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BRIEF REPORT

FGF-2 increases osteogenic and chondrogenic differentiation potentials of human mesenchymal stem cells by inactivation of TGF-β signaling

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Abstract Human mesenchymal stem cells (hMSCs) are able to self-replicate and differentiate into a variety of cell types including osteoblasts, chondrocytes, adipocytes, endothelial cells, and muscle cells. It was reported that fibroblast growth factor-2 (FGF-2) increased the growth rate and multidifferentiation potentials of hMSCs. In this study, we investigated the genes involved in the promotion of osteogenic and chondrogenic differentiation potentials of hMSCs in the presence of FGF-2. hMSCs were maintained in the medium with FGF-2. hMSCs were harvested for the study of osteogenic or chondrogenic differentiation potential after 15 days' culture. To investigate osteogenic differentiation, the protein levels of alkaline phosphatase (ALP) and the mRNA expression levels of osteocalcin were measured after the induction of osteogenic differentiation. Moreover, the investigation for chondrogenic differentiation was performed by measuring the mRNA expression levels of type II and type X collagens after the induction of chondrogenic differentiation. The expression levels of ALP, type II collagen, and type X collagen of hMSCs cultured with FGF-2 were significantly higher than control. These results suggested that FGF-2 increased osteogenic and chondrogenic differentiation potentials of hMSCs. Furthermore, microarray analysis was performed after 15 days' culture in the medium with FGF-2. We found that the overall insulin-like growth factor-I (IGF-I) and transforming growth factor-β (TGF-β) signaling pathways were inactivated by FGF-2. These results suggested that the inactivation of IGF-I and TGF-β signaling promotes osteogenic and chondrogenic differentiation potential of hMSCs in the presence of FGF-2.

Keywords Mesenchymal stem cells · Fibroblast growth factor-2 · Insulin-like growth factor-I · Transforming growth factor-β · Osteogenic differentiation · Chondrogenic differentiation

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Introduction

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 (Caplan et al. 2001; Pittenger et al. 1999; Wakitani et al. 1995). Based on these qualities of MSCs, the regeneration of bone and cartilage has been studied (Ochi et al. 2004; Petite et al. 2000). Fibroblast growth factor-2 (FGF-2) is

involved in angiogenesis and tissue repair. It was reported that the abilities of hMSCs to differentiate into osteoblasts and chondrocytes decreased with long-term subculture in vitro; however, FGF-2 increased the potential for osteogenic, chondrogenic, and adipogenic differentiation of human MSCs (hMSCs) (Kakudo et al. 2007; Quarto et al. 2006; Solchaga et al. 2005; Tsutsumi et al. 2001). Our previous studies showed that the ability of hMSCs proliferation was decreased by long-term subculture in vitro, during which the expression level of transforming growth factor-β (TGF-β) mRNA was increased (Sawada et al. 2006). Furthermore, we also showed that FGF-2 suppressed the decrease of hMSCs proliferation by down-regulation of TGF-β2 (Ito et al. 2007). In this study, we investigated the relation between the TGF-β signaling and the promotion of osteogenic and chondrogenic differentiations of hMSCs induced by FGF-2.

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 5,000 cells/cm^2 with or without FGF-2 (BD Biosciences, Bedford, MA). The first passage was regarded as the third generation because the cells were secondary cultures when they were obtained. The cells were maintained in humidified incubators at 37 °C with 5% CO_2. FGF-2 was added to the culture medium at the final concentration of 1 ng/ml, and the medium were changed every 2–3 days.

Cell differentiation

The culture medium was replaced with a specific differentiation-inducing medium after hMSCs were cultured in the medium with or without FGF-2 for 15 days. For osteogenic differentiation, hMSCs were cultured in Differentiation Basal Medium-Osteogenic medium (Cambrex Bio Science Walkersville) at 3,100 cells/cm^2 and maintained for 21 days. For chondrogenic differentiation, hMSCs were cultured in Differentiation Basal Medium-Chondrogenic medium (Cambrex Bio Science Walkersville) supplemented with 10 ng/ml TGF-β3 at 2.5×10^5 cells per 15 ml polypropylene tube and maintained for 21 days. The medium was changed every 2–3 days.

Quantitative real-time RT-PCR

Total RNA was extracted from hMSCs using Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. The first-strand cDNA was synthesized from 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Tokyo, Japan). Using the cDNAs as templates, PCRs of GAPDH, type II collagen, and type X collagen were performed for 40 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 osteocalcin: denaturation at 95 °C for 10 s, annealing at 62 °C for 15 s, and extension at 72 °C for 6 s using the LightCycler Real-time PCR System (Roche Diagnostics). The primers for GAPDH, type II collagen, and type X collagen from a LightCycler-Primer Set (Search LC GmbH, Heidelberg, Germany) were used. The primer for osteocalcin was from a LightCycler-Primer/Probes Set (Search LC GmbH).

Protein levels of alkaline phosphatase

After the induction of osteogenic differentiation, the protein levels of alkaline phosphatase of hMSCs were determined using Osteolinks-BAP (DS Pharma Biomedical Co., Ltd., Osaka, Japan).

DNA microarray analysis

Total RNA was isolated using Isogen following the manufacturer's protocol after hMSCs were cultured in the medium with or without FGF-2 for 15 days. One microgram of total RNA was used in each microarray experiment. cDNA and cRNA were made using Affymetrix's Two-Cycle cDNA Synthesis and IVT Labeling Kits (Affymetrix Inc., Santa Clara, CA). cRNAs were hybridized to an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. After hybridization, GeneChips were washed and

stained using the GeneChip Fluidics station and scanned in a GeneChip Scanner. Gene expression data were loaded into GeneSpring 7.3 (Agilent Technologies, Santa Clara, CA), then normalized and filtered by the flags of Present or Marginal and the expression levels. Furthermore, the passed genes were performed by Ingenuity Pathway Analysis.

Statistical analysis

Statistical evaluation was performed with the Student's t test. A p-value of less than 0.05 was considered significant. Values are presented as means ± SD.

Results

FGF-2 increased the potentials for the osteogenic and chondrogenic differentiation of hMSCs

To investigate the effects of FGF-2 on osteogenic and chondrogenic differentiation, hMSCs were maintained in MSCGM medium with or without FGF-2 for 15 days, then osteogenic or chondrogenic differentiation of the hMSCs was induced individually for 21 days (Fig. 1A). Then we measured the protein expression levels of alkaline phosphatase (ALP) and the mRNA expression levels of osteocalcin to investigate the osteogenic differentiation potential. We also measured type II collagen and type X collagen to investigate the chondrogenic differentiation potential. FGF-2 increased the protein expression level of ALP (Fig. 1B). FGF-2, however, did not affect the mRNA expression levels of osteocalcin (Fig. 1C). On the other hand, FGF-2 increased the mRNA expression levels of type II collagen and type X collagen (Fig. 1D and 1E). These results suggested that FGF-2 increased the potentials for osteogenic and chondrogenic differentiation of hMSCs.

Inactivation of TGF-β signaling contributed to the increase of osteogenic and chondrogenic differentiation potentials in the presence of FGF-2

To determine the genes that contribute to the increase of differentiation potentials of hMSCs by FGF-2, we extracted the genes of hMSCs that were up-regulated (>2 fold) or down-regulated (<1/2 fold) by FGF-2 using microarray analysis. It was performed before the induction of osteogenic and chondrogenic differentiation in hMSCs. Seven-hundred and fourteen

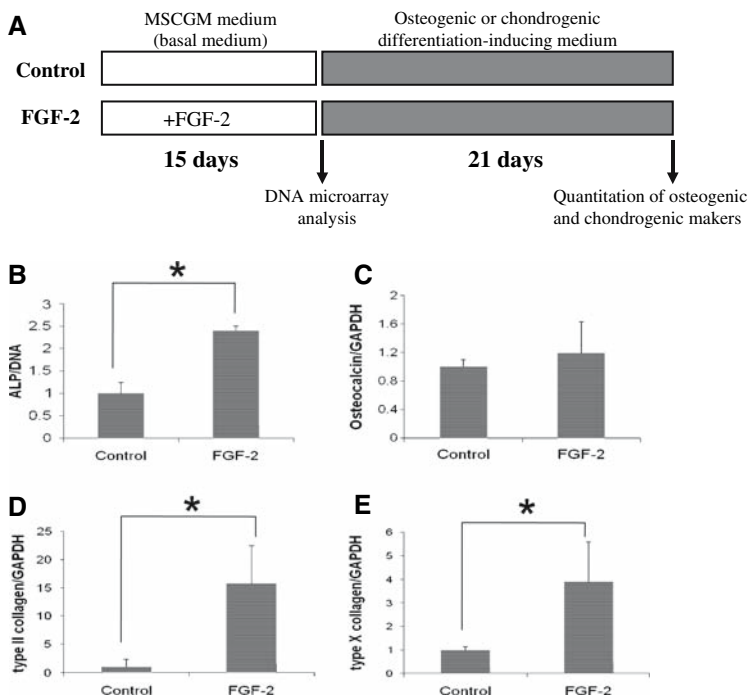

Fig. 1 Experimental protocol and quantitation of osteogenic and chondrogenic markers. hMSCs were maintained in the medium with or without FGF-2 for 15 day, and osteogenic or chondrogenic differentiation of hMSCs was induced for 21 days (**A**). Then, the protein levels of ALP (**B**), the mRNA expression levels of osteocalcin (**C**), type II collagen (**D**) and type X collagen (**E**) were measured

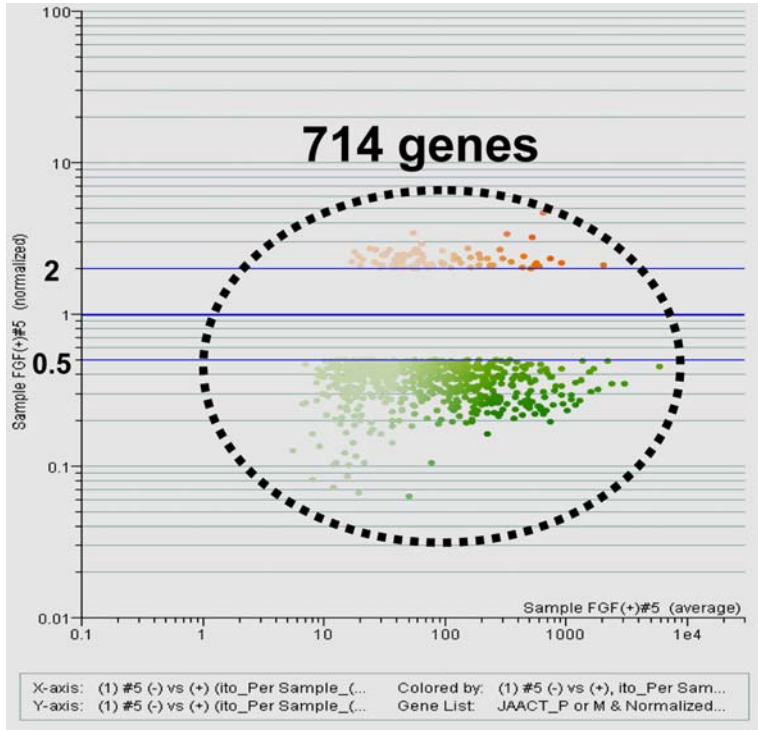

Fig. 2 Genes up-regulated (>2 fold) and down-regulated (<1/2 fold) by FGF-2 in hMSCs. hMSCs were maintained in the medium with or without FGF-2 for 15 days. Then, total RNA were extracted from the hMSCs and microarray analysis were performed. The *x*-axis showed the fold-change of FGF-2 against Control. The *y*-axis showed the raw expression levels of hMSCs cultured in the medium with FGF-2

genes were extracted (Fig. 2), and the canonical pathways of these genes were investigated using Ingenuity Pathway Analysis. As a result, IGF-I and TGF-β signaling genes were found to be included in the extracted genes (Fig. 3, see, red arrows). IGF-I signaling pathway (7 mapped genes out of 67) and TGF-β signaling pathway (6 mapped genes out of 59) were found to be at upper rank. Furthermore, the overall IGF-I and TGF-β signaling pathway was inactivated by FGF-2 (Figs. 4 and 5).

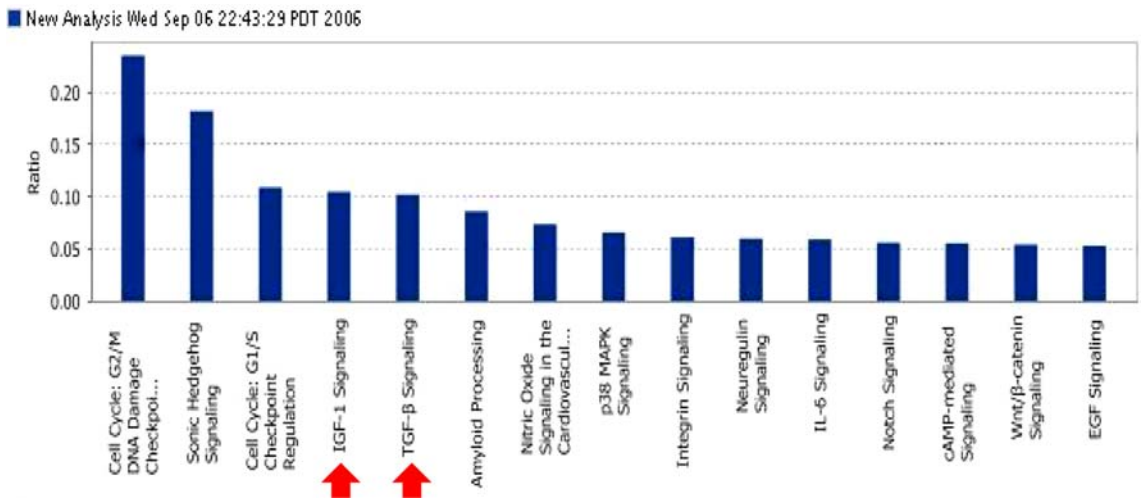

Fig. 3 Pathway analysis of genes up-regulated and down-regulated by FGF-2 in hMSCs. Pathway analysis of up-regulated and down-regulated genes by FGF-2 (Fig. 2) was performed by Ingenuity Pathway Analysis. The *y*-axis showed the ratio of genes mapped in Fig. 2 against all of genes belongs to each canonical pathway

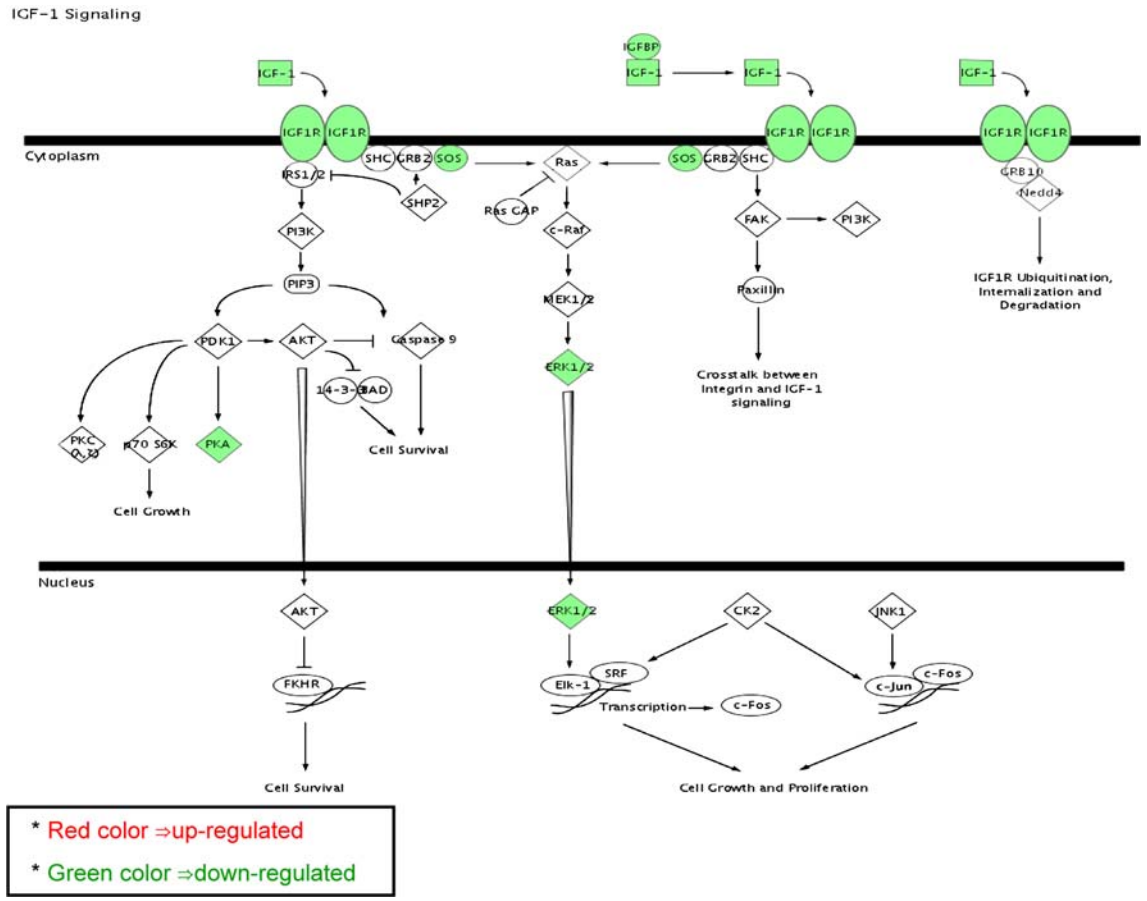

Fig. 4 Genes up-regulated and down-regulated by FGF-2 in IGF-I signaling pathway. Genes up-regulated and down-regulated by FGF-2 (Fig. 2) were mapped with the IGF-I signaling pathway by Ingenuity Pathway Analysis. The red color showed up-regulated genes and green color showed down-regulated genes

Discussion

hMSCs are found in adult human bone marrow, and cells obtained from patients until late adulthood still exhibit osteogenic potency (Leskela et al. 2003). In fact, hMSCs may maintain differentiation capacity in vivo throughout life. However, after 50 days' culture of hMSCs with or without FGF-2, the protein expression levels of ALP and the mRNA expression levels of osteocalcin, type II collagen, and type X collagen were significantly lower than after 15 days' culture of hMSCs (data not shown). Those differentiation makers were up-regulated by FGF-2 after 15 days' culture of hMSCs (Fig. 1), but not after 50 days' culture of hMSCs (data not shown). These results suggested that the osteogenic and chondrogenic differentiation potentials of hMSCs were decreased by long-term subculture in vitro, as shown in Fig. 6. Furthermore, in our previous study, we have suggested that the mRNA expressions of TGF-βs increased by long-term culture (Sawada et al. 2006). The decrease of osteogenic and chondrogenic differentiation potentials by long-term culture may be involved in the increase of TGF-βs levels.

Our data in this study (Fig. 1B–E) were in agreement with the report that FGF-2 increased the potential for chondrogenic and osteogenic differentiation of hMSCs (Tsutsumi et al. 2001). Moreover, since the exposure of adipose-derived stem cells to FGF-2 before the induction of differentiation enhanced the adipogenesis (Kakudo et al. 2007), the treatment by FGF-2 before induction may increase the osteogenic and chondrogenic differentiation potentials of hMSCs.

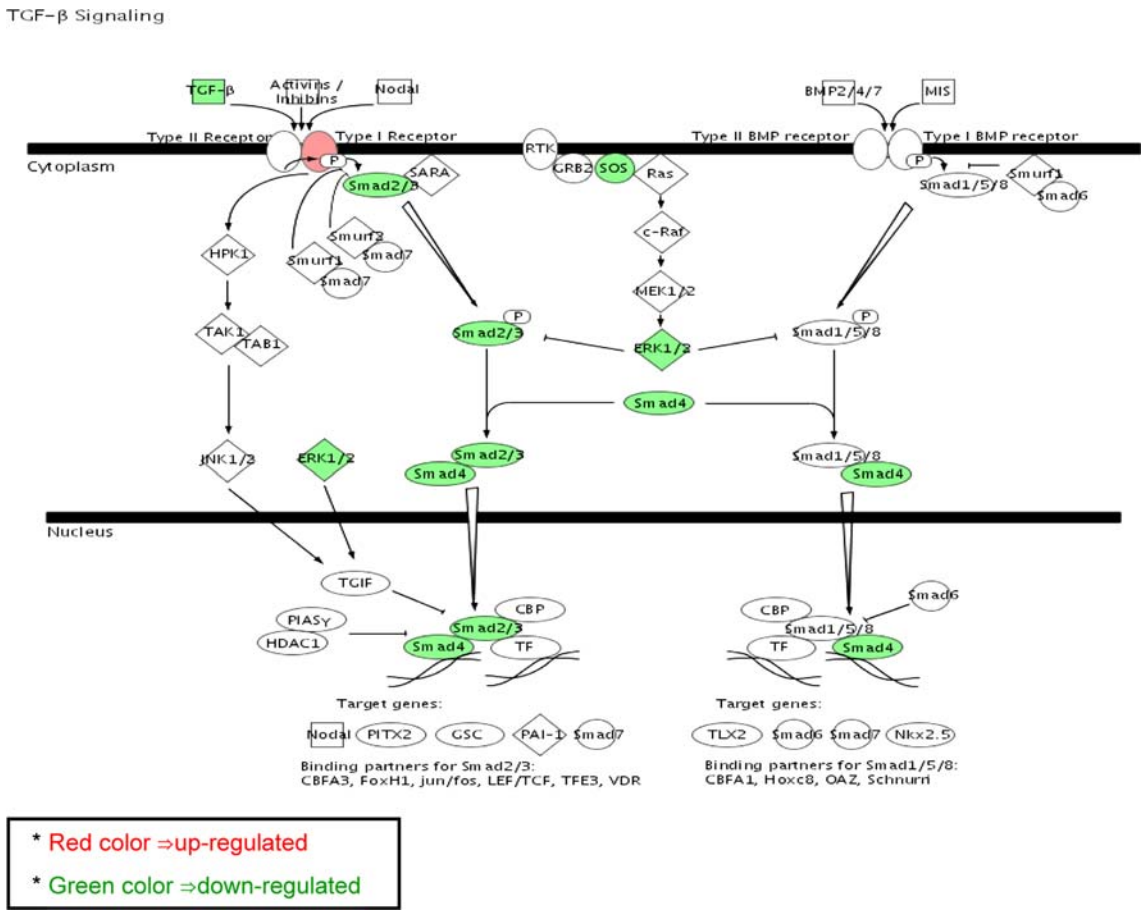

Fig. 5 Genes up-regulated and down-regulated by FGF-2 in TGF-β signaling pathway. Genes up-regulated and down-regulated by FGF-2 (Fig. 2) were mapped with the TGF-β signaling pathway by Ingenuity Pathway Analysis. The red color showed up-regulated genes and green color showed down-regulated genes

In this study, we extracted 714 genes that were up-regulated or down-regulated by FGF-2 (Fig. 2), and investigated which canonical pathway they were involved. As a result, cell cycle signaling pathways were ranked first, second and third (Fig. 3). Cyclin-dependent kinase inhibitors included in those signaling pathways were down-regulated by FGF-2 (data not shown). This result suggests that cell cycle were activated by FGF-2, in agreement with our previous study (Ito et al. 2007, p. 108). In previous study, it was reported that mitogen activated protein kinase (MAPK) and Wnt modulated the differentiation potential of adult stem cells (Solchaga et al. 2005). In this study, IGF-I and TGF-β signaling genes were included in the 714 genes (Fig. 3), and the overall IGF-I and TGF-β signaling pathway was inactivated (Fig. 4 and 5). Furthermore, since it was reported that TGF-β decrease osteoprogenitor fraction in cultures of human bone marrow stromal cells (Walsh et al. 2003), inactivation of TGF-β signaling pathway may be important for the increase of differentiation potentials of hMSCs.

In conclusion, we consider that the exposure of hMSCs to FGF-2 before the induction of differentiation enhanced osteogenic and chondrogenic differentiation potentials by inactivation of IGF-I and TGF-β signaling. However, more studies will be needed for explanation the molecular mechanisms that inactivation of IGF-I and TGF-β signaling by FGF-2 enhance osteogenesis and chondrogenesis of hMSCs.

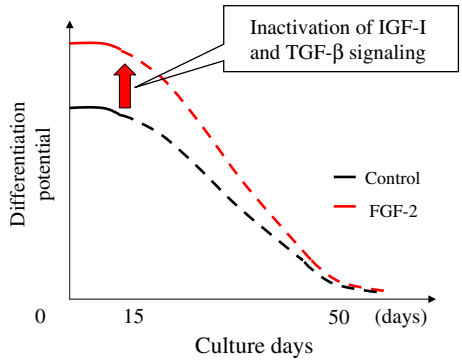

Fig. 6 Our hypothesis that FGF-2 increases the osteogenic and chondrogenic differentiation potentials of hMSCs by inactivation of IGF-I and TGF-β signaling

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