して最も実用に近いものの1つであろう.

しかしその反面，幹細胞は多分化能と同時に自己複製能を持つ細胞である[9]ため，正常細胞でありながら増殖能力を持つという点で癌細胞と共通の性質を持つともいえる．そのような背景の中，Rubioら[10]により脂肪組織由来のヒト間葉系幹細胞を長期間（4—5ヵ月）*in vitro* で培養すると自然に形質転換（癌化）するという報告がなされた．一方最近，間葉系幹細胞の由来によるその性質の違いについて，骨髄，臍帯血，脂肪組織由来の間葉系幹細胞をそれぞれ比較することによって示した報告[11]もあり，脂肪組織由来のヒト間葉系幹細胞が自然に形質転換するという上記の報告[10]が直ちに骨髄由来の間葉系幹細胞やさらには他の体性幹細胞も同様な変化を起こすということにはならないが，やはりその危険性に対して注意を払う必要はあるであろう．特に，体性幹細胞を細胞組織利用医療機器や細胞治療に用いるためには，生体内から取り出したのち *in vitro* で培養しある程度の細胞数を得なければならない．このため，少なくとも *in vitro* での培養中に幹細胞の性質ができるだけ変化しないことが望ましい．幹細胞を用いた細胞組織利用医療機器や細胞治療の実用化に向けて *in vitro* での培養中における幹細胞の安全性評価法の早期確立が重要課題であろう.

その第一歩として，筆者らは現在，幹細胞の *in vitro* での培養中に起こる遺伝子発現レベルの変化について検討を行っている．その理由としては，幹細胞におけるいくつかの遺伝子発現について調べることでその安全性を評価できる系を最終的に確立できれば，誰でも簡便に評価できるためであり，幹細胞を用いた細胞組織利用医療機器等の開発の促進につながることを期待している．本稿では，幹細胞の自己複製制御機構を探るために骨髄由来ヒト間葉系幹細胞の *in vitro* での培養中に起こる遺伝子発現レベルの変化について検討した結果を紹介する.

2. ヒト骨髄由来間葉系幹細胞の増殖能について

ヒト骨髄由来間葉系幹細胞（hMSC; Cambrex 社より2継代目の凍結細胞を購入）を *in vitro* で培養していくと，通常その増殖能は次第に低下していく (Fig. 1)．細胞を採取した個体による増殖速度の差はみられるものの，そのほとんどが培養期間2ヵ月を超えると増殖速度は低下し始め，4—5ヵ月になるとほとんど増殖しなくなってくる．増殖速度が低下した幹細胞は Senescence associated β-galactosidase (SA-β-Gal) staining によって細胞中に老化している細胞が含まれていることが確認された．増殖因子を添加した培地を用いた培養も行っているが，増殖速度は上昇するものの培養期間による速度の変化は増殖因子を加えていない細胞と同様であり，長期間培養しても無限増殖する幹細胞の存在は現在の所筆者らは確認していない.

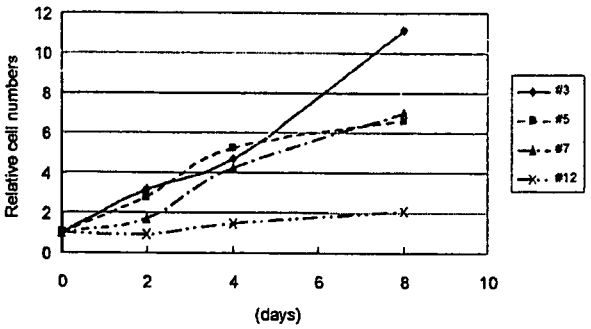


Fig. 1. Proliferation of hMSC in 3rd, 5th, 7th, and 12th Passages[16]

hMSC were seeded at 1.7×10^5 cells/Φ60 mm dish (6000 cells/cm^2), and cells were counted after 2, 4, and 8 days. The initial cell number (0 day) is expressed as 1, and the other cell numbers (2, 4, and 8 days) are relative to that of day 0. $n=3$.

3. *In vitro* 培養における hMSC の遺伝子発現レベルの変化について

上述したように，hMSC は *in vitro* での培養を続けることによってその増殖能は低下してくる．また細胞の形態等の変化もみられており，培養期間中に遺伝子の発現に変化が生じる可能性が示唆された．そこで，*in vitro* 培養期間の長さによる hMSC の遺伝子発現レベルの変化について調べるために，まず DNA アレイ解析（BD Atlas™ Human Cancer 1.2 Array）を行った．培養期間1ヵ月程度の細胞と2ヵ月以上の細胞とで比較検討した．それぞれの遺伝子の機能による分類単位での変化について Table 1 に示した．こちらはあくまで全体的な傾向を示しているため，それぞれの分類に含まれる個々の遺伝子の発現の変化がすべて同じという訳ではないが，hMSC は *in vitro* での培養を続けることによって遺伝子発現レベルの変化が起こることは確認された．hMSC の培養中に癌化といった形質転換が起こっていないことを確かめる指標を探るために「細胞増殖」という点に着目し，また上記の DNA アレイ解析結果も踏まえて，次に個々の遺伝子の発現レベル

Table 1. Comparison of Gene Expressions in hMSC (1 Month Culture) and hMSC (Over 2 Months)

The genes concerned with the following functions were up-regulated with the culture term
- Cell cycle
- Cell adhesion receptors/proteins
- Immune system proteins
- Oncogenes and tumor suppressors
- Stress response proteins
- DNA binding and chromatin proteins
- Cell receptors (by ligands)
- Cell receptors (by activities)
- Intracellular transducers/effectors/modulators
- DNA synthesis, recombination, and repair

The genes concerned with the following functions were down-regulated with the culture term
- Membrane channels and transporters
- Metabolism
- Translation
- Apoptosis associated proteins
- RNA processing, turnover, and transport
- Protein turnover
- Cytoskeleton/motility proteins

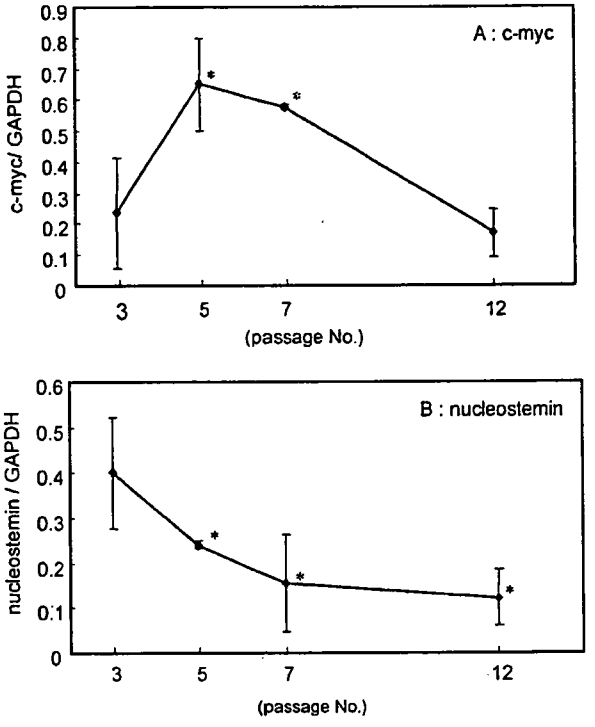


Fig. 2. Effect of *In vitro* Culture Length on the mRNA Expressions of c-myc (A) and Nucleostemin (B) in hMSC[16]
Expressions of the two genes relative to GAPDH in confluent cultures of hMSC in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative RT-PCR. Mean values with standard deviations from three independent experiments are presented. Asterisks denote statistically significant differences compared with the 3rd passage ($*p<0.05$).

の変化について hMSC の培養期間を 4 点取り検討した．まず，癌遺伝子の 1 つであり細胞の増殖機能に係わる c-myc，幹細胞と癌細胞の両者の増殖に係わる nucleostemin，様々なシグナル伝達経路や発癌に係わるといわれている Wnt-8B について検討したところ，c-myc 及び nucleostemin (Fig. 2) は hMSC の培養期間の長さに依存してそれぞれの発現レベルは低下した．一方，Wnt-8B についてはどの培養期間においてもその発現は認められなかった．さらに，細胞増殖，分化，アポトーシス，細胞外マトリックス形成，免疫抑制そして発癌などの制御に係わる TGFβ について検討した．TGFβ には 3 つの分子種が存在 (TGFβ1, 2, 3) し，TGFβ は細胞表面にある 3 つのタイプの受容体 (TGFβRI, II, III) を通じてシグナルを細胞内へ伝達する．TGFβRI と TGFβRII はセリン-チロシンキナーゼで，TGFβRIII はベータグリカンとして知られている．[12] TGFβ はまず TGFβRII に直接か又は TGFβRIII を介して結合し，TGFβRII によって TGFβRI を刺激することで細胞内 TGFβ シグナル伝達系がスタートする．活性化された TGFβRI が Smad2 若しくは Smad3 をリン酸化したのち，シグナルを核内へと伝え c-myc のような TGFβ に依存する遺伝子の転写を制御する．[13,14] そのため，TGFβ の 3 種類の分子種と 3 タイプの受容体及び Smad3 についても hMSC の培養期間によるその発現レベルの変化について調べた．TGFβ1 及び TGFβ2 は *in vitro* での培養を続けることによってその発現レベルが上昇したが，TGFβ3 は変化しなかった (Fig. 3)．受容体についてはタイプ I は上昇したが，タイプ II 及び III は変化がみられなかった (Fig. 3)．Smad3 は TGFβ1, β2 及び TGFβRI と同様に上昇した．以上の結果から hMSC の培養中の遺伝子発現について，TGFβ→c-myc へのシグナル伝達系に係わる因子についての変化を Fig. 4 にまとめた．hMSC は *in vitro* での培養を続ける過程で，TGFβ→TGFβRI→Smad3→c-myc の経路で細胞周期停止が起こり，細胞の増殖が抑制されるのかもしれない．

以上の結果から，hMSC を *in vitro* で培養することによって通常はその増殖能が徐々に低下していき，その間の遺伝子発現の変化からも上皮系の他の

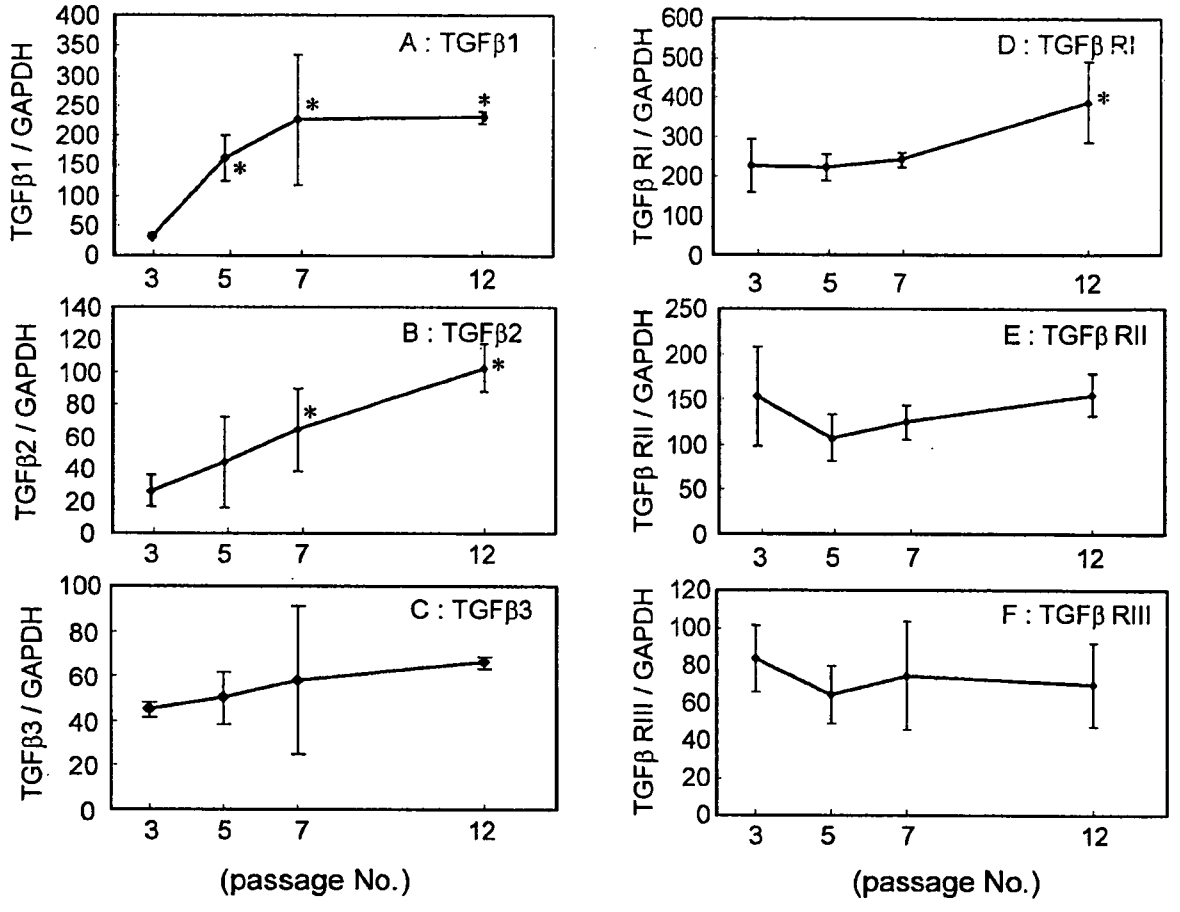


Fig. 3. Effect of *In vitro* Culture Length on mRNA Expressions of TGFβ1 (A), TGFβ2 (B), TGFβ3 (C), TGFβRI (D), TGFβRII (E), and TGFβRIII (F) in hMSC[16]

Expressions of the four genes, relative to GAPDH, in confluent cultures of hMSC in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative real time RT-PCR. Mean values with standard deviations from three independent experiments are presented. Asterisks denote statistically significant differences compared with the 3rd passage ($*p<0.05$).

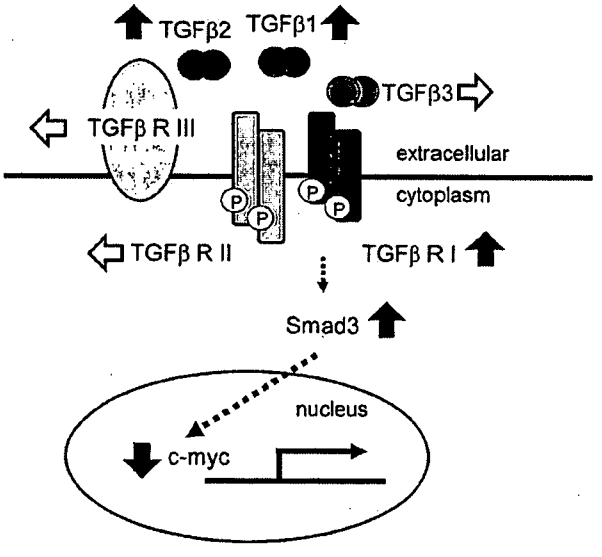


Fig. 4. Changes in the Expressions of TGFβ Signaling Genes during hMSC *In vitro* Culture for Three Months

細胞と同様なメカニズムで細胞増殖抑制が起こっていると考えられる．もしもこのような細胞の変化が「正常」な変化であると考えた場合，hMSC が培養中に形質転換等の望ましくない変化を起こした場合には違った発現パターンがみられる可能性があり，本研究で検討した遺伝子が幹細胞における培養中の変化に対する安全性評価の 1 つの指標となり得るかもしれない．現在，遺伝子発現の変化における hMSC の個体差を考慮し個体差を超えた共通性を見い出すために，複数の個体由来の hMSC を研究対象とし，また細胞の癌化や老化という観点からも更なる検討を行っている．

4. 幹細胞と癌細胞における遺伝子発現の比較について

幹細胞の癌化について，その危険性を評価するためには「自然に癌化した」幹細胞との比較検討が必

要であると思われる．しかし，現段階でそのような幹細胞は得られていない．そのため，筆者らはこれまでにライン化された数種類の癌細胞と幹細胞(hMSC)を，特に「細胞増殖」や「発癌」に係わると考えられている遺伝子についてその発現を比較した．「細胞増殖」に係わる遺伝子についてはその発現が上皮系の癌細胞では幹細胞よりも高いものもいくつか認められたが，肉腫細胞との比較ではその限りではないものもあり，未だ検討の余地が大きく残されている．つまり，幹細胞と癌細胞の違いを明らかにし幹細胞の癌化の指標となる遺伝子を決定するためにはさらなる検討を必要としている．特に現在，癌幹細胞の存在も広く認められてきており，[9] 組織幹細胞と癌幹細胞との共通性や特異性を明らかにしていくことが望まれる．それが最終的に幹細胞の癌化のメカニズムを探る1つのきっかけとなるであろう．

5. おわりに

再生医療を目的とする幹細胞の研究は，わが国でも非常に盛んに行われている．特に間葉系幹細胞を用いることによる有効性については，骨・軟骨再生から心筋梗塞治療に至るまで幅広い臨床分野で報告されている．また，先頃厚生労働省より「ヒト幹細胞を用いる臨床研究に関する指針」が公布され，幹細胞を用いた細胞組織利用医療機器についてさらなる臨床研究の発展が期待される．しかし一方で，実際に幹細胞を用いる際の安全性について評価する明確な基準は今のところ制定されていない．幹細胞の調製の際に無菌的に取り扱うための基準等は定められているものの，幹細胞自身の安全性さらに生体内へ移植したのちの癌化等を含む安全性を担保するための評価法については確立しておらず，その早期確立が求められている．また幹細胞が将来的に「細胞組織利用医療機器」としての材料となるためにはautoだけでなくalloも視野に入れていかなければならず，より一層早急な対応が望まれる．現在，幹細胞の調製段階においてMSC及び繊維芽細胞のマーカー遺伝子の発現をみることによって骨髄間葉系幹細胞の均一性を検査する方法をKatoら[15]が提案しており，われわれは同様な方法で細胞の癌化に対する安全性評価法を確立できたら幹細胞の調製時にその均一性と安全性を同時に簡便に評価できるのではないか考え，そのマーカー遺伝子の探索を行っている．細胞組織利用医療機器として移植された幹細胞が生体内で癌化等の望ましくない変化を起こさないかどうか確認するためには，本来ならば10年単位の非常に長期的な観察が必要であろう．しかし現時点である程度癌化の予測ができるような評価系を確立しなければ，最新の技術によって支えられた「細胞組織利用医療機器」という次世代の医療機器の開発を妨げることになってしまう．そのため，筆者らは幹細胞の安全性評価法の早期確立を目指して，第一段階として移植前の *in vitro* 培養中の細胞の変化について検討し，幹細胞の増殖能に関する性質を探ることで，そこから逸脱しないという形での基準作りを試みている．本稿で述べた研究内容はその第一歩である．「細胞組織利用医療機器」の実現のために少しでも貢献できるように現在も検討を続けている．

REFERENCES

1) Jiang Y., Jahagirdar B. N., Reinhardt R. L., Schwartz R. E., Keene C. D., Ortiz-Gonzalez X. R., Reyes M., Lenvik T., Lund T., Blackstad M., Du J., Aldrich S., Lisberg A., Low W. C., Largaespada D. A., Verfaillie C. M., *Nature*, **418**, 41–49 (2002).
2) Rosenthal N., *N. Engl. J. Med.*, **349**, 267–274 (2003).
3) Korbling M., Estrov Z., *N. Engl. J. Med.*, **349**, 570–582 (2003).
4) Hishikawa K., Miura S., Marumo T., Yoshioka H., Mori Y., Takato T., Fujita T., *Biochem. Biophys. Res. Commun.*, **317**, 1103–1107 (2004).
5) Horwitz E. M., Gordon P. L., Koo W. K. K., Marx J. C., Neel M. D., McNall R. Y., Muul L., Hofmann T., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 8932–8937 (2002).
6) Petersen B. E., Bowen W. C., Patrene K. D., Mars W. N., Sullivan A. K., Murase N., Boggs S. S., Greenberger J. S., *Science*, **284**, 1168–1170 (1999).
7) Mangi A. A., Noiseux N., Kong D., He H., Rezvani M., Ingwall J. S., Dzau V. J., *Nat. Med.*, **9**, 1195–1201 (2003).
8) Strauer B. E., Brehm M., Zeus T., Kostering M., Hernandez A., Sorg R. V., Kogler G., Wernet P., *Circulation*, **106**, 1913–1918

(2002).
9) Pardal R., Clarke M. F., Morrison S. J., *Nat. Rev. Cancer*, **3**, 895–902 (2003).
10) Rubio D., Garcia-Castro J., Martin M. C., Fuente R., Cigudosa J. C., Lloyd A. C., Bernad A., *Cancer Res.*, **65**, 3035–3039 (2005).
11) Kern S., Eichler H., Stoeve J., Kluter H., Bieback K., *Stem Cells*, **24**, 1294–1301 (2006).
12) Lopez-Casillas F., Cheifetz S., Doody J., Andres J. L., Lane W. S., Massague J., *Cell*, **67**, 785–795 (1991).
13) Massague J., Wotton D., *EMBO J.*, **19**, 1745–1754 (2000).
14) Moustakas A., Souchelnytskyi S., Heldin C.-H., *J. Cell Sci.*, **114**, 4359–4369 (2001).
15) Ishii M., Koike C., Igarashi A., Yamanaka K., Pan H., Higashi Y., Kawaguchi H., Sugiyama M., Kamata N., Iwata T., Matsubara T., Nakamura K., Kurihara H., Tsuji K., Kato Y., *Biochem. Biophys. Res. Commun.*, **332**, 297–303 (2005).
16) Sawada R., Ito T., Tsuchiya T., *J. Artif. Organs*, **9**, 179–184 (2006).

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 359 (2007) 108–114
BBRC
www.elsevier.com/locate/ybbrc

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

Tomomi Ito [a,b], Rumi Sawada [a], Yoko Fujiwara [b], Yousuke Seyama [b], Toshie Tsuchiya [a,*]

[a] Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan

Received 9 May 2007
Available online 21 May 2007

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

* Corresponding author. Fax: +81 3 3700 9196.
E-mail address: tsuchiya@nihs.go.jp (T. Tsuchiya).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.05.067

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).

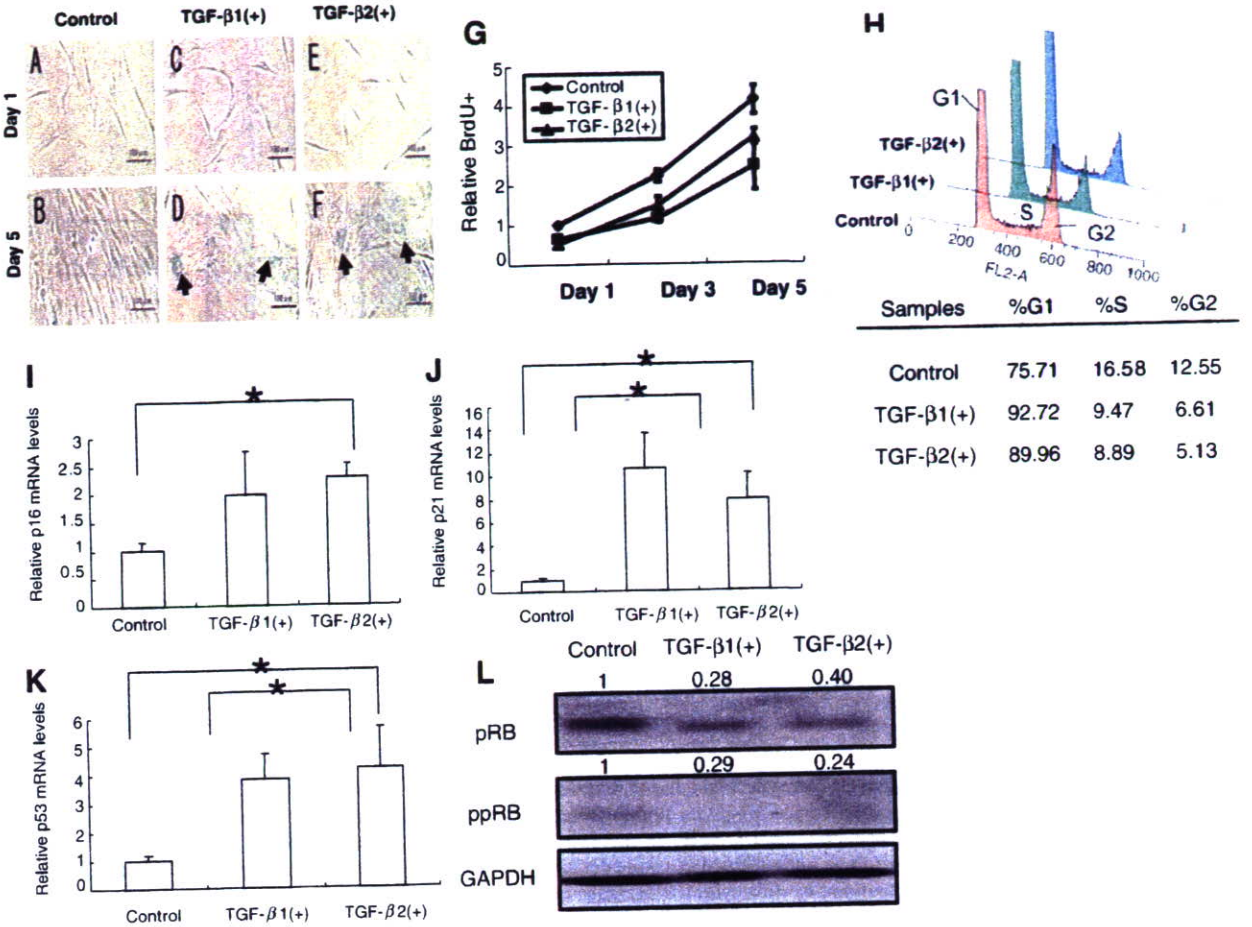


Fig. 1. TGF-β1 and β2 induce cellular senescence through G1 cell cycle arrest in hMSCs. hMSCs were maintained as an untreated control or treated with 5 ng/ml TGF-β1 or TGF-β2 for 5 days. (A–F) hMSCs were performed SA-β-Gal staining 1 or 5 days after TGF-β treatment (Day 1 or 5). The arrows in (D) and (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.

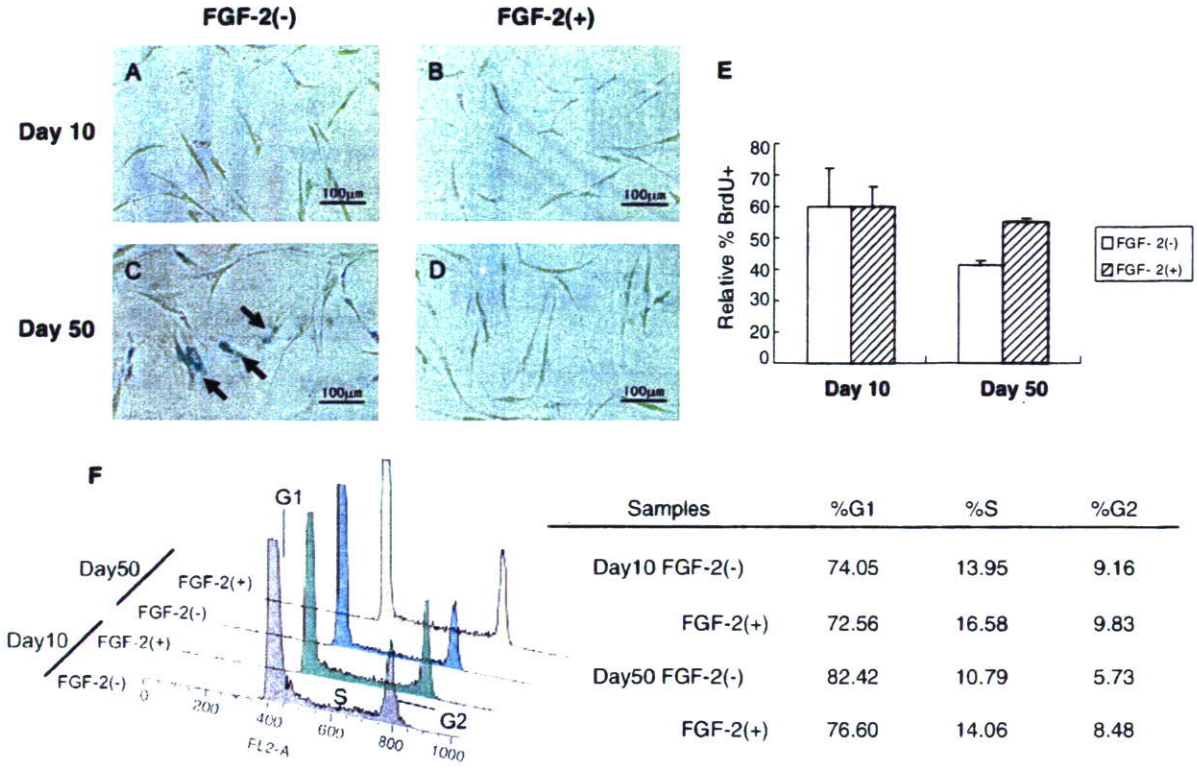
The mRNA expression levels of $p16^{INK4a}$, $p21^{Cip1}$, and p53 and the expression levels of pRB in hMSCs were measured after culture for 10 or 50 days in FGF-2(−) or FGF-2(+). After 50 days' culture in FGF-2(−), the mRNA expression levels of $p16^{INK4a}$, $p21^{Cip1}$, and p53 were significantly 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



Samples	%G1	%S	%G2
Day10 FGF-2(-)	74.05	13.95	9.16
FGF-2(+)	72.56	16.58	9.83
Day50 FGF-2(-)	82.42	10.79	5.73
FGF-2(+)	76.60	14.06	8.48

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).

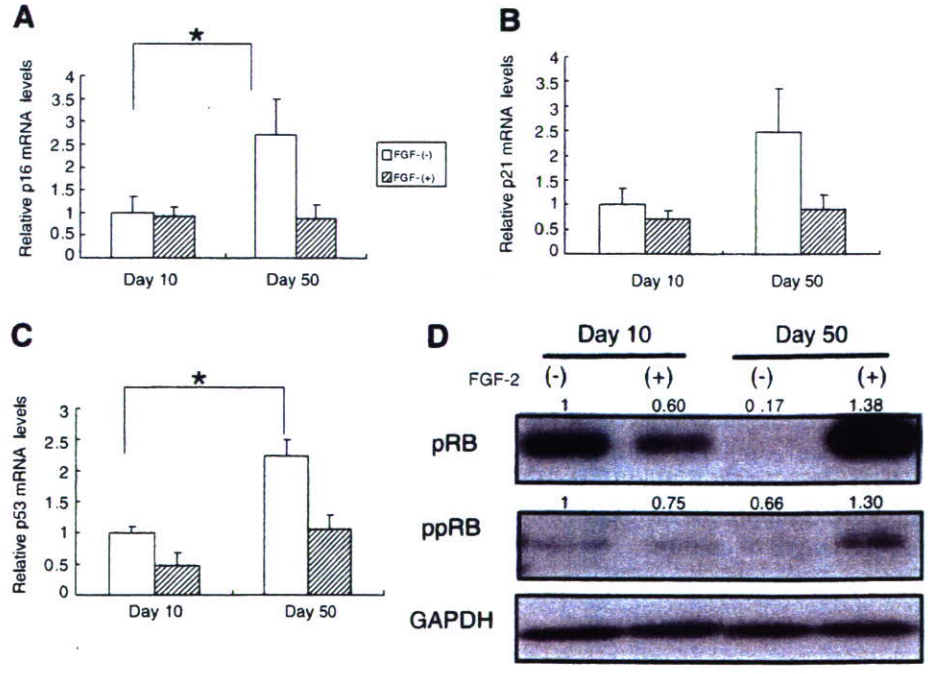


Fig. 3. FGF-2 suppresses G1 cell cycle arrest due to passaging. hMSCs were maintained in the medium in the presence or absence of FGF-2 (1 ng/ml), and total RNAs and proteins were extracted when approaching confluence. (A–C) $p16^{INK4a}$ (A), p53 (B), and $p21^{Cip1}$ (C) mRNA expression levels were 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.

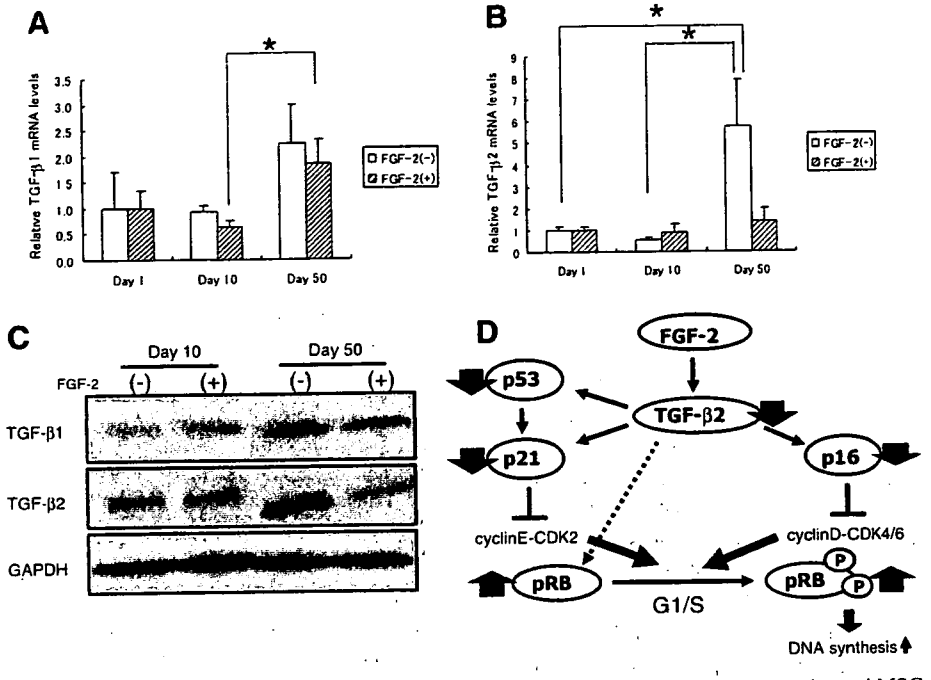


Fig. 4. FGF-2 increases TGF-β1 mRNA expression levels, but does not increase TGF-β2 during long-term culture. hMSCs were maintained in the medium in the presence or absence of FGF-2 (1 ng/ml), and total RNAs and proteins were extracted when the cells approached confluence. (A,B) TGF-β1 and β2 mRNA expression 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.

References

[1] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.

[2] A.I. Caplan, S.P. Bruder, Mesenchymal stem cells: building blocks for molecular medicine in the 21st century, Trends Mol. Med. 7 (2001) 259–264.

[3] S. Gojo, N. Gojo, Y. Takeda, T. Mori, H. Abe, S. Kyo, J. Hata, A. Umezawa, In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells, Exp. Cell Res. 288 (2003) 51–59.

[4] S. Wakitani, T. Saito, A.I. Caplan, Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine, Muscle Nerve 18 (1995) 1417–1426.

[5] J.P. Darwin, Marrow stromal cells as stem cells for nonhematopoietic tissues, Science 276 (1997) 71–74.

[6] H. Ohgushi, A.I. Caplan, Stem cell technology and bioceramics: from cell to gene engineering, J. Biomed. Mater. Res. 48 (1999) 913–927.

[7] H. Petite, V. Viateau, W. Bensaid, A. Meunier, C. Pollak, M. Bourguignon, K. Oudina, L. Sedel, G. Guillemin, Tissue-engineered bone regeneration, Nat. Biotechnol. 18 (2000) 959–963.

[8] M. Ochi, N. Adachi, N. Hiroo, S. Yamada, Y. Ito, M. Agung, Articular cartilage repair using tissue engineering technique-novel approach with minimally invasive procedure, Artif. Organs 28 (2004) 28–32.

[9] M. Mastrogiacomo, R. Cancedda, R. Quarto, Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells, Osteoarthritis Cartilage 9 (Suppl. A) (2001) S36–S40.

[10] I. Martin, A. Muraglia, G. Campanile, R. Cancedda, R. Quarto, Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow, Endocrinology 138 (1997) 4456–4462.

[11] G. Bianchi, A. Banfi, M. Mastrogiacomo, R. Notaro, L. Luzzatto, R. Cancedda, R. Quarto, Ex vivo enrichment of mesenchymal stem cell progenitors by fibroblast growth factor 2, Exp. Cell Res. 287 (2003) 98–105.

[12] L.A. Solchaga, K. Penick, J.D. Porter, V.M. Goldberg, A.I. Caplan, J.F. Welter, FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells, J. Cell Physiol. 203 (2005) 398–409.

[13] S. Tsutsumi, A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi, Y. Kato, Retention of multilineage differentiation potential of mesenchymal stem cells during proliferation in response to FGF, Biochem. Biopys. Res. Commun. 288 (2001) 413–419.

[14] K. Miyazono, H. Suzuki, T. Imamura, Regulation of TGF-β signaling and its roles in progression of tumors, Cancer Sci. 94 (2003) 230–234.

[15] M. Laiho, J.A. DeCaprio, J.W. Ludlow, D.M. Livingston, J. Massague, Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation, Cell 62 (1990) 175–185.

[16] J. Charles, M.R. Jeames, CDK inhibitors: positive and negative regulators of G1-phase progression, Genes & Development 13 (1999) 1501–1512.

[17] J. Massague, S.W. Blain, R.S. Lo, TGF-β signaling in growth control, cancer, and heritable disorders, Cell 103 (2000) 295–309.

[18] W. Zhou, I. Park, M. Pins, J.M. Kozlowski, B. Jovanovic, J. Zhang, C. Lee, K. Ilio, Dual regulation of proliferation and growth arrest in prostatic stromal cells by transforming growth factor-β1, Endocrinology 144 (2003) 4280–4284.

[19] K. Itahana, G. Dimri, J. Campisi, Regulation of cellular senescence by p53, Eur. J. Biochem. 268 (2001) 2784–2791.

[20] G.P. Dimri, X. Lee, G. Basile, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, M. Peacocke, J. Campisi, A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. USA 92 (1995) 9363–9367.

[21] W.S. el-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, B. Vogelstein, WAF1, a potential mediator of p53 tumor suppression, Cell 75 (1993) 817–825.

[22] J. LaBaer, M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, E. Harlow, New function activities for the p21 family of CDK inhibitors, Genes. Dev. 11 (1997) 847–862.

[23] K. Itahana, G.P. Dimri, E. Hara, Y. Itahana, Y. Zou, P.Y. Desprez, J. Campisi, A role for p53 in maintaining and establishing the quiescence growth arrest in human cells, J. Biol. Chem. 277 (2002) 18206–18214.

[24] T. Kiyono, S.A. Foster, J.I. Koop, J.K. McDougall, D.A. Galloway, A.J. Klingelhutz, Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalize human epithelial cells, Nature 396 (1998) 84–88.

[25] J.G. Rheinwald, W.C. Hahn, M.R. Ramsey, J.Y. Wu, Z. Guo, H. Tsao, M. Luca, C. Catricala, K.M. O'Toole, A two-stage, p16 (INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status, Mol. Cell Biol. 22 (2002) 5157–5172.

[26] C.M. Beausejour, A. Krtolica, F. Galimi, M. Narita, S.W. Lowe, P. Yaswen, J. Campisi, Reversal of human cellular senescence: roles of the p53 and p16 pathways, EMBO J. 22 (2003) 4212–4222.

[27] R.Sawada, T. Ito, T. Tsuchiya, Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells, J. Artif. Organs 9 (2006) 179–184.
[28] D. Wang, J.S. Park, J.S. Chu, A. Krakowski, K. Luo, D.J. Chen, S. Li, Proteomic profiling of bone marrow mesenchymal stem cells upon transforming growth factor β1 stimulation, J. Biol. Chem. 279 (2004) 43725–43734.
[29] H.V. Leskela, J. Risteli, S. Niskanen, J. Koivunen, K.K. Ivaska, P. Lehenkari, Osteoblast recruitment from stem cells does not decrease by age at late adulthood, Biochem. Biopys. Res. Commun. 311 (2003) 1008–1013.
[30] K. Ito, A. Hirao, F. Arai, S. Matsuoka, K. Takubo, I. Hamaguchi, K. Nomiyama, K. Hosokawa, K. Sakurada, N. Nakagata, Y. Ikeda, T.W. Mak, T. Suda, Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells, Nature 431 (2004) 997–1002.
[31] G. Untergasser, R. Gander, C. Lilg, E.P. Lepperdinger, P. Berger, Profiling molecular targets of TGF-β1 in prostate fibroblast-to-myofibroblast transdifferentiation, Mech. Ageing Dev. 126 (2005) 59–69.
[32] R. Tremain, M. Marko, V. Kinnimulki, H. Ueno, E. Bottinger, A. Glick, Defects in TGF-β signaling overcome senescence of mouse keratinocytes expressing *v-ras*^{Ha}, Oncogene 19 (2000) 1698–1709.
[33] R.A. Weinberg, The retinoblastoma protein and cell cycle control, Cell 81 (1995) 323–330.
[34] R.E. Herrera, T.P. Makela, R.A. Weinberg, TGF beta-induced growth inhibition in primary fibroblasts requires the retinoblastoma protein, Mol. Biol. Cell. 7 (1996) 1335–1342.
[35] Z. Yan, S. Hsu, S. Winawer, E. Friedman, Transforming growth factor beta 1 (TGF-beta1) inhibits retinoblastoma gene expression but not pRB phosphorylation in TGF-beta 1-growth stimulated colon carcinoma cells, Oncogene 7 (1992) 801–805.
[36] J. Lu, Z. Lu, P. Reinach, J. Zhang, W. Dai, L. Lu, M. Xu, TGF-beta2 inhibits AKT activation and FGF-2-induced corneal endothelial cell proliferation, Exp. Cell Res. 312 (2006) 3631–3640.
[37] Q.M. Chen, V.C. Tu, J. Liu, Measurements of hydrogen peroxide induced premature senescence: senescence-associated beta-galactosidase and DNA synthesis index in human diploid fibroblasts with down-regulated p53 or Rb, Biogerontology 1 (2000) 335–339.

Cytotechnology (2008) 56:1–7
DOI 10.1007/s10616-007-9092-1

BRIEF REPORT

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

Tomomi Ito · Rumi Sawada · Yoko Fujiwara · Toshie Tsuchiya

Received: 29 December 2006 / Accepted: 1 September 2007 / Published online: 18 October 2007

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

T. Ito · R. Sawada · T. Tsuchiya (✉)
Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
e-mail: tsuchiya@nihs.go.jp

T. Ito · Y. Fujiwara
Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan

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

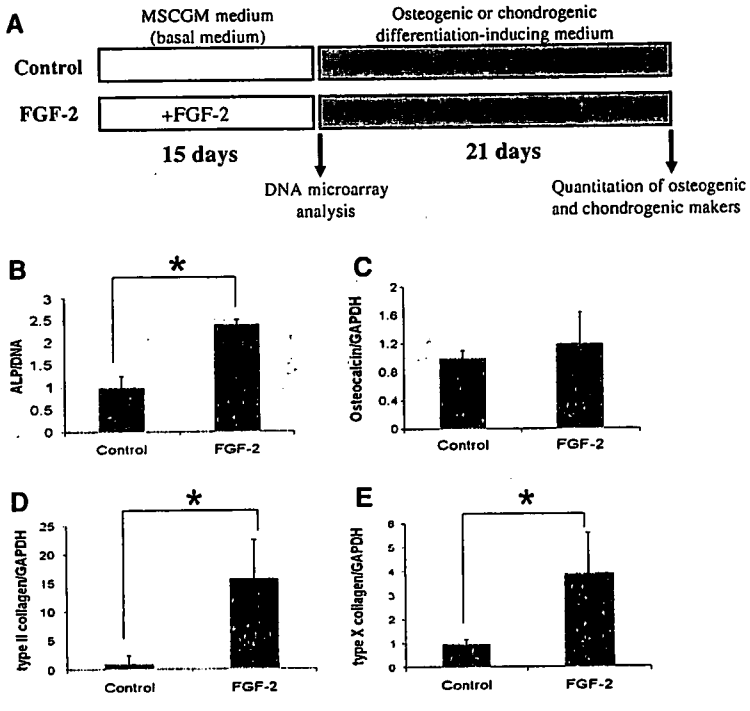


Fig. 1 Experimental protocol and quantitation of osteogenic and chondrogenic markers. hMSCs were maintained in the medium with or without FGF-2 for 15 day, and osteogenic or chondrogenic differentiation of hMSCs was induced for 21 days (**A**). Then, the protein levels of ALP (**B**), the mRNA expression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

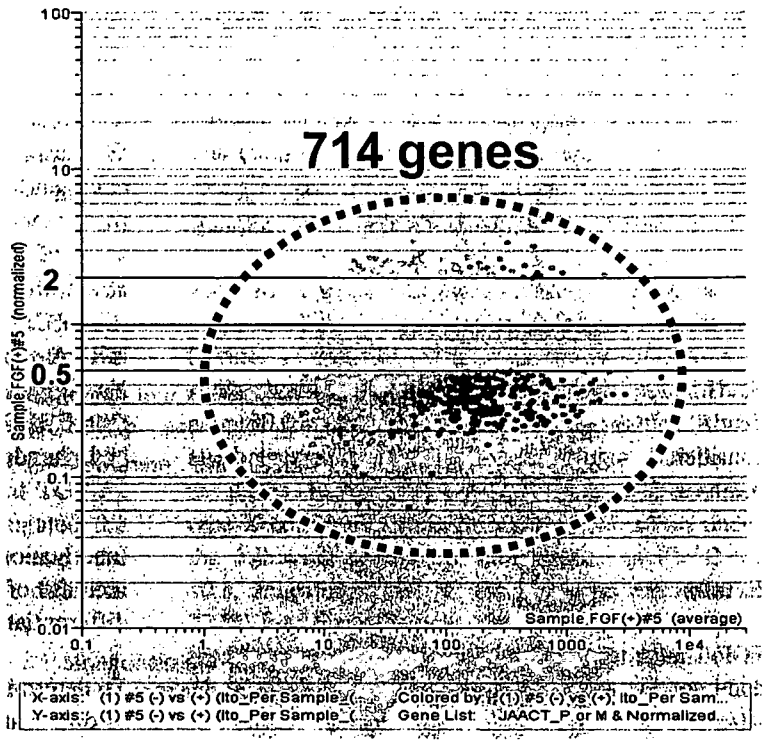


Fig. 2 Genes up-regulated (>2 fold) and down-regulated (<1/2 fold) by FGF-2 in hMSCs. hMSCs were maintained in the medium with or without FGF-2 for 15 days. Then, total RNA were extracted from the hMSCs and microarray analysis were performed. The *x*-axis showed the fold-change of FGF-2 against Control.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

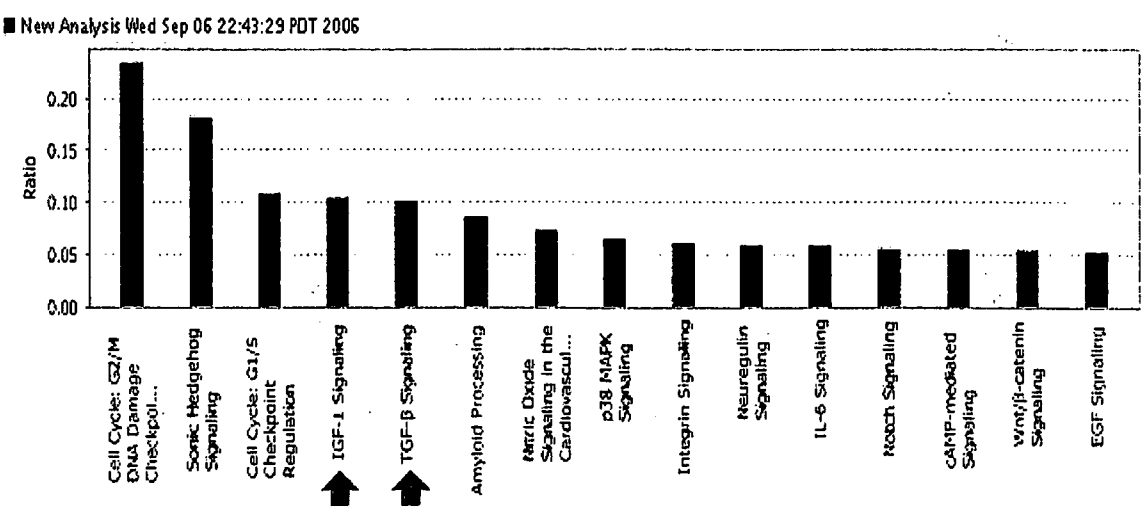


Fig. 3 Pathway analysis of genes up-regulated and down-regulated by FGF-2 in hMSCs. Pathway analysis of up-regulated and down-regulated genes by FGF-2 (Fig. 2) was performed by Ingenuity Pathway Analysis. The *y*-axis showed the ratio of genes mapped in Fig. 2 against all of genes belongs to each canonical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

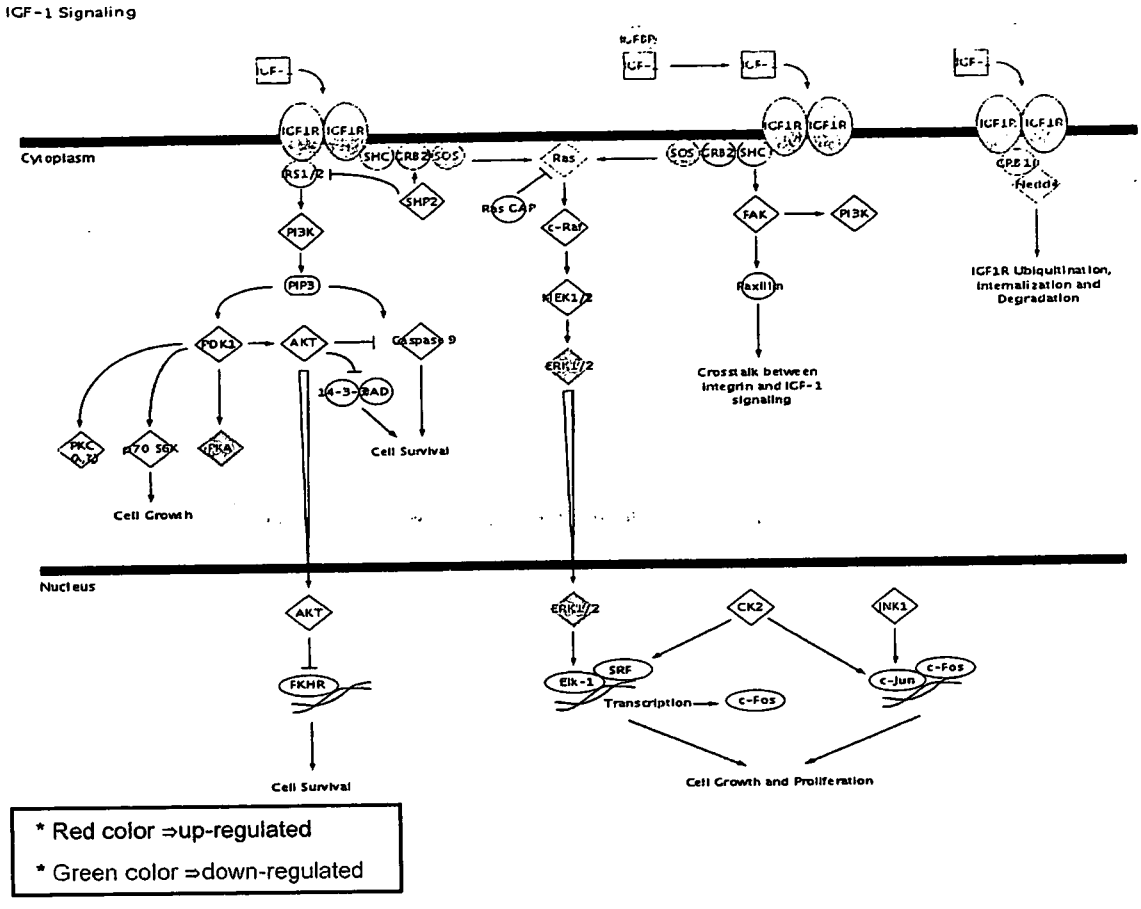


Fig. 4 Genes up-regulated and down-regulated by FGF-2 in IGF-I signaling pathway. Genes up-regulated and down-regulated by FGF-2 (Fig. 2) were mapped with the IGF-I signaling pathway by Ingenuity Pathway Analysis. The red color showed up-regulated genes and green color showed down-regulated genes

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.

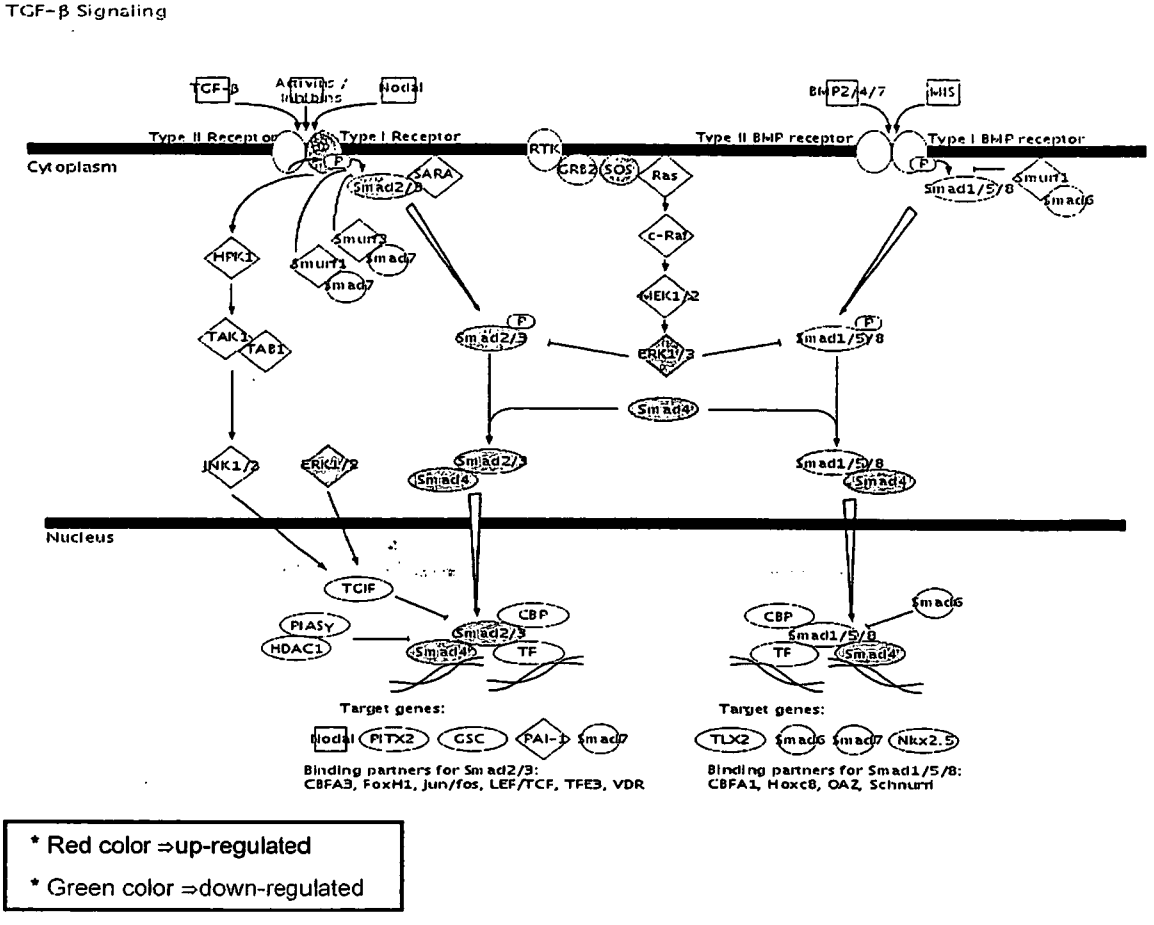


Fig. 5 Genes up-regulated and down-regulated by FGF-2 in TGF-β signaling pathway. Genes up-regulated and down-regulated by FGF-2 (Fig. 2) were mapped with the TGF-β signaling pathway by Ingenuity Pathway Analysis. The red color showed up-regulated genes and green color showed down-regulated genes

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.

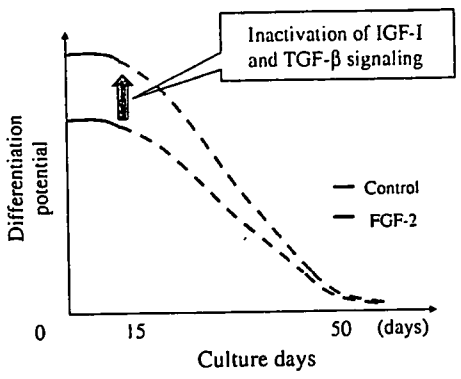


Fig. 6 Our hypothesis that FGF-2 increases the osteogenic and chondrogenic differentiation potentials of hMSCs by inactivation of IGF-I and TGF-β signaling

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid from the Japan Human Sciences Foundation.

References

Caplan AI, Bruder SP (2001) Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 7:259–264

Ito T, Sawada R, Fujiwara Y, Seyama Y, Tsuchiya T (2007) FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF-β2. Biochem Biophys Res Commun 359:108–114

Kakudo N, Shimotsuma A, Kusumoto K (2007) Fibroblasts growth factor-2 stimulates adipogenic differentiation of human adipose-derived stem cells. Biochem Biophys Res Commun 359:239–244

Leskelä HV, Risteli J, Niskanen S, Koivunen J, Ivaska KK, Lehenkari P (2003) Osteoblast recruitment from stem cells does not decrease by age at late adulthood. Biochem Biophys Res Commun 311:1008–1013

Ochi M, Adachi N, Nobuto H, Yanada S, Ito Y, Agung M (2004) Articular cartilage repair using tissue engineering technique-novel approach with minimally invasive procedure. Artif Organs 28:28–32

Petite H, Viateau V, Bensaïd W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G (2000) Tissue-engineered bone regeneration. Nat Biotechnol 18:959–963

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Quarto N, Longaker MT (2006) FGF-2 inhibits osteogenesis in mouse adipose tissue-derived stromal cells and sustains their proliferative and osteogenic potential state. Tissue Eng 12:1405–1418

Sawada R, Ito T, Tsuchiya T (2006) Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during *in vitro* culture in comparison with cancer cells. J Artif Organs 9:179–184

Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF (2005) FGF-2 enhances the mitotic and chondrogenic potentials human adult bone marrow-derived mesenchymal stem cells. J Cell Physiol 203:398–409

Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, Takagishi K, Kato Y (2001) Retention of multilineage differentiation potential of mesenchymal stem cells during proliferation in response to FGF. Biochem Biopys Res Commun 288:413–419

Wakitani S, Saito T, Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417–1426

Walsh S, Jefferiss C, Stewart K, Beresford JN (2003) TGF-beta 1 limits the expression of osteoprogenitor fraction in cultures of human bone marrow stromal cells. Cell Tissue Res 311:187–198

TISSUE ENGINEERING
Volume 13, Number 10, 2007

DOI: 10.1089/ten.2006.0340

Selection of Common Markers for Bone Marrow Stromal Cells from Various Bones Using Real-Time RT-PCR: Effects of Passage Number and Donor Age

AKIRA IGARASHI, M.S.,[1,2] KAZUMI SEGOSHI, M.S.,[1,2] YUHIRO SAKAI, M.S.,[1,2] HAIOU PAN, Ph.D.,[1,2] MASAMI KANAWA, Ph.D.,[1] YUKIHITO HIGASHI, Ph.D.,[3] MASARU SUGIYAMA, Ph.D.,[4] KOZO NAKAMURA, Ph.D.,[5] HIDEMI KURIHARA, Ph.D.,[6] SATORU YAMAGUCHI, M.D.,[7] KOICHIRO TSUJI, Ph.D.,[2] TAKESHI KAWAMOTO, Ph.D.,[1] and YUKIO KATO, Ph.D.[1,2]

ABSTRACT

Bone marrow stromal cells (BMSCs) are valuable in tissue engineering and cell therapy, but the quality of the cells is critical for the efficacy of therapy. To test the quality and identity of transplantable cells, we identified the molecular markers that were expressed at higher levels in BMSCs than in fibroblasts. Using numerous BMSC lines from tibia, femur, ilium, and jaw, together with skin and gum fibroblasts, we compared the gene expression profiles of these cells using DNA microarrays and low-density array cards. The differentiation potential of tibia and femur BMSCs was similar to that of iliac BMSCs, and different from jaw BMSCs, but all BMSC lines had many common markers that were expressed at much higher levels in BMSCs than in fibroblasts; several BMSC markers showed discrete expression patterns between jaw and other BMSCs. The common markers are probably useful in routine tests, but their efficacy may depend upon the passage number or donor age. In our study the passage number markedly altered the expression levels of several markers, while donor age had little effect on them. Considering the effects of *in vivo* location of BMSCs and passage, magnitude of increase in expression levels, and interindividual differences, we identified several reliable markers—LIF, IGF1, PRG1, MGP, BMP4, CTGF, KCTD12, IGFBP7, TRIB2, and DYNC1I1—among many candidates. This marker set may be useful in a routine test for BMSCs in tissue engineering and cell therapy.

INTRODUCTION

BONE MARROW STROMAL CELLS (BMSCs) isolated from the ilium and some other bones have osteogenic, chondrogenic, and adipogenic potential,[1–11] and transplantation of BMSCs on various scaffolds, including collagen, calcium phosphate compounds, and poly (DL-lactic-co-glycolic acid), enhanced regeneration in bone and/or cartilage defects in both animal models and clinical studies.[12–26] Usually the scaffolds can be chemically and physically defined, whereas

[1]Department of Dental and Medical Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.
[2]Two Cells Co. Ltd., Hiroshima, Japan.
[3]Department of Cardiovascular Physiology and Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.
[4]Department of Oral Health Research, Faculty of Dentistry, Hiroshima University, Hiroshima, Japan.
[5]Department of Orthopedic Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.
[6]Department of Periodontal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.
[7]Department of General Surgical Science (Surgery I), Graduate School of Medicine, Gunma University, Maebashi, Japan.