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FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF-β2

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Abstract

Human mesenchymal stem cells (hMSCs) are able to both self-replicate and differentiate into a variety of cell types. Fibroblast growth factor-2 (FGF-2) stimulates the growth of hMSCs *in vitro*, but its mechanisms have not been clarified yet. In this study, we investigated whether cellular senescence was involved in the stimulation of hMSCs growth by FGF-2 and the expression levels of transforming growth factor-β1 and -β2 (TGF-βs). Because hMSCs were induced cellular senescence due to long-term culture, FGF-2 decreased the percentage of senescent cells and suppressed G1 cell growth arrest through the suppression of $p21^{Cip1}$, p53, and $p16^{INK4a}$ mRNA expression levels. Furthermore, the levels of TGF-βs mRNA expression in hMSCs were increased by long-term culture, but FGF-2 suppressed the increase of TGF-β2 mRNA expression due to long-term culture. These results suggest that FGF-2 suppresses the hMSCs cellular senescence dependent on the length of culture through down-regulation of TGF-β2 expression.

Keywords: Human mesenchymal stem cells; FGF-2; TGF-β; Cellular senescence; Cyclin-dependent kinase inhibitors; RB

Mesenchymal stem cells (MSCs) are able to self-replicate and differentiate into a variety of cell types such as osteoblasts, chondrocytes, adipocytes, and smooth muscle cells [1–5]. These capacities of MSCs have been used in studies of bone and cartilage regeneration [6–8]. One of the sources for human MSCs (hMSCs) is adult bone marrow. However, the ratio of hMSCs in adult bone marrow is about one per one-hundred-thousand nucleated cells [6], and the volume of bone marrow obtainable is limited. To secure the numbers of hMSCs required for the regeneration of tissues, hMSCs obtained from bone marrow need to be expanded *in vitro*.

Fibroblast growth factor-2 (FGF-2) is a cell growth factor involved in angiogenesis and tissue repair. FGF-2 maintains human bone marrow stromal cells in an immature state during *in vitro* expansion [9–11]. In hMSCs, FGF-2 enhances growth and maintains the potential for multidifferentiation [12,13]. Thus, it is thought that FGF-2 is one of the effective factors in the regeneration of tissues.

Transforming growth factor-βs (TGF-βs) are multifunctional proteins that regulate cell growth, differentiation, migration, extracellular matrix production, angiogenesis, and immunosuppression [14]. TGF-βs arrest the cell growth of epithelial cells and blood cells in the G1 phase through inhibition of G1 cyclin-dependent kinases (CDKs) [15,16]. It is reported that TGF-βs down-regulate the c-myc oncogene and up-regulate the CDK inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ [17,18].

Cellular senescence is one of the tumor suppressor functions of normal human cells [19]. Senescent cells induce cell growth arrest in the G1 phase and a change in morphology and metabolism. Some of the senescence-associated changes that are common to many different cell types include cellular enlargement, increased lysosome biogenesis, and expression of a β-galactosidase that has a pH optimum of 6 (senescence-associated β-galactosidase or

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SA-β-Gal) [20]. It is thought that two mechanisms of cellular senescence exist: intrinsic senescence dependent on telomere shortening and extrinsic senescence independent of it. The former is induced by activation of p53 and an increase of expression levels of $p21^{Cip1}$, a well-recognized p53 target gene [19,21–23]. The latter is induced by various culture stresses and the up-regulation of $p16^{INK4a}$ expression [24–26]. Activation of cyclin-CDK complex by suppression of expression of the CDK inhibitors $p21^{Cip1}$, p53, $p16^{INK4a}$ promotes phosphorylation of retinoblastoma proteins (pRB). Phosphorylation of pRB is required for the progress from the cellular G1 phase to the S phase.

Our previous studies have shown that hMSCs growth was decreased and the level of TGF-β mRNA expression increased during long-term subculture *in vitro* [27]. In this study, we investigated whether the decrease of growth ability in long-term culture involves cellular senescence through changes in the expressions of TGF-β and the CDK inhibitors. Moreover, we attempted to stimulate hMSCs growth using FGF-2 and investigated whether FGF-2 affected cellular senescence and the expressions of TGF-βs, cell growth suppression factors.

Materials and methods

Cell culture. hMSCs were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and seeded in MSCGM medium (Cambrex Bio Science Walkersville) at 5000 cells/cm^2 with or without 1 ng/ml FGF-2 (BD Biosciences, Bedford, MA). FGF-2 was also added when the culture medium was changed every 2–3 days. The concentration of FGF-2 used in this study was based on a previous report [13]. The cells were maintained in humidified incubators at 37 °C with 5% CO_2.

TGF-β treatment. TGF-β1 and TGF-β2 (human, recombinant) were purchased from Sigma (St. Louis, MO). TGF-β1 or TGF-β2 at 5 ng/ml was added to the culture medium without FGF-2 for 5 days. The concentration of TGF-βs used was determined by a previously published study [28].

SA-β-Gal staining. SA-β-Gal staining was performed using a Senescence-associated β-Galactosidase Staining Kit (Cell Signaling, Beverly, MA) following the manufacturer's protocol.

BrdU incorporation. The incorporation of BrdU during DNA synthesis was measured using a Cell Proliferation ELISA kit with BrdU (Roche Diagnostics, Penzberg, Germany) following the manufacturer's protocol.

Flow cytometry analysis. hMSCs were removed from the culture dish with trypsin/EDTA (Cambrex Bio Science Walkersville), then stained using a CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA) following the manufacturer's protocol. Propidium iodide (PI) fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo (Tree Star, Inc., Ashland, OR).

Quantitative real-time RT-PCR. PCRs of p53, TGF-β1, and TGF-β2 were performed for 35 cycles under the following conditions: denaturation at 95 °C for 10 s, annealing at 68 °C for 10 s, and extension at 72 °C for 16 s; of p16: 95 °C for 10 s, 60 °C for 10 s, 72 °C for 6 s; and p21: 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s, using the LightCycler real-time PCR System (Roche Diagnostics, Tokyo, Japan). The primers for p53, TGF-β1, TGF-β2, and GAPDH were from a LightCycler-Primer Set (Search LC GmbH, Heidelberg, Germany). The primers for p16 and p21 were 5′-CACTCACGCCCTAAGC-3′ and 5′-GCAGTGTGACTCAAGAGAA-3′, and 5′-TTGATTAGCAGCGGAACA-3′ and 5′-GGAGAAACGG GAACCAG-3′, respectively.

Western blotting. A mouse monoclonal antibody against pRb and a rabbit polyclonal antibody against phospho-pRb were purchased from Cell Signaling (Beverly, MA). The rabbit polyclonal antibodies against TGF-β1, TGF-β2, and GAPDH were purchased from Santa Cruz Biotechnology, Inc. The bands were quantified using ImageQuant™ TL (GE Healthcare UK Ltd., Buckingham, England).

Results

TGF-β induced cellular senescence in hMSCs

To investigate the effects of TGF-β on cellular senescence, hMSCs were cultured in MSCGM medium supplemented with TGF-β1 or TGF-β2, and then SA-β-Gal staining was performed and incorporation of BrdU, an analog of thymidine, was measured. One day after TGF-β1 or TGF-β2 treatment, hMSCs had a fibroblast-like morphology (Fig. 1C and E) similar to the control (Fig. 1A). Five days after TGF-β1 or TGF-β2 treatment, hMSCs had acquired a depressed morphology, and some of them were stained blue by SA-β-Gal staining (Fig. 1D and F, arrows). In the control, however, stained cells were rarely observed (Fig. 1B). Five days after TGF-β1 and TGF-β2 treatment, BrdU incorporation had decreased in comparison with the control (Fig. 1G). Furthermore, to confirm whether TGF-βs induced G1 cell growth arrest in hMSCs, cell cycle analysis was performed using flow cytometry. As shown in Fig. 1H, TGF-β1 and TGF-β2 increased the percentage of cells in G1 phase, and decreased it in S and G2 phases. Then, the mRNA expression levels of $p16^{INK4a}$, $p21^{Cip1}$, and p53, CDK inhibitors of the G1 phase, and the protein expression levels of pRB were measured after 5 days of TGF-β1 or TGF-β2 treatment. TGF-βs increased all three mRNA expression levels (Fig. 1I–K). On the other hand, the phosphorylated pRB (ppRB) expression was decreased by both TGF-βs (Fig. 1L). These results suggest that cellular senescence of hMSCs is induced through G1 growth arrest by TGF-β1 and TGF-β2.

FGF-2 suppressed hMSCs cellular senescence

To investigate whether stimulation of hMSCs growth by FGF-2 was involved in the suppression of cellular senescence, hMSCs were stained with SA-β-Gal after culture with or without FGF-2 (FGF-2(+) or FGF-2(−)) for 10 or 50 days. After 10 days' culture in FGF-2(−), hMSCs had a fibroblast-like morphology, and 20.5% of the cells were stained blue by SA-β-Gal (Fig. 2A); however, after 50 days' culture, hMSCs had developed a depressed morphology, and 57.6% of the cells were stained blue (Fig. 2C, arrows). After 10 or 50 days' culture in FGF-2(+), hMSCs morphology had a fibroblast-like morphology; moreover, 35.8% or 27.3% of the cells were stained blue, respectively (Fig. 2B and D). BrdU incorporation into hMSCs after 50 days' culture was 40% lower than after 10 days' culture in FGF-2(−) but not in FGF-2(+) (Fig. 2E). Furthermore, we investigated the effects of FGF-2 on the cell cycle. After 50 days' culture in FGF-2(−), the number of cells in the G1 phase was increased, but not after culture in FGF-2(+) (Fig. 2F).

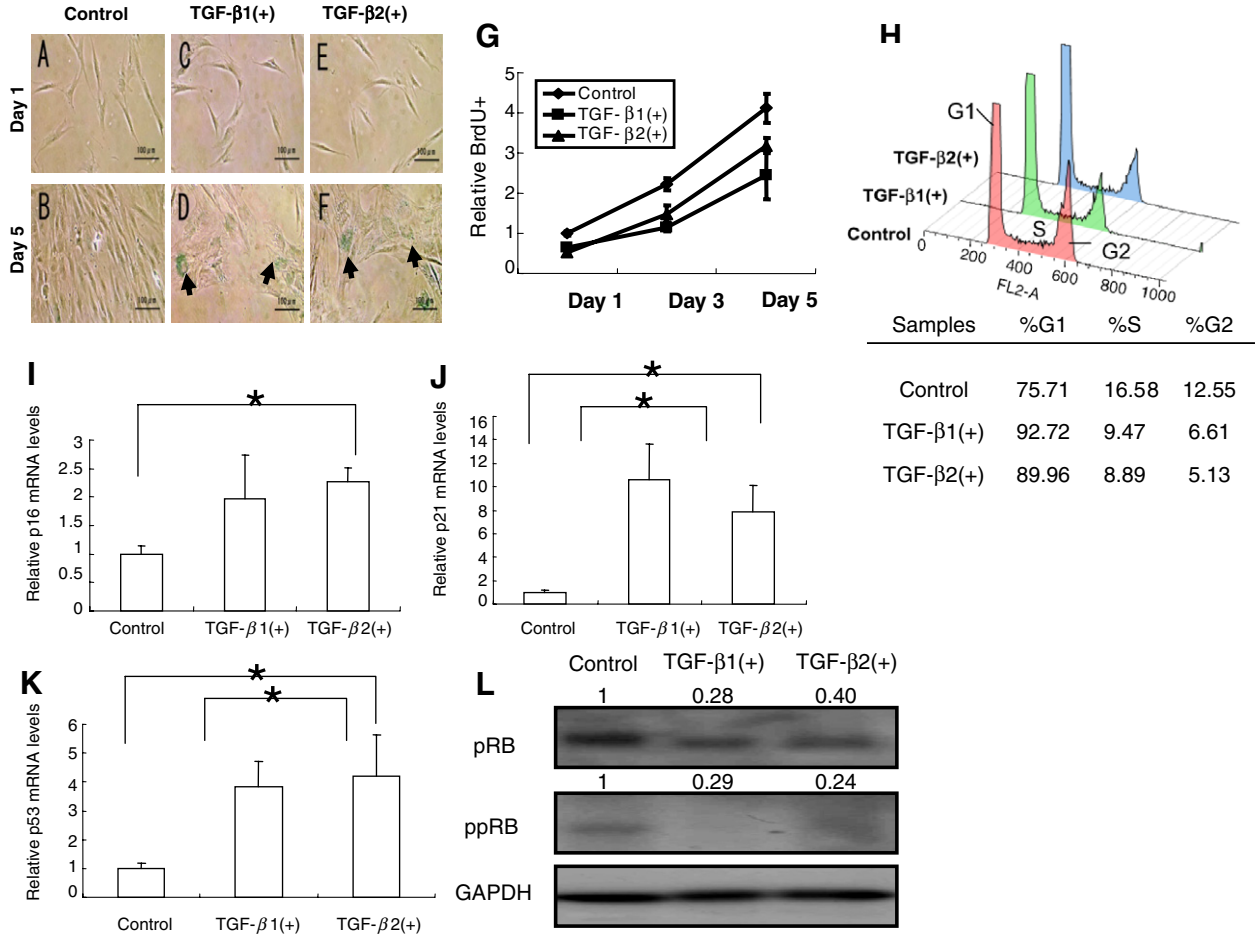

Fig. 1. TGF-β1 and β2 induce cellular senescence through G1 cell cycle arrest in hMSCs. hMSCs were maintained as an untreated control or treated with 5 ng/ml TGF-β1 or TGF-β2 for 5 days. (A–F) hMSCs were performed SA-β-Gal staining 1 or 5 days after TGF-β treatment (Day 1 or 5). The arrows in (D) and (F) indicate the senescent cells stained blue. The scale bar is 100 μm. (G) BrdU incorporation into hMSCs was assayed at Days 1, 3, and 5. Each point represents quantities relative to the untreated control at Day 1. (H) After 3 days' culture with or without TGF-β1 or TGF-β2, cells were removed from the culture dish with trypsin/EDTA, fixed, stained for DNA with PI, and analyzed by flow cytometry (*y*-axis, cell count; *x*-axis, PI intensity). (I–K) $p16^{INK4a}$ (A), p53 (B), and $p21^{Cip1}$ (C) mRNA expression levels measured using real time RT-PCR. The relative levels of gene expression of target mRNA were normalized to GAPDH expression. Values are means ± SD of three experiments ($^{*}P < 0.05$). (L) total pRB and phospho-pRB proteins detected using Western blot analysis.

The mRNA expression levels of $p16^{INK4a}$, $p21^{Cip1}$, and p53 and the expression levels of pRB in hMSCs were measured after culture for 10 or 50 days in FGF-2(−) or FGF-2(+). After 50 days' culture in FGF-2(−), the mRNA expression levels of $p16^{INK4a}$, $p21^{Cip1}$, and p53 were significantly higher than after 10 days' culture, but not after culture in FGF-2(+) (Fig. 3A–C). On the other hand, after 50 days' culture in FGF-2(−), the expression levels of total pRB and ppRB were decreased compared with after 10 days' culture, but not after culture in FGF-2(+) (Fig. 3D). These results suggest that FGF-2 suppresses hMSCs cellular senescence depending on the length of culture.

FGF-2 influenced TGF-β mRNA expression in hMSCs

To investigate the effects of FGF-2 on TGF-β mRNA and protein expression levels in hMSCs, their levels were measured after culture for 1, 10, or 50 days in FGF-2(−) or FGF-2(+). After culture for 50 days in both FGF-2(−) and FGF-2(+), TGF-β1 mRNA expression levels of hMSCs had increased in comparison with culture for 1 and 10 days (Fig. 4A). On the other hand, TGF-β2 mRNA expression levels were higher after 50 days' culture in FGF-2(−) than after 10 days, but not after culture in FGF-2(+) (Fig. 4B). Comparing 50 days' culture with 10 days' culture, the changes of TGF-β1 and TGF-β2 protein expression levels paralleled the results of mRNA expression levels (Fig. 4C). These results suggest that FGF-2 has no effect on TGF-β1 expression levels in hMSCs, but inhibits the increase of TGF-β2 expression, depending on the length of culture.

Discussion

hMSCs are one of the human tissue stem cells, and they maintain the homeostasis of bone and cartilage. hMSCs

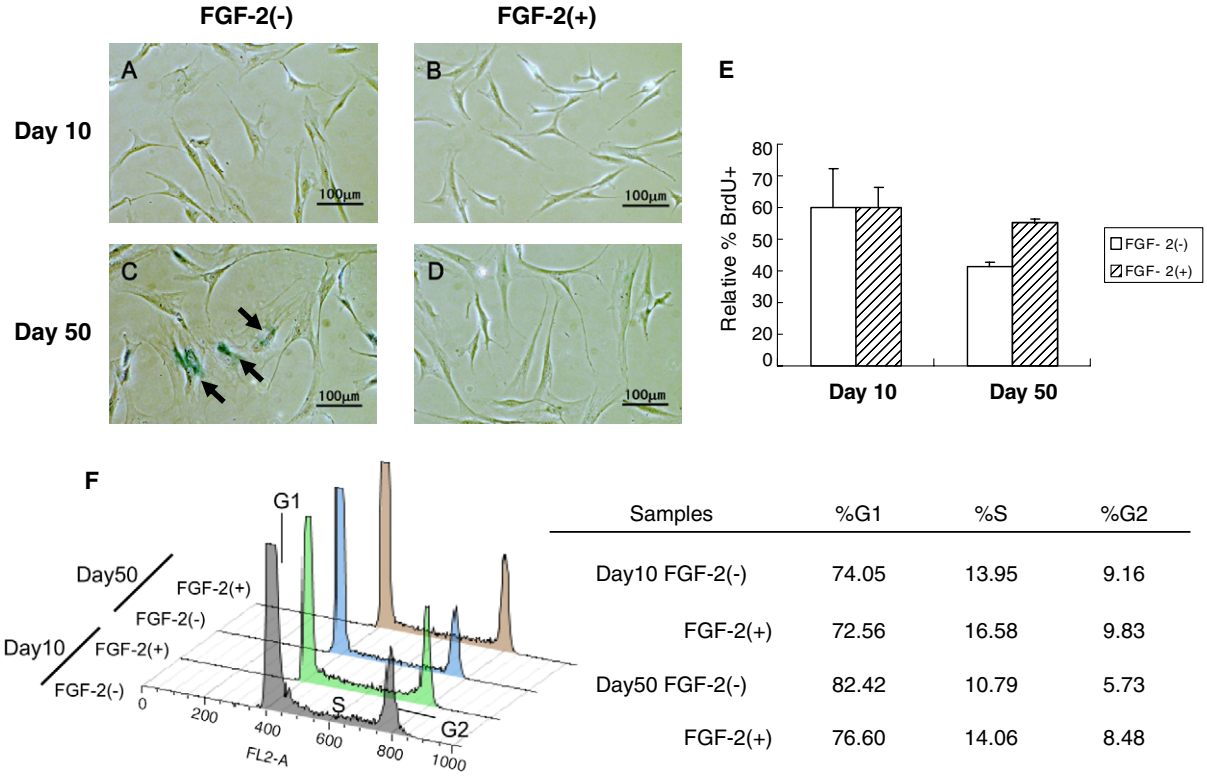

Fig. 2. FGF-2 suppresses cellular senescence through G1 cell cycle arrest due to long-term culture. hMSCs were maintained in the medium in the presence or absence of FGF-2 (1 ng/ml). (A–D) hMSCs were performed SA-β-Gal staining after culture for 10 days or 50 days (Day 10 or 50). The arrows in (C) indicate senescent cells that stained blue. The scale bar is 100 μm. (E) BrdU incorporation into hMSCs was assayed at Days 10 and 50. Each bar represents quantities relative to Day 10 and is average ± SD of three wells. (F) hMSCs were detached from the culture dish with trypsin/EDTA after culture at Days 10 and 50, fixed, stained for DNA with PI, and analyzed by flow cytometry (*y*-axis, cell count; *x*-axis, PI intensity).

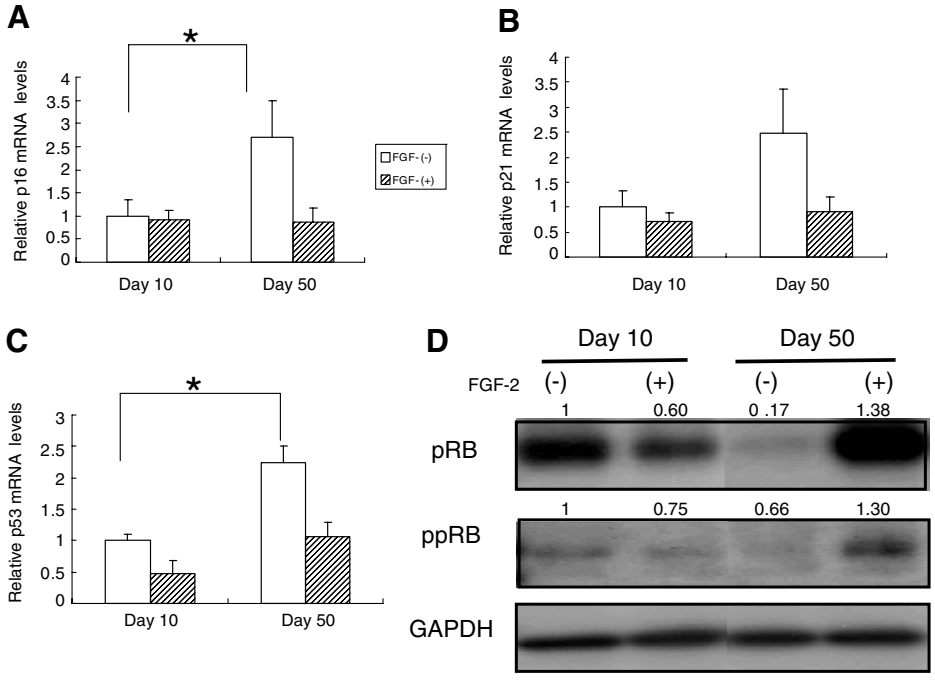

Fig. 3. FGF-2 suppresses G1 cell cycle arrest due to passaging. hMSCs were maintained in the medium in the presence or absence of FGF-2 (1 ng/ml), and total RNAs and proteins were extracted when approaching confluence. (A–C) $p16^{INK4a}$ (A), p53 (B), and $p21^{Cip1}$ (C) mRNA expression levels were measured using real time RT-PCR at Days 10 and 50. (D) Total pRB and phospho-pRB proteins detected using Western blot analysis at Days 10 and 50.

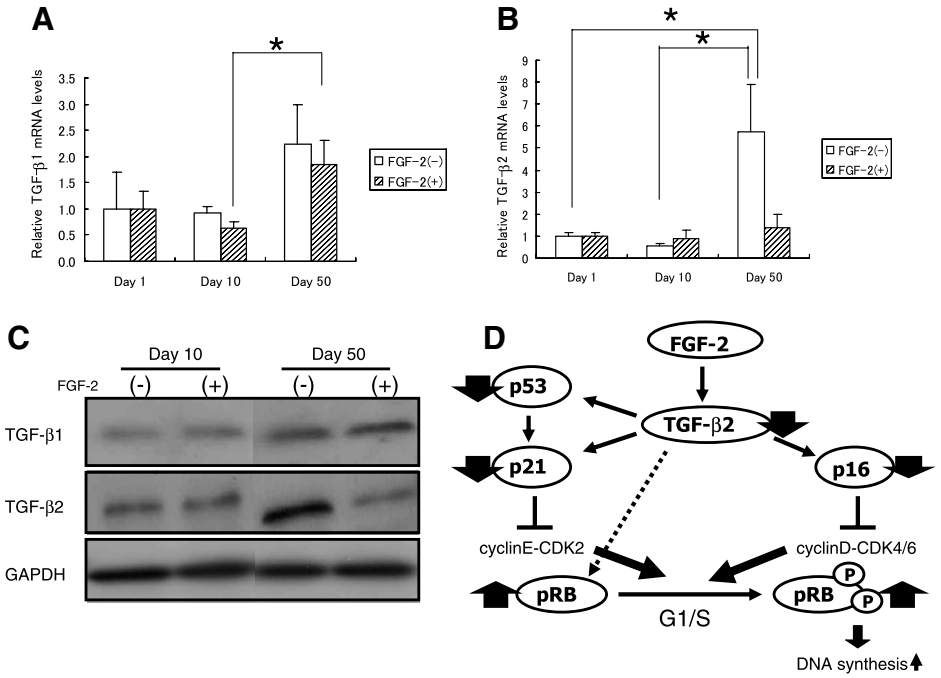

Fig. 4. FGF-2 increases TGF-β1 mRNA expression levels, but does not increase TGF-β2 during long-term culture. hMSCs were maintained in the medium in the presence or absence of FGF-2 (1 ng/ml), and total RNAs and proteins were extracted when the cells approached confluence. (A,B) TGF-β1 and β2 mRNA expression levels were measured using real time RT-PCR at Days 1, 10, and 50. (C) TGF-β1 and β2 protein expression levels were detected using Western blot analysis at Days 10 and 50.

are found in adult human bone marrow, and those obtained from patients until late adulthood still exhibit osteogenic potency [29]. Thus, it is thought that hMSCs maintain self-renewal and differentiation capacity *in vivo* throughout life. However, our previous studies have shown that the self-renewal potency of hMSCs is decreased by long-term culture *in vitro* [27]. The results of this study suggest that cellular senescence was induced in hMSCs (Fig. 2) following G1 cell growth arrest through increases of $p16^{INK4a}$, $p21^{Cip1}$, and p53 mRNA expression levels (Figs. 2 and 3) due to long-term culture *in vitro*. Since it was reported that cellular senescence was induced by the stress of culture [30], hMSCs would also be subject to finite proliferation due to many unknown stresses in our studies.

It was reported that TGF-β1 induced changes in hMSC morphology [28]. In the present study, after 5 days' treatment with TGF-β1 and TGF-β2 (TGF-βs), hMSCs were spread out, some of the cells were stained blue by SA-β-Gal staining (Fig. 1A–F), and they had decreased DNA replicative potential (Fig. 1G). In human prostate stromal cells, TGF-β1 induced similar morphological changes, but had no effect on cellular senescence [31]. However, we hypothesize that the changes in hMSC morphology induced by TGF-βs were due to cellular senescence because the conditions of our study differed from those of previous studies: hMSCs were treated with 5 ng/ml TGF-βs for 5 days in this study (Fig. 1), whereas hMSCs were treated with 1 ng/ml TGF-β1 for 3 days in the previous study [31]. Moreover, the responses to TGF-β stimulation may depend on the kind of cell.

We considered that the cellular senescence induced by TGF-βs is involved in G1 growth arrest through the increase of CDK inhibitors ($p16^{INK4a}$, $p21^{Cip1}$, and p53). We observed that the number of cells in the G1 phase (Fig. 1) and the mRNA expression levels of $p16^{INK4a}$, $p21^{Cip1}$, and p53 were increased by TGF-βs (Fig. 2A–C). It has been reported that TGF-β increased the expression levels of $p21^{Cip1}$ [17] but not $p16^{INK4a}$ [18,32]. However, the increase of $p16^{INK4a}$ expression levels was important for the irreversible stop of the cell cycle [26]. Based on our results in this study, we support the latter report.

The phosphorylation of pRB, which is regulated by CDK inhibitors, accompanies the G1/S transition [16,33]. We also showed that the expression levels of total pRB and ppRB were decreased by TGF-βs treatment (Fig. 2D). It has been reported that TGF-β1 decreased RB gene expression [34] moreover, TGF-β1 inhibited pRB phosphorylation [15,35]. We suspect that ppRB expression decreased for two reasons: the relative decrease due to the inhibition of total pRB expression by TGF-βs and the decrease of pRB phosphorylation due to the increase of CDK inhibitors by TGF-βs.

FGF-2, one of the cell growth factors, efficiently increases the number of hMSCs [12,13], but its mechanisms have unknown yet. In this study, we showed that the cell growth arrest in the G1 phase was suppressed by FGF-2 through the suppression of $p16^{INK4a}$, $p21^{Cip1}$, and p53 mRNA expression levels and the increase of ppRB expression levels (Figs. 2 and 3). Furthermore, the expression levels of TGF-β1 and β2 mRNA were increased by long-term

culture (Fig. 4A and B), in agreement with our previous studies [27]. However, FGF-2 suppressed the increase of TGF-β2 mRNA expression levels (Fig. 4B). FGF-2 also consistently suppressed the increase of TGF-β2 protein expression levels in long-term culture (Fig. 4C). It was reported that TGF-β2 inhibited FGF-2-induced proliferation of corneal endothelial cells [36]. Based on our results and that report, we consider that FGF-2 suppressed cellular senescence through down-regulation of TGF-β2 expression in hMSCs (Fig. 4D).

After 50 days' culture with FGF-2, pRB protein expression levels were remarkably increased, ppRB expression levels were up-regulated (Fig. 3D), and TGF-β2 expression was down-regulated as well (Fig. 4B). Moreover, TGF-βs remarkably decreased the pRB expression levels and induced cellular senescence (Fig. 1). It was also reported that senescence in cells induced reduction of RB protein levels [37]. Therefore, the increase of pRB expression levels when FGF-2 suppressed hMSC senescence may be involved in the down-regulation of TGF-β2 (Fig. 4D).

In conclusion, long-term culture induced cellular senescence by arresting cell growth in the G1 phase and increasing expression levels of TGF-βs in hMSCs. On the other hand, FGF-2 suppressed cellular senescence and down-regulated TGF-β2 expression in hMSCs. We consider that the suppression of TGF-β2 expression is important in the suppression of cellular senescence of hMSCs by FGF-2. However, after 150 days' culture, hMSCs no longer maintained self-renewal capacity, and the expression levels of TGF-β2 were increased in spite of the addition of FGF-2 (data not shown). These results suggest that FGF-2 delayed the decrease of self-renewal capacity due to long-term culture in hMSCs. TGF-β2 may be useful for the maintenance of self-renewal capacity in hMSCs. The data in this study will advance the knowledge of hMSC biology, and allow us to realize safe and efficient clinical applications of hMSCs.

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