

Material and Methods

Cell culture

Primary MEFs were isolated from E12.5 pregnant C57/BL6 mice. The embryos were isolated after anesthesia and the head, limbs, and internal organs were removed. After washing with phosphate-buffered saline (PBS), the embryo was cut with scissors and then digested in trypsin for 15 minutes at 37°C followed by centrifugation at 800 g for 5 minutes. The cell precipitation was resuspended with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and cultured in a 10-cm dish in an atmosphere with 5% CO_2 and 21% O_2. When the cells grew to near confluence, they were labeled as Passage 1 and divided into 3 dishes after cell number

counting. The animal experiments were approved by the Ethic Committee of Hubei University of Medicine.

SA-β-Gal staining

The cells cultured in 6-well plates were fixed with 4% paraformaldehyde for 5 minutes followed by washing with PBS and then staining with SA-β-Gal staining kit at 37°C overnight as instructed by the manufacturer (Beyotime Institute of Biotechnology, Haimen, China).

RNA sequencing

The cells were lysed with TRIzol reagents. The lysis was frozen in liquid nitrogen and sent to Genewiz Company (Genewiz Co., Suzhou, China) in dry ice for transcriptome sequencing. RNA isolation, library construction, sequencing, genome mapping, and gene expression analysis were performed by the Genewiz Company based on the Illumina HiSeq2000 platform.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol reagent from the cells cultured in 6-cm dish followed by concentration determination. RNA (2 μg) was reverse transcribed into cDNA with the RevertAid reverse transcription kit (Thermo Fisher Scientific Co., MA, USA). RT-qPCR was run on a Biorad-CFX96 machine. The primers used in this study were as follows. FGF2: 5-GCG ACC CAC ACG TCA AAC TA (forward), 5-CTT AGA AGC CAG CAG CCG T-3 (reverse). GAPDH: 5-GGC AAA TTC AAC GGC ACA GT-3 (forward), 5-GGC CTC ACC CCA TTT GAT GT-3 (reverse). The relative mRNA levels were calculated with $2^{-\Delta\Delta Ct}$ method.

Western blot

The cells were harvested with RIPA lysis buffer. Protein concentration was determined with (bicinchoninic acid) BCA kit (Beyotime Institute of Biotechnology, Haimen, China); 100 ug of each protein samples was loaded and separated with SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred onto PVDF (polyvinylidene difluoride) membrane. After blocking in 5% non-fat milk for 2 hours, the membranes were incubated with primary antibodies overnight at 4°C. The membranes were then washed with TBST (tris-buffered saline and Tween 20) and incubated with secondary antibody for 1 hour at room temperature. After washing with TBST again, the membranes were developed in ECL (enhanced chemiluminescence) solution. The FGF2 rabbit polyclonal antibody was purchased from Thermo Fisher Scientific Co. (MA, USA, Cat no. OSG00015W) and the GAPDH primary antibody was purchased from Abcam Co. (MA, USA, Cat no. ab8245).
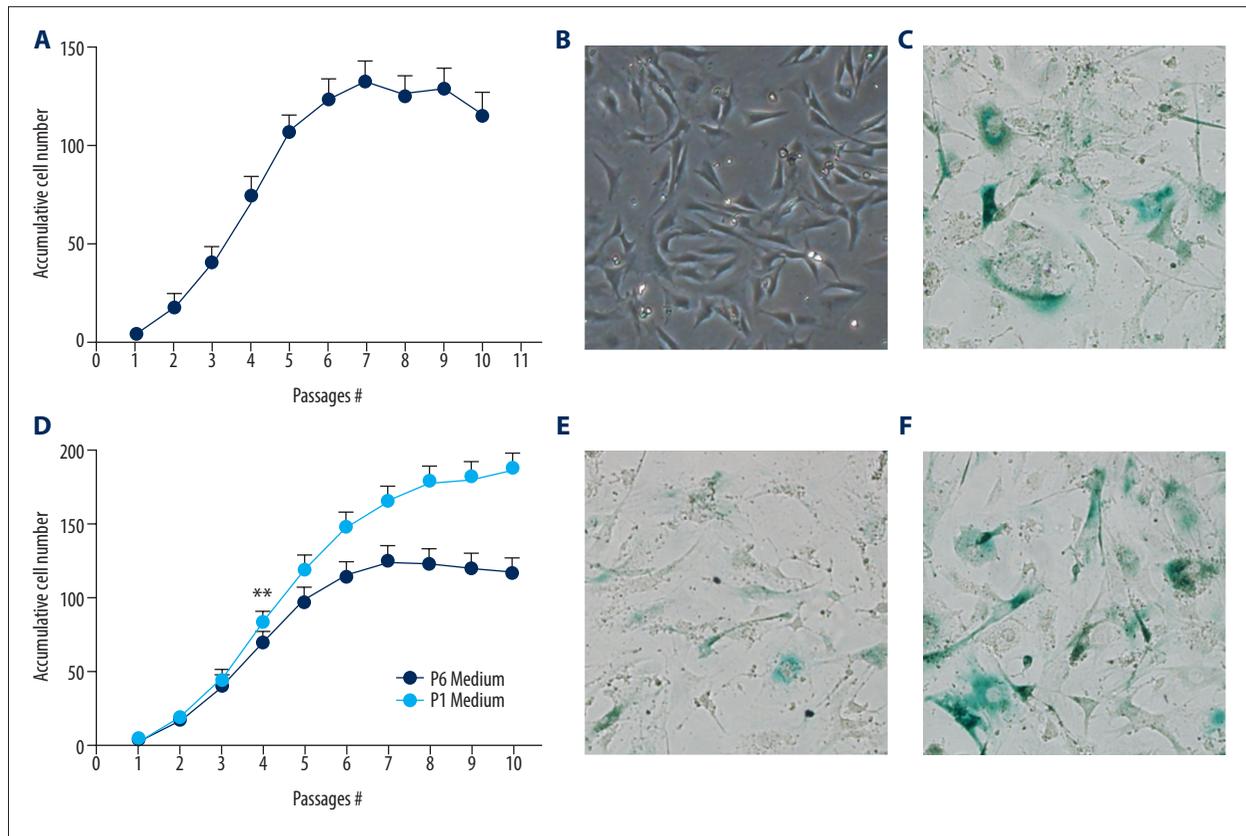
Figure 1. The supernatant of primary mouse embryonic fibroblasts (MEFs) had anti-senescence function. (**A**) Proliferation dynamic of primary MEFs. (**B**) MEFs of Passage 1. (**C**) MEFs of Passage 9, SA-β-Gal staining showed that they entered senescence. (**D**) Proliferation dynamic of primary MEFs grown in the culture medium collected from Passage 1 (P1) or Passage 6 (P6) MEF supernatant. From Passage 4, cell number of MEFs in P1 medium significantly exceeded that in P6 medium (Student's *t*-test, ** *P*<0.01). (**E, F**) SA-β-Gal staining of Passage 9 MEFs grown in P1 (**E**) or P6 medium (**F**).

FGF2 gene cloning

The *FGF2* gene was cloned from HEK-293 cells. RNA from HEK-293 cells was reverse-transcribed into cDNA. The *FGF2* gene was amplified from the cDNA by PCR. The forward primer was: 5-GCTCTAGA TGG CAG CCG GGA GCA TC-3, and the reverse primer was: 5-GCGAATTC AGC TCT TAG CAG ACA TTG G-3. The PCR product was inserted into pCDH-CMV-MCS-EF1-Puro vector. To increase FGF2 secretion, nucleotides of Igκ signal peptide (N- METDTLLLWV LLLWVPGSTG D-C) were added at the 5-end of the *FGF2* gene. The plasmid was sequenced to guarantee its proper construction.

Adipogenesis analysis

Cells cultured in adipocyte differentiation medium (dexamethasone 2.0 μM, insulin 20 μg/mL, indomethacin 400 μM, and IBMX 1.0 mM) were washed with PBS and then fixed in 4% paraformaldehyde for 10 minutes. Lipid substance was stained with Oil-Red following the manufacturer's instruction (Solarbio Co., Beijing, China).

Statistics

All the experiments were repeated at least 3 times. Data was presented as mean±standard deviation (SD). Student's *t*-test was used to compare the difference between 2 groups.

Results

The supernatant of primary MEF had anti-senescence function

Freshly isolated MEFs proliferated rapidly at the first 5 passages, slowing down in the following passages (Figure 1A). At Passage 9, the MEFs showed characteristics of senescence: cell cycle arrest, enlarged cell volume, pseudopodia forming and SA-β-Gal positive staining (Figure 1B, 1C). To compare the difference of the secretome between primary MEFs and serial passaged MEFs, we collected the culture supernatant of Passage 1 and Passage 6 MEFs and mix them with fresh DMEM (with 10% FBS) at 1: 1 volume ratio separately (marked as P1 or P6 medium). We then cultured
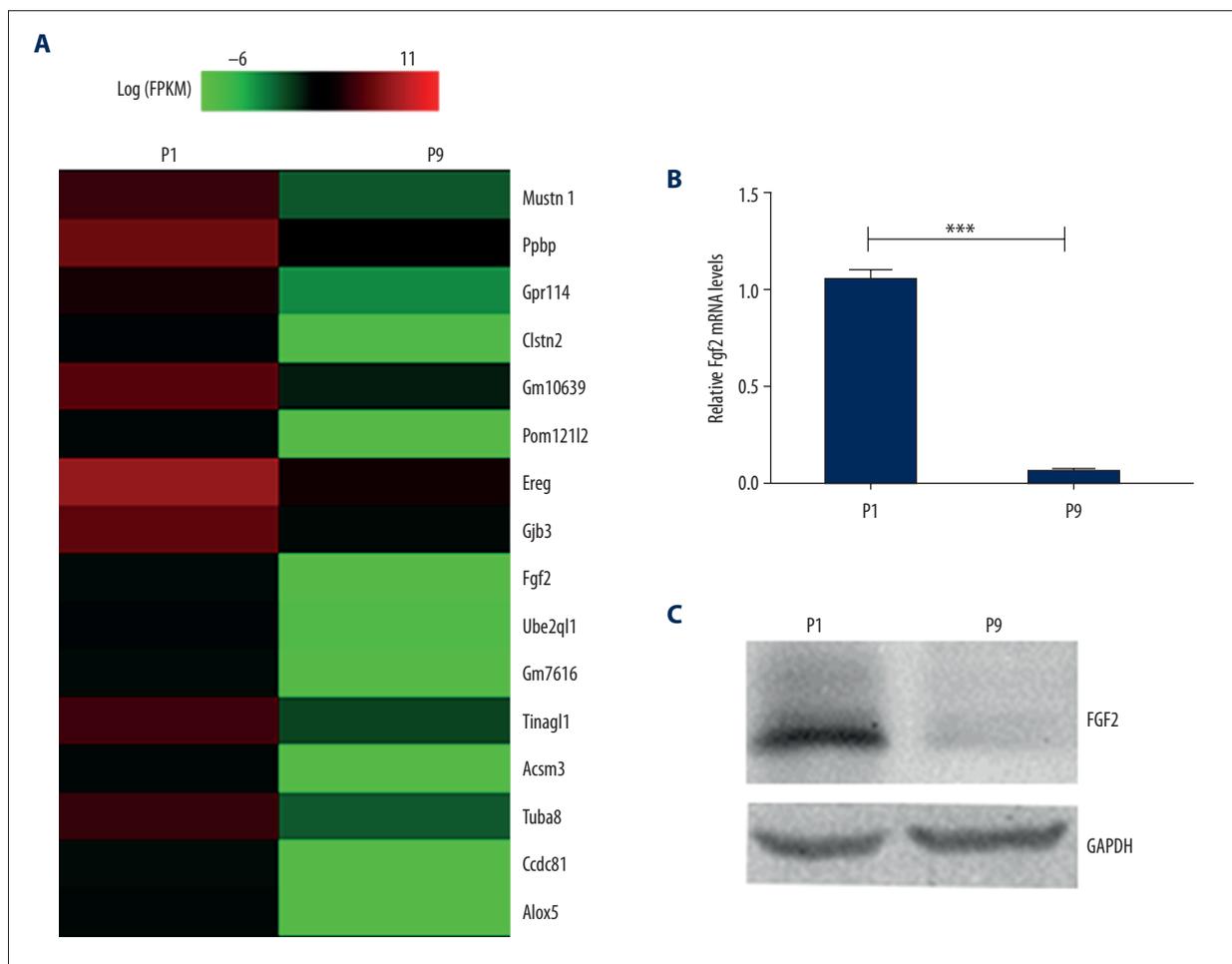
Figure 2. FGF2 synthesis was decreased in senescent mouse embryonic fibroblasts (MEFs). (**A**) RNA-Seq revealed the dramatic decrease of FGF2 expression in senescent MEFs. (**B**) Decrease of FGF2 was further verified by RT-qPCR and (**C**) western blot. (Student's *t*-test, *** *P*<0.001.)

primary MEFs in P1 or P6 medium for 9 passages to compare the senescent phenotype. Figure 1D shows that MEFs in P1 medium proliferated more rapidly than in P6 medium. SA-β-Gal staining also showed that the senescent phenotype of Passage 9 MEF in P1 medium was much weaker when compared to P6 medium (Figure 1E, 1F). These results indicated that the supernatant of primary MEFs had anti-senescence function, and such function disappeared as MEFs experienced several cell cycles.

FGF2 expression was decreased in senescent MEFs

To identify the anti-senescence molecules secreted by primary MEFs, we collected Passage 1 and senescent Passage 9 MEFs and performed RNA-sequencing experiments to compare the gene expression difference between them. There were 497 genes whose expression in Passage 1 MEFs were more than 10-fold the expression in senescent Passage 9 cells. Among them, the level of FGF2 in Passage 1 MEFs was 22-fold more than in Passage 9 MEFs (Figure 2A). The downregulation of

FGF2 in senescent Passage 9 MEFs was further verified by RT-qPCR and western blot assays (Figure 2B, 2C).

Exogenous FGF2 could alleviate cell senescence

To investigate the function of FGF2 in MEF senescence, we cloned the *FGF2* gene from HEK 293 cells. A signal peptide of Igκ was added before *FGF2* gene to increase its secretion. We then transfected the FGF2 over-expression plasmids into HEK 293 cells and a GFP vector was used as a control. The culture supernatant from FGF2 over-expression or control cells was collected and mixed with fresh DMEM (with 10% FBS) at 1: 1 volume ratio separately (marked as FGF2 medium or control medium). We used the FGF2 medium or control medium to culture primary MEFs to Passage 9 and then stained them for SA-β-Gal activity. As shown in Figure 3, the Passage 9 MEFs cultured in FGF2 medium were significantly rescued from senescence as compared to control medium. This result suggested that exogenous FGF2 could postpone the senescence of MEFs.
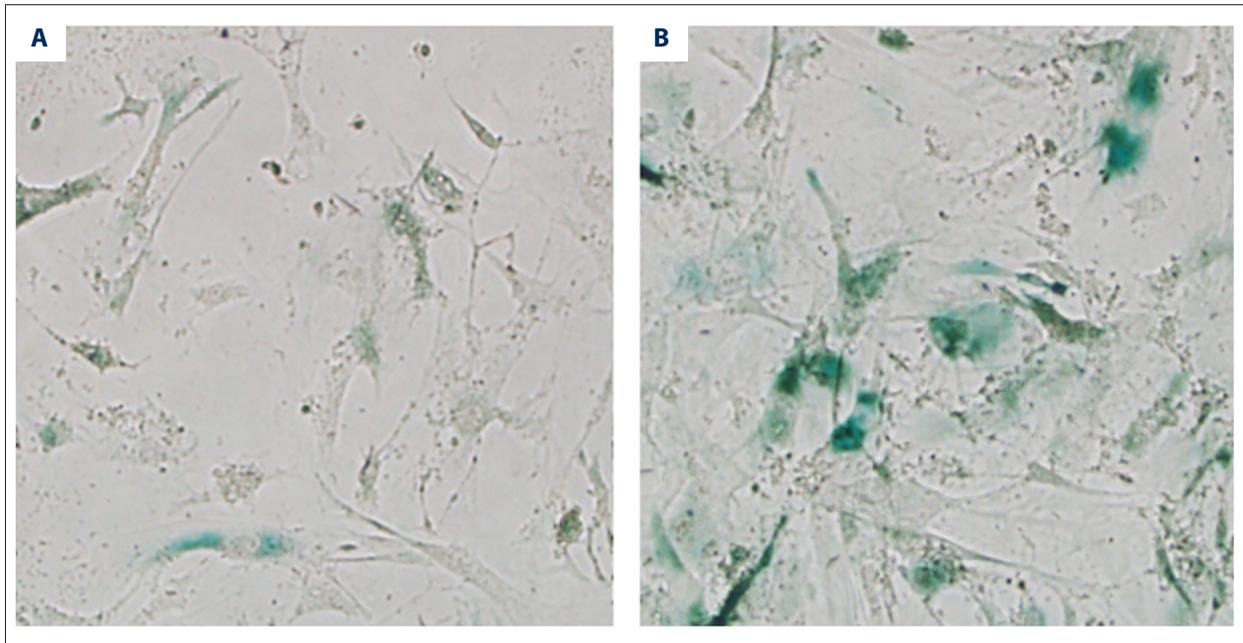
Figure 3. Exogenous FGF2 could alleviate mouse embryonic fibroblast (MEF) senescence. Primary MEFs were cultured in medium supplemented with exogenous FGF2 (**A**) or control medium (**B**) to Passage 9 and then stained with SA-β-Gal kit.

Exogenous FGF2 maintained MEF stemness.

MEFs have pluripotent differentiation ability. To investigate the influence of FGF2 on the adipocyte differentiation of MEFs, we treated MEFs of Passage 3 with FGF2 medium or control medium for 4 days in mixture with 2x adipocyte differentiation medium (at 1: 1 volume ratio). The cells were then fixed and stained with Oil-Red. As shown in Figure 4, the adipogenic differentiation ability of MEFs cultured in FGF2 medium was enhanced compared to control medium. This indicated that the stemness of MEFs was better maintained by exogenous FGF2.

Discussion

Cellular senescence can be triggered by various stresses that include telomere erosion resulting from repeated cell division, mitochondrial deterioration, oxidative stress, severe DNA damage, and activation of certain oncogenes. Here we found that FGF2 expression was downregulated when MEFs entered senescent stated. On the other hand, FGF2 had anti-senescence function as shown that exogenous FGF could alleviated cell senescence. Thus, the deficiency of FGF2 formed a positive feedback loop as MEF dividing, which greatly accelerated the MEF senescence process. These results might explain why *in vitro* cultured MEFs senescence prematurely as compared to human embryonic fibroblast, although they have long telomere and baseline expression of telomerase.

Fibroblast growth factor (FGF) family proteins play key roles in stem cell growth and surviving during embryogenesis, tissues repair and carcinogenesis [15–17]. Previous reports showed that the synthesis of FGF2 was decreased in various mouse and human cells in an age-dependent manner [18,19] and supplement of exogenous FGF2 could increase the proliferation and suppress the senescence of human mesenchymal stem cells [12,20,21]. However, the deregulation of FGF2 in the premature senescence of MEFs is unknown. This might be because the secretion of endogenous FGF2 protein was inefficient due to the lack of signal peptide and it was hard to be detected in the out-cellular space. In this study, we also found that exogenous FGF2 could enhance the adipocyte differentiation ability of MEFs. However, Chakkalakal et al. reported that increasing FGF2 signaling in muscle stem cells resulted in the loss of quiescence and diminished regenerative capacity [22]. Thus, the stemness preservation function of FGF2 was tissue-specific.

Although cellular senescence has been studied most extensively in cultured cells, there are broad appreciation for cellular senescence to occur *in vivo* [7]. As senescent cells accumulated in tissues when the organism ages, their metabolic behaviors and gene expression profiles had been inevitably linked to a number of age-related physiologic and pathologic changes. Given the impact of cellular senescence in the age-associated processes, there has been much interest in understanding how to modulate senescence for therapeutic purposes. It has been shown that blood from young mice can positively impact aged animals, such as improving cognitive function. Villeda et al. [23] and Sinha et al. [24] showed in their heterochronic parabiosis
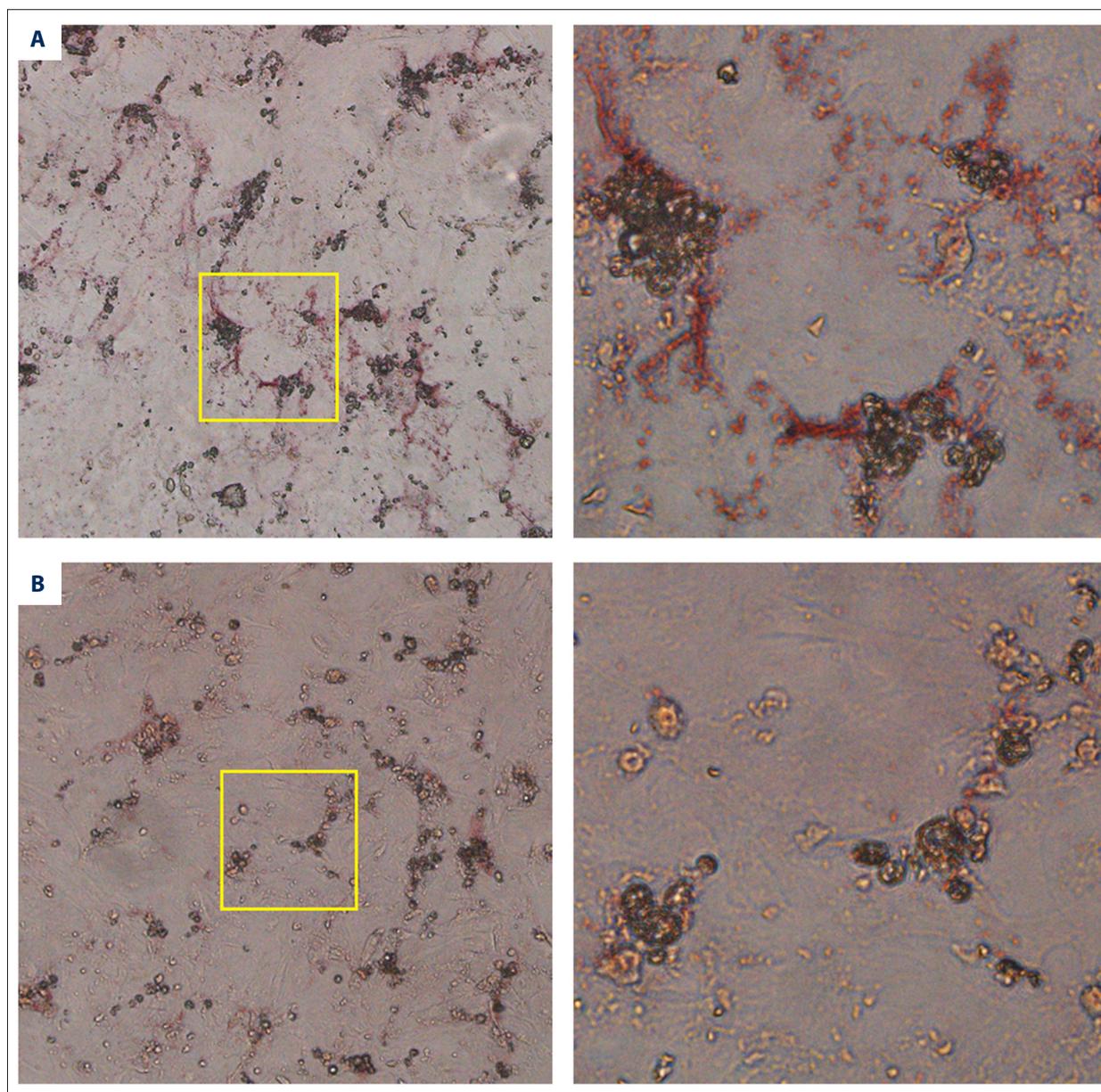
Figure 4. Exogenous FGF2 maintained mouse embryonic fibroblast (MEF) stemness. Passage 3 MEFs were cultured in adipocyte differentiation medium supplemented with exogenous FGF2 (**A**) or control medium (**B**) for 4 days and then stained with Oil-Red. (**Left**, 100× magnification: **right**, 200× magnification.)

experiments that muscle stem cells, liver cells, and hippocampal neurons could gain benefits from exposure to blood from young mice. However, the specific rejuvenation molecules in the blood from young mice, and their sources, still remained elusive. Here we showed that both primary MEFs and FGF2-stimulated HEK293 cells could secrete molecules with anti-senescence function. Our results hold the promise that primary MEFs or MEFs derived substrate (such as NIH-3T3 cell with ectopic expression of FGF2) may be another source of rejuvenation reagent instead of the blood of young mice blood used to treat senescence or senescence-associated symptoms.

Conclusions

Taken together, our results showed that the spontaneous senescence of *in vitro* cultured MEFs was at least partly caused by FGF2 deficiency. Supplement of FGF2 could not only postpone the appearance of senescence but also help in maintaining the stemness of MEFs.

Conflict of interest

None.

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