effects of PGA synthesized with and without an inorganic tin catalyst. In this study, the biocompatibility of PGA with and without a tin catalyst was investigated, using human articular chondrocytes (HAC) in a micromass culture system.

MATERIALS AND METHODS

Medium and polymers used for cell culture

Chondrocyte growth medium was obtained commercially from BioWhittaker (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (M_w = 1500) and without a catalyst (PGA) (M_w = 1100) were custom-made (TAKI chemicals, Kakogawa, Japan) and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical, St. Louis, MO, USA).

Cells and culture methods

Human articular chondrocytes (HAC) of the knee joint was commercially obtained from BioWhittaker. High-density micromass cultures were started by spotting 4×10^5 cells in 20 μL of medium onto Costar 24-well tissue culture microplates (Costar type 3526, Corning). After a 2 h attachment period at 37°C in a CO_2 incubator, culture medium (1 mL/well) was added to each well. Media were supplemented with DMSO (0.8 μL/mL), PGA, and PGA(Sn) (50 μg/mL). HAC cultured with DMSO was used as the control. The cultures were continued for 4 weeks with a medium change twice a week. At least four cultures were performed for each sample.

Cell proliferation study

Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[28] After the culture period, cells were fixed with 100% methanol at room temperature, followed by application of 0.1% crystal violet in methanol. After a proper wash, cells were again incubated in methanol; 100 μL from each well was transferred to a new 96-well plate, and the absorbance was measured at a wavelength of 590 nm, using an ELISA reader (Bio-Tek Instruments, Winooski, VT). Blank values were subtracted from experimental values to eliminate background readings.

Differentiation assay

Cell differentiation assay was performed by alcian blue (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[29] Following crystal violet staining, the cells were washed with methanol and then 3% acetic acid. Cultures were then stained with 1% (v/v) alcian blue in 3% acetic acid, pH 1.0. The cartilage proteoglycans were extracted with 4*M* guanidine hydrochloride (GH), and the bound dye was measured at wavelength of 600 nm, using an ELISA reader (Bio-Tek Instruments). Fresh 4*M* GH served as the blank. Blank values were subtracted from experimental values to eliminate background readings.

Analytical assays

Commercially available assay kits (collagen and glycosaminoglycan [GAG] assay kits, Biocolor, Newtownabbey, Northern Ireland) were used for the measurement of collagen and sulfated GAGs within the cultured cells, as previously described.[30]

Briefly, for the GAG assay, GAG was extracted from the cultured cells using a solvent system of 4*M* guanidine-HCl, 0.5*M* sodium acetate, pH 6, with 1 m*M* benzamidine-HCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 m*M* *N*-ethylmaleimide (NEM). Incubation was carried out at 4°C on an orbital shaker for a 12- to 20-h period. After the extraction, the samples were centrifuged, and blyscan dye reagent (composed of 1,9-dimethyl methylene blue in an organic buffer) was mixed with the supernatant. The GAG–dye complex was collected by centrifugation. The dye bound to the pellet was subsequently solubilized by mixing it with a dissociation reagent. The absorbance of the samples was measured at a wavelength of 656 nm, using a UV spectrophotometer. A calibration solution containing chondroitin-4 sulfate was used to obtain the standard curve for this experiment.

The total collagen concentration (acid- and pepsin-soluble fractions) of the cultured chondrocytes was also measured. The acid-soluble collagen was removed by adding 0.5*M* acetic acid to the cultured cells, followed by centrifugation. The remaining pepsin-soluble collagen was subsequently extracted from the cultured cells. A pepsin solution (1 mg/10 mg tissue sample; Sigma) was added to the cells, and they were incubated overnight at 37°C. Both the acid- and pepsin-soluble collagen samples were further separated for assay by mixing with Sircol dye reagent for 30 min in a mechanical shaker, and the collagen–dye complex was collected by centrifugation. The dye bound to the collagen pellet was solubilized with an alkaline reagent, and the absorbance of the samples was measured at a wavelength of 540 nm, using a UV spectrophotometer. A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment.

Real-time polymerase chain reaction

To detect the presence of collagen type II and aggrecan, single-stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT), using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently, real-time polymerase chain reaction (PCR) was done using a LightCycler system with LightCycler FastStart DNA Master SYBR Green I

(Roche Diagnostics, Penzberg, Germany). The LightCycler™- Primer set (Roche Diagnostics) was used for quantitative detection of the collagen type II and aggrecan genes, and also for quantitation of a housekeeping gene, Glyceraldehide-3-phosphate dehydrogenase (GAPDH), according to the manufacturer's instructions. An initial denaturation step at 95°C for 10 min was followed by amplification and extension steps for 35 cycles (95°C for 10 s, 68°C for 10 s, 72°C for 16 s) with final extension step at 58°C for 10 s. The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

Statistical study

Student's t tests were used to assess whether differences observed between the polymers treated and the control samples were statistically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD (standard deviation) except in figure 3. Four samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

RESULTS

Cell proliferation

Chondrocyte proliferation was quantified by crystal violet staining and expressed as a percentage of the average control value (Fig. 1). Cell proliferation was increased 1.8-fold ($p < 0.05$) in PGA(Sn)-treated cultures compared with that of the control culture, whereas cell proliferation in PGA-treated cultures was almost identical to the DMSO-treated control culture.

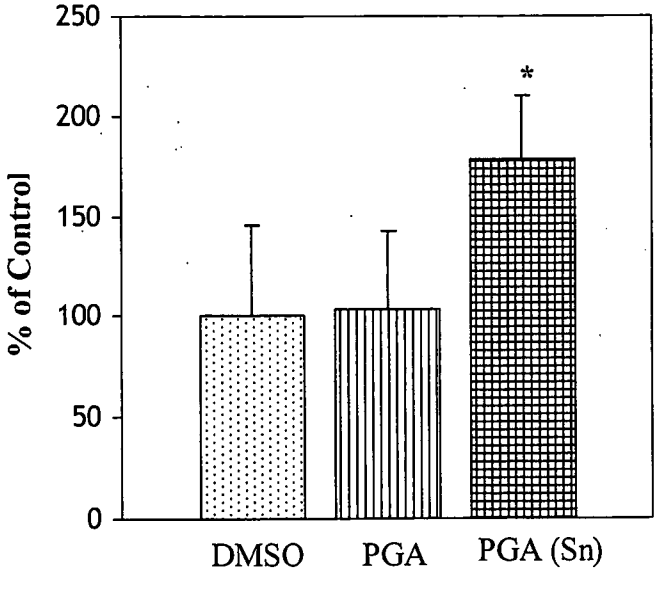

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

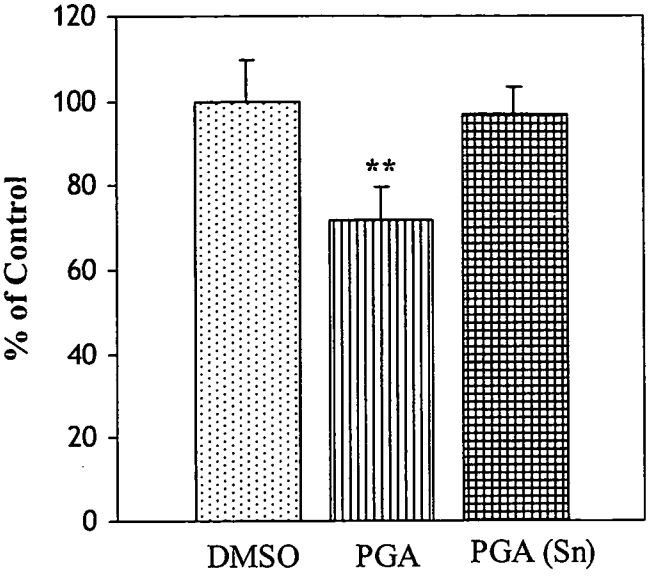

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

Cell differentiation

Chondrocyte differentiation was estimated by alcian blue staining and the amounts were expressed as a percentage of the average control value, which was calculated as 100%. Chondrocytes treated with PGA revealed a 0.71-fold ($p < 0.01$) decrease in cell differentiation compared with that of the control culture. At the same time, cultures treated with PGA(Sn) showed a slight, but nonsignificant, decrease in cell differentiation (Fig. 2).

Extracellular matrix gene expression

Extracellular matrix gene expression was quantitatively measured by real-time PCR. Here, compared with that of the control culture, the collagen type II gene was more strongly expressed ($p < 0.01$) in PGA(Sn) than in PGA-treated cultured chondrocytes [Fig. 3(A)]. Aggrecan gene expression was inhibited in the latter, but no difference was observed between the former and the control culture [Fig. 3(B)].

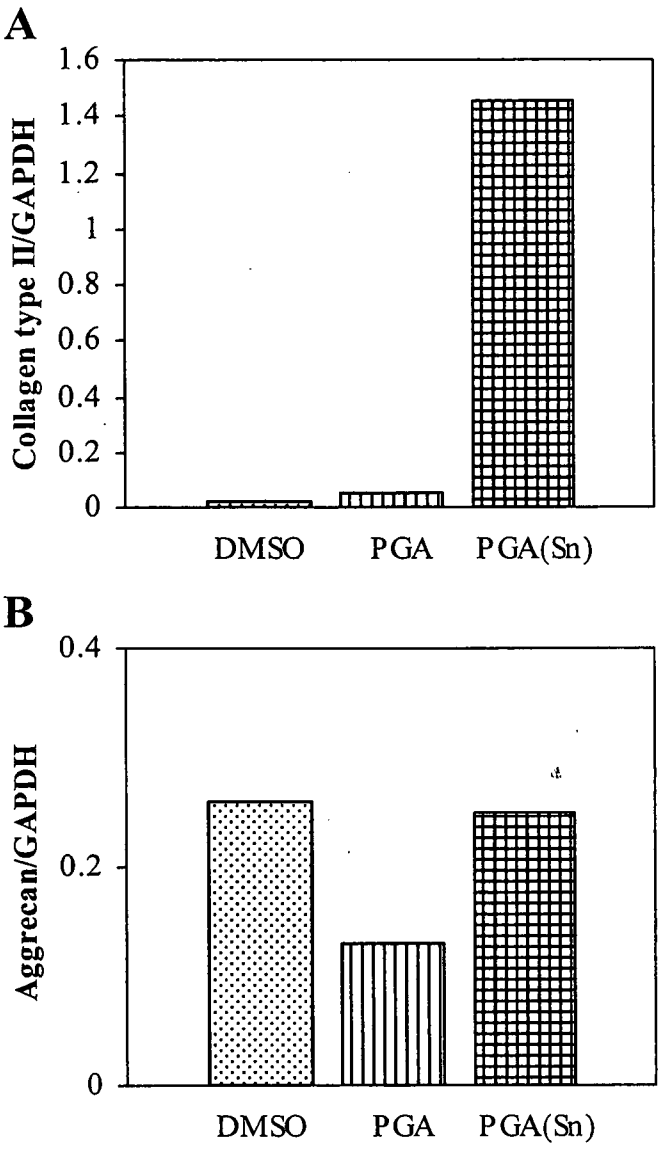

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between the PGA(Sn) and the control. All experiments were run in quadruplicate for two separate times.

Measurement of collagen type II protein

The amount of pepsin-soluble and cartilage-specific collagen type II protein was increased in both PGA and PGA(Sn) treated chondrocytes on comparing with that of the control culture(Fig. 4). However, this increase was more in the latter than in the former case.

Measurement of total collagen

Quantitative estimations of both acid- and pepsin-soluble total collagen revealed a decrease in PGA(Sn)-treated cultures compared with that of the control

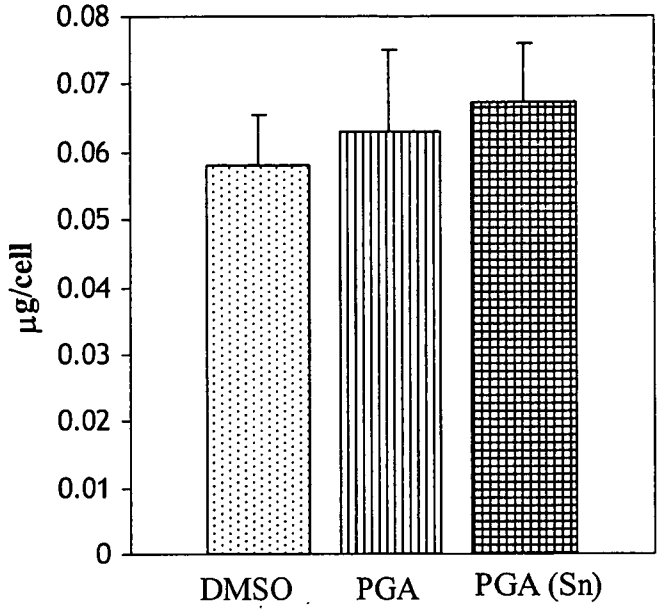

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

(Fig. 5). Simultaneously, there was a slight increase in the amount of total collagen in PGA-treated cultures compared with that of the control sample.

Estimation of sulfated glycosaminoglycan concentration

Evaluation of the amount of sulfated GAG showed a decrease in PGA(Sn)-treated cultured cells com-

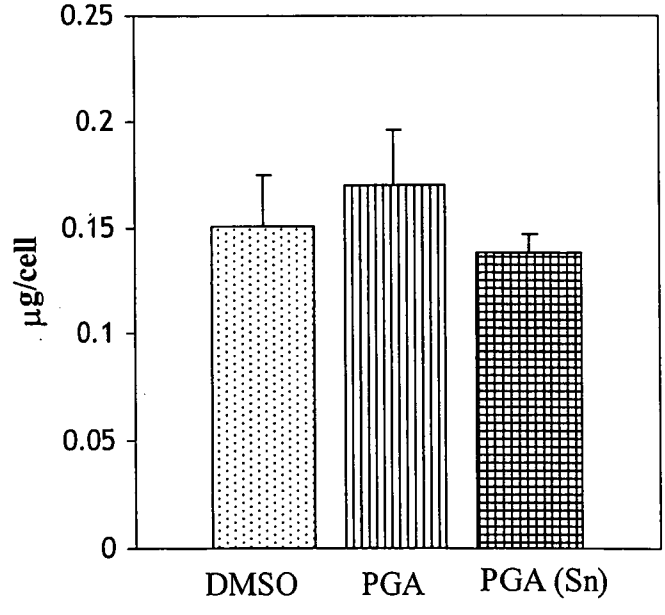

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

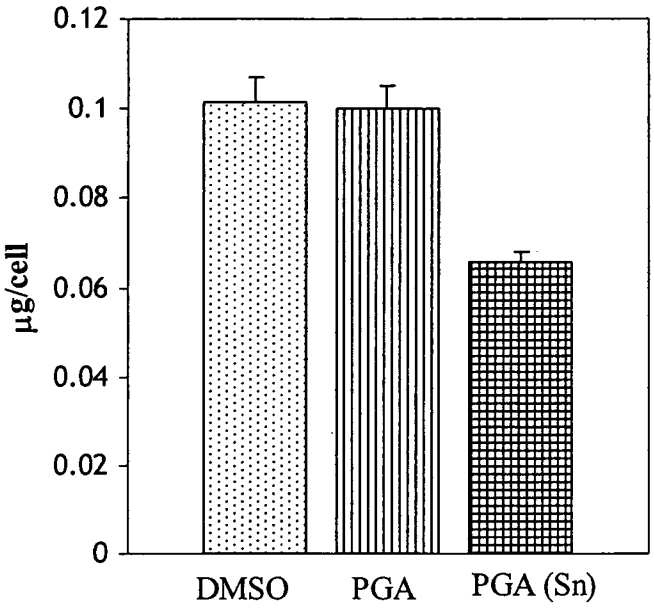

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

pared with that of the control (Fig. 6). However, in the same experiment, almost no difference in this amount was observed between the PGA-treated culture and the control.

DISCUSSION

Attempts to identify a perfectly biocompatible and biodegradable polymer have been ongoing over the past decade. An ideal biomaterial should fulfill its purpose satisfactorily and then biodegrade to obviate any risk of foreign body reaction.[31] Synthetic biodegradable polymers, especially those belonging to the polyester family, have played an important role in a number of tissue engineering efforts. PGA, an aliphatic polyester, can be degraded in two ways: by hydrolysis and by nonspecific esterases and carboxypeptidases, followed by either excretion in the urine or entrance into the tricarboxylic acid cycle.[32]

Several different catalysts, namely organotin, antimony, zinc, and lead, are used in the polymerization process to synthesize high molecular weight PGA. Different tin compounds were observed to produce general cytotoxic effects in rabbit articular cartilage in monolayer culture, [33] and bone is suggested to be the critical organ in inorganic tin toxicity in rats.[26] Therefore, in this study, we aspired to evaluate the chondrogenic effects of HAC with PGA synthesized with and without an inorganic tin catalyst, with the aim of clarifying the biocompatibility of inorganic tin as a catalyst for future clinical use.

It was reported that oral administration of certain tin compounds at specific concentrations exerted stimulatory effects on chondrocyte proliferation in the rat.[33] Consistent with this, the proliferation assay performed in our study also showed that HAC with PGA(Sn) had stimulatory effects on chondrocyte proliferation in micromass culture (Fig. 1). On the other hand, PGA neither stimulated nor inhibited the chondrocyte proliferation, and thus, inorganic tin as catalyst seemed to play a stimulatory role in HAC proliferation. In our experiment, PGA with inorganic tin as the catalyst caused almost no change in cell differentiation, but PGA-treated cultures did show a significant decrease when compared with that of the control (Fig. 2). Furthermore, quantitative estimation of extracellular matrix gene expression by real-time PCR confirmed that the cartilage-specific protein, collagen type II, was more strongly expressed in PGA(Sn)- than in PGA-treated cultured chondrocytes [Fig. 3(A)]. However, the expression of the aggrecan gene was inhibited in the PGA culture, but no difference was observed between the PGA(Sn) and the control cultures [Fig. 3(B)].

It was reported that oral administration of inorganic tin caused a decrease in the proliferation of chondrocytes, accompanied by suppression of DNA synthesis with subsequent inhibition in collagen synthesis in rat.[34] On the contrary, our results showed enhancement of proliferation, expression of the collagen type II gene, and amount of collagen type II protein by *in vitro* culture of HAC with PGA(Sn). We speculated that difference in the route of administration might be the cause of these diverse effects of inorganic tin compound. As mentioned earlier, monolayer culture of rabbit articular cartilage with tin compounds caused inhibition in the synthesis of core proteins, followed by a decrease in the synthesis of sulfated GAG.[33] In agreement with this result, our report also showed a decrease in the amount of sulfated GAG by culture of HAC with PGA(Sn). A study performed in our laboratory using HAC in a micromass culture system has already shown that PGA synthesized with organic tin catalyst caused a decrease in cell proliferation, but a significant increase in cell differentiation [29] and was completely contradictory to our present results. The molecular weight of PGA(Sn), and the type of tin product such as $SnCl_2$ and dibutyl tin were thought to be the key factor of different effects of chondrogenesis on HAC.

To the best of our knowledge, no other study has yet investigated the chondrogenic effects of PGA with inorganic tin as a catalyst, using HAC in a micromass culture system. This study is the first to show the biological action of inorganic tin as catalyst in PGA on human articular chondrogenesis in a micromass culture system. Our observation revealed that low concentration of inorganic tin when used in the polymer

of PGA showed enhancing effects of tin compounds on chondrocytes in comparison to without tin polymer because of increase in the permeability of inorganic tin under the presence of PGA. However, further study is required for the application of this PGA(Sn) in clinical practice.

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—Reviews—

細胞組織利用医療機器に用いられる幹細胞の品質及び安全性評価について

澤田留美,* 伊藤友実, 土屋利江

Safety Evaluation of Tissue Engineered Medical Devices Using Normal Human Mesenchymal Stem Cells

Rumi SAWADA,* Tomomi ITO, and Toshie TSUCHIYA
Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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Several recent studies demonstrated the potential of bioengineering using somatic stem cells in regenerative medicine. Adult human mesenchymal stem cells (hMSCs) derived from bone marrow have the pluripotency to differentiate into cells of mesodermal origin, *e.g.*, bone, cartilage, adipose, and muscle cells; they, therefore, have many potential clinical applications. On the other hand, stem cells possess a self-renewal capability similar to cancer cells. For safety evaluation of tissue engineered medical devices using normal hMSCs, in this study, we investigated the expression levels of several genes that affect cell proliferation in hMSCs during *in vitro* culture. We focused on the relationship between the hMSC proliferation and their transforming growth factor β (TGFβ) signaling during *in vitro* culture. The proliferation rate of hMSCs gradually decreased and cellular senescence was observed for about 3 months. The mRNA expressions of TGFβ1, TGFβ2, and TGFβ receptor type I (TGFβRI) in hMSCs increased with the length of cell culture. The mRNA expressions of Smad3 increased, but those of c-myc and nucleostemin decreased with the length hMSCs were in *in vitro* culture. In addition, the expression profiles of the genes which regulate cellular proliferation in hMSCs were significantly different from those of cancer cells. In conclusion, hMSCs derived from bone marrow seldom underwent spontaneous transformation during 1—2 months *in vitro* culture for use in clinical applications. In hMSCs as well as in epithelial cells, growth might be controlled by the TGFβ family signaling.

Key words——human mesenchymal stem cells; tissue engineered medical devices; proliferation; transforming growth factor β

1. はじめに

様々な疾病などに起因した組織や器官の機能不全に対して，組織再生又は機能回復を目指した「再生医療」が，現在注目されている．その手段としてこれまでに，幹細胞や人工素材を用いた医療機器の開発や細胞治療などについて多くの研究がなされている．胚性幹細胞（ES細胞）は全能性を持つが受精卵を用いることから倫理的問題が大きいのに対し，体性幹細胞はES細胞のような倫理的問題がない点が再生医療や細胞治療のツールとして利用されている大きな理由であろう．中でも骨髄に含まれる間葉系幹細胞，造血幹細胞，血管内皮前駆細胞は，様々な臨床分野での応用が期待されている．また，骨髄や臍帯血中には間葉系幹細胞や造血幹細胞よりもさらに上の段階で多くの細胞系への分化能を持った細胞（multipotent adult progenitor cells; MAPCs）が存在することも報告されている.[1] さらに骨髄中だけでなくそれぞれの組織に特異的な幹細胞（肝，心筋，神経，上皮など）の存在も知られている．骨髄由来の間葉系幹細胞は骨，軟骨，脂肪，筋肉へ分化可能な細胞として広く知られている[1-5]が，さらに，神経細胞[3]や肝細胞,[1,6] 心筋,[7,8] 皮膚など胚を越えた分化も報告されており，整形外科の分野のみならず動脈硬化症，心筋梗塞，肝硬変，糖尿病などの治療への応用も期待されている．骨髄間葉系幹細胞は採取も比較的容易で *in vitro* での培養技術も確立されているため，細胞組織利用医療機器の材料と

国立医薬品食品衛生研究所療品部（〒158-8501 東京都世田谷区上用賀 1-18-1)
*e-mail: rsawada@nihs.go.jp
本総説は，日本薬学会第126年会シンポジウムS4で発表したものを中心に記述したものである．

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.

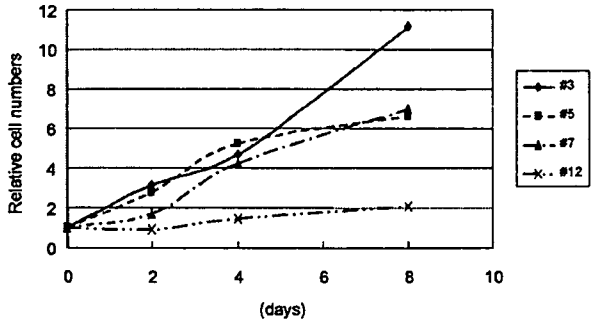

Fig. 1. Proliferation of hMSC in 3rd, 5th, 7th, and 12th Passages[16]

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

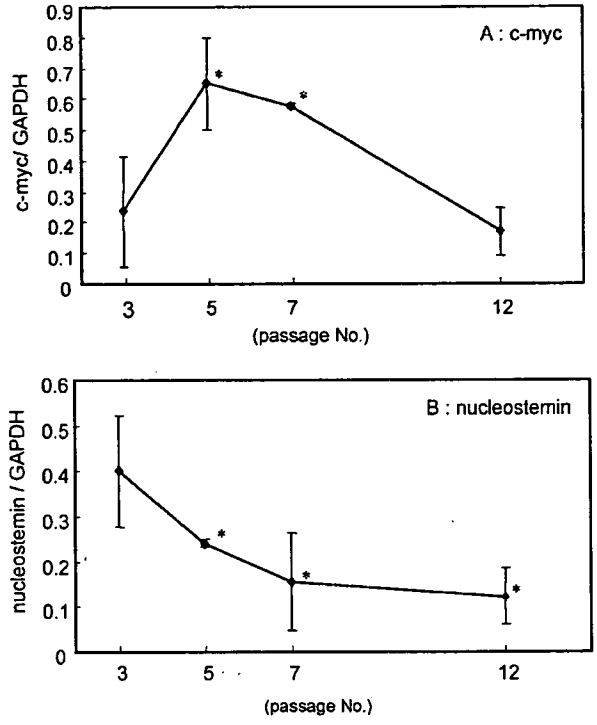

Fig. 2. Effect of *In vitro* Culture Length on the mRNA Expressions of c-myc (A) and Nucleostemin (B) in hMSC[16]

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

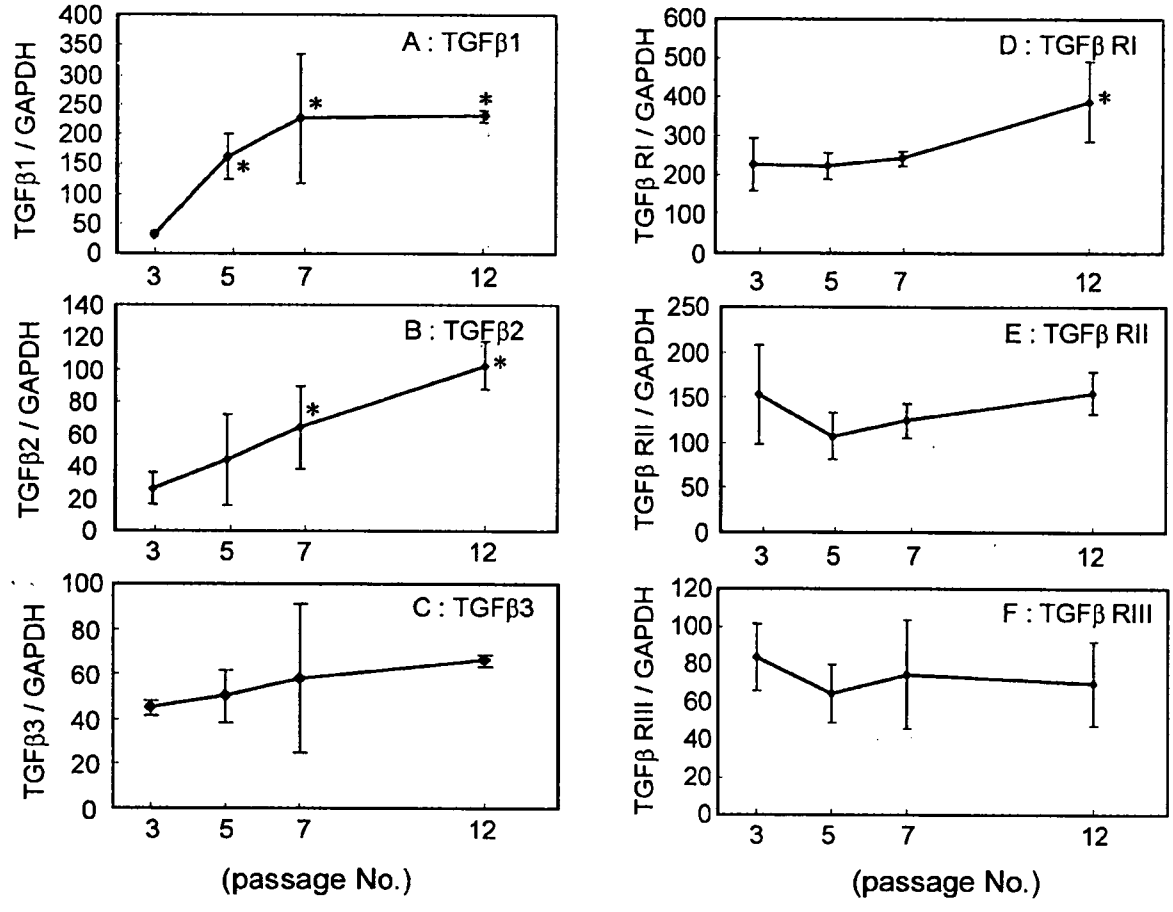

Fig. 3. Effect of *In vitro* Culture Length on mRNA Expressions of TGFβ1 (A), TGFβ2 (B), TGFβ3 (C), TGFβRI (D), TGFβRII (E), and TGFβRIII (F) in hMSC[16]

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 (*$p<0.05$).

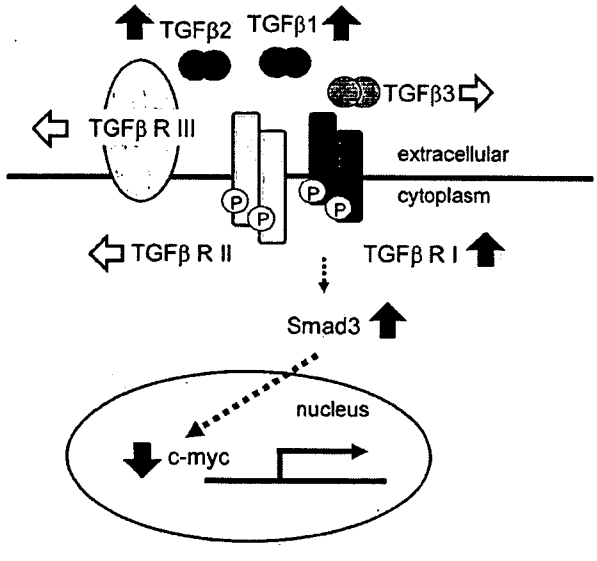

Fig. 4. Changes in the Expressions of TGFβ Signaling Genes during hMSC *In vitro* Culture for Three Months

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．

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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

Tomomi Ito · Rumi Sawada · Yoko Fujiwara · Toshie Tsuchiya

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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

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

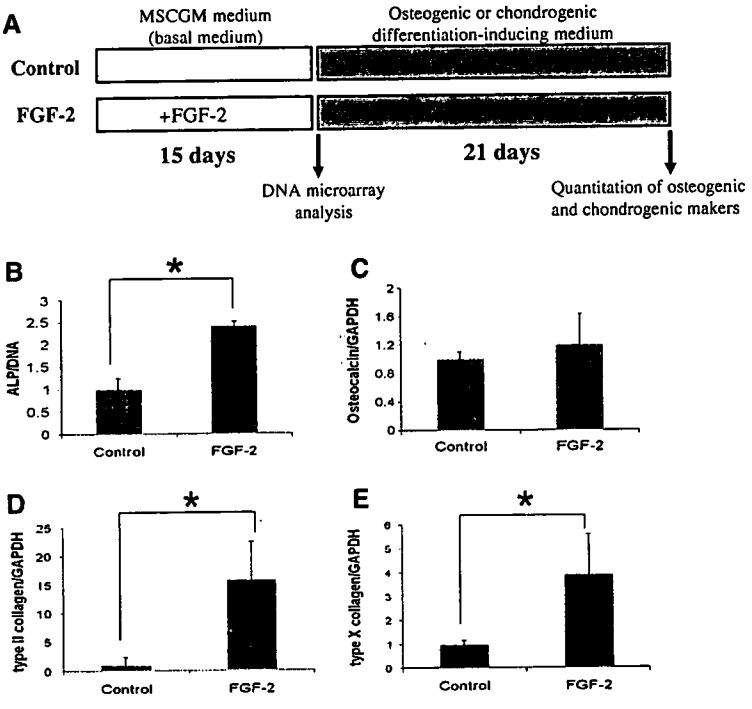

Fig. 1 Experimental protocol and quantitation of osteogenic and chondrogenic markers. hMSCs were maintained in the medium with or without FGF-2 for 15 day, and osteogenic or chondrogenic differentiation of hMSCs was induced for 21 days (**A**). Then, the protein levels of ALP (**B**), the mRNA expression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

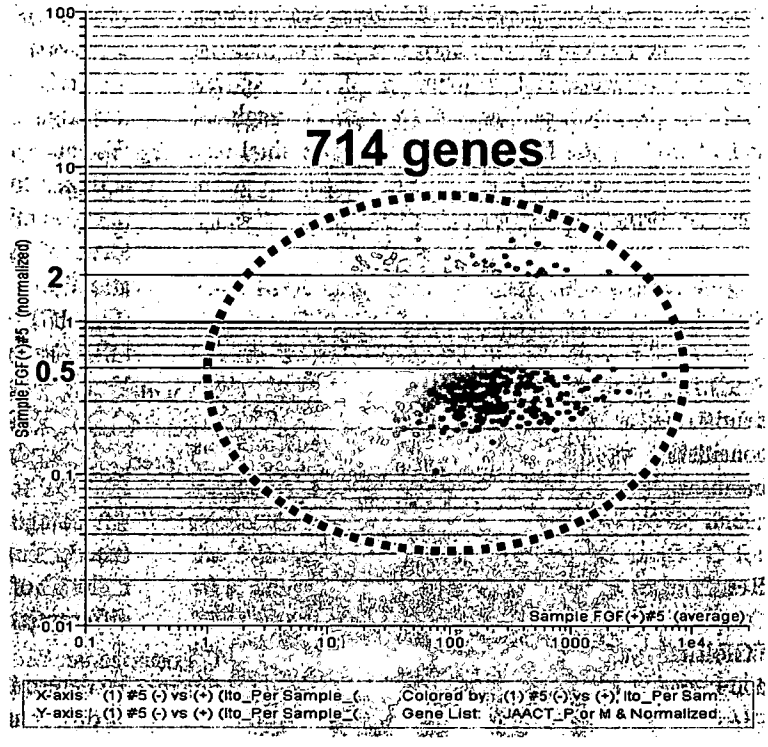

Fig. 2 Genes up-regulated (>2 fold) and down-regulated (<1/2 fold) by FGF-2 in hMSCs. hMSCs were maintained in the medium with or without FGF-2 for 15 days. Then, total RNA were extracted from the hMSCs and microarray analysis were performed. The *x*-axis showed the fold-change of FGF-2 against Control.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

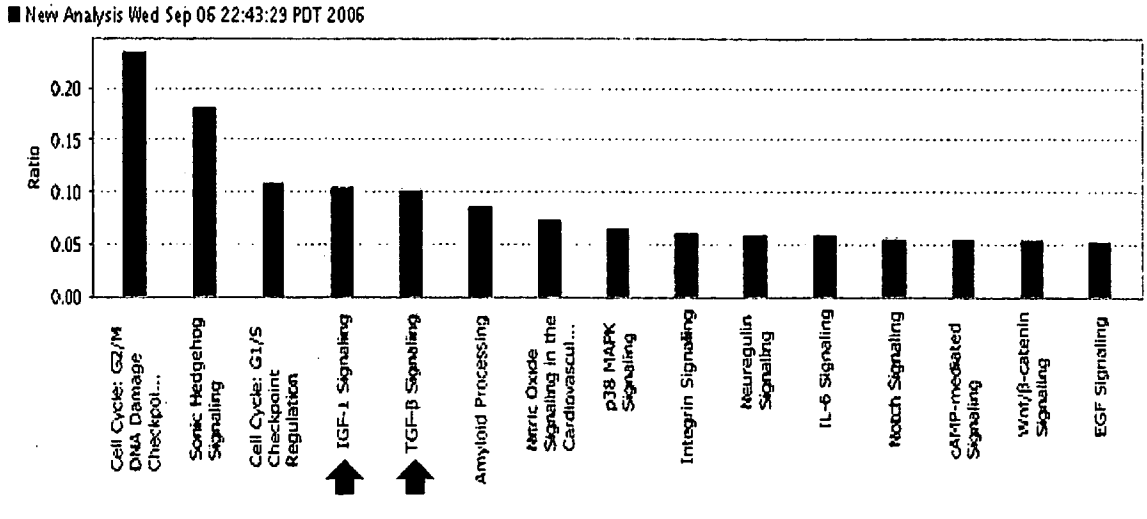

Fig. 3 Pathway analysis of genes up-regulated and down-regulated by FGF-2 in hMSCs. Pathway analysis of up-regulated and down-regulated genes by FGF-2 (Fig. 2) was performed by Ingenuity Pathway Analysis. The *y*-axis showed the ratio of genes mapped in Fig. 2 against all of genes belongs to each canonical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

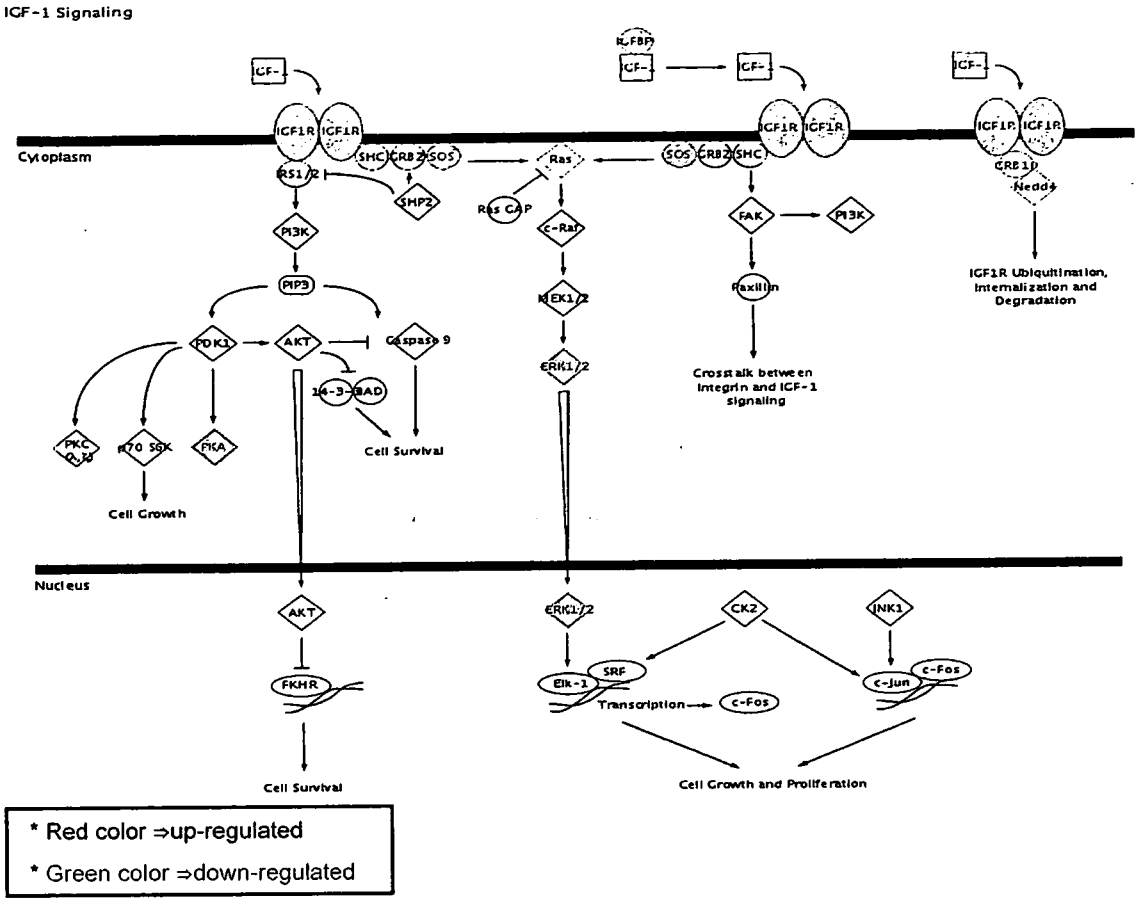

Fig. 4 Genes up-regulated and down-regulated by FGF-2 in IGF-I signaling pathway. Genes up-regulated and down-regulated by FGF-2 (Fig. 2) were mapped with the IGF-I signaling pathway by Ingenuity Pathway Analysis. The red color showed up-regulated genes and green color showed down-regulated genes

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.

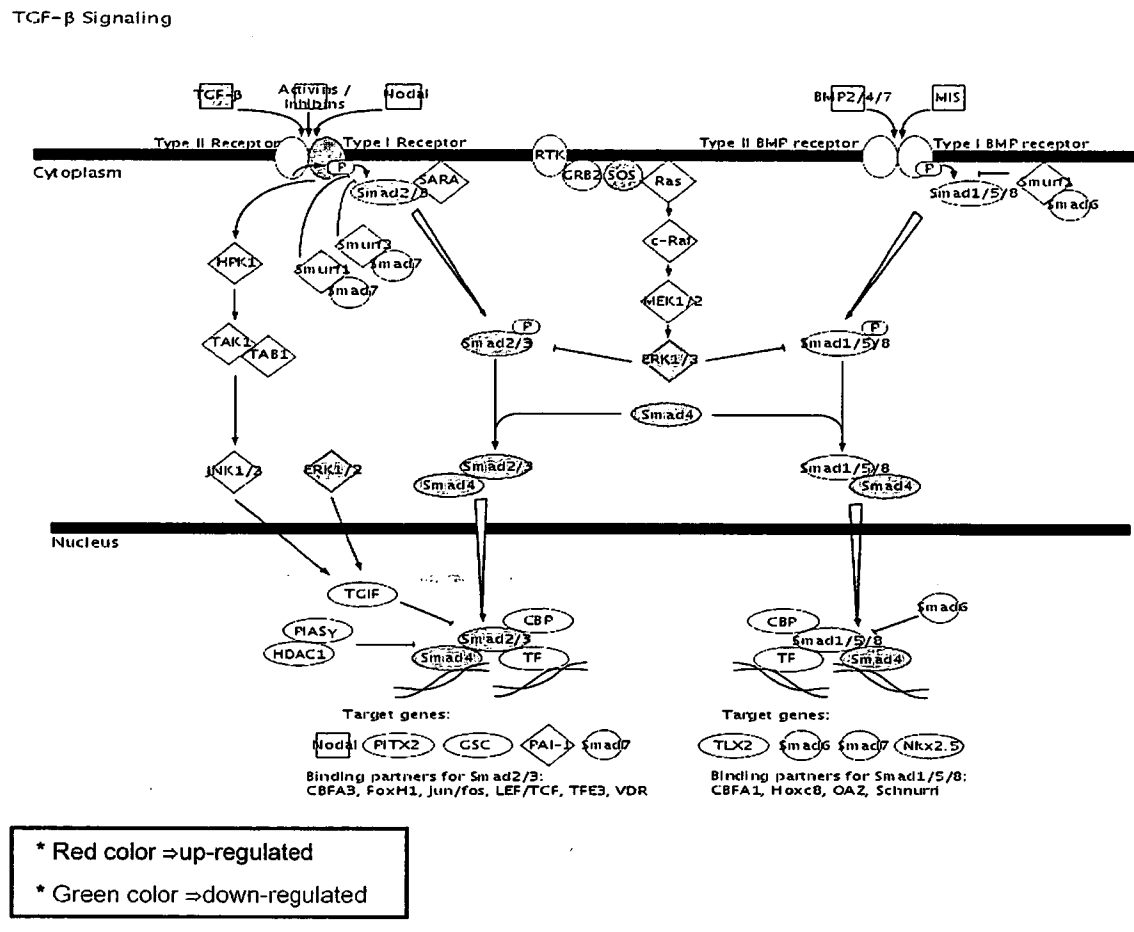

Fig. 5 Genes up-regulated and down-regulated by FGF-2 in TGF-β signaling pathway. Genes up-regulated and down-regulated by FGF-2 (Fig. 2) were mapped with the TGF-β signaling pathway by Ingenuity Pathway Analysis. The red color showed up-regulated genes and green color showed down-regulated genes

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.

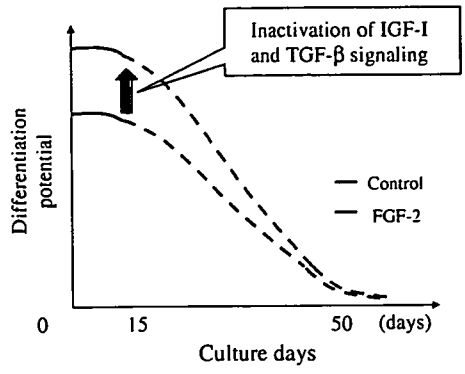

Fig. 6 Our hypothesis that FGF-2 increases the osteogenic and chondrogenic differentiation potentials of hMSCs by inactivation of IGF-I and TGF-β signaling

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3　間葉系幹細胞の特質

加藤幸夫[*1]，五十嵐　晃[*2]，清水正和[*3]，久保裕嗣[*4]

3.1　はじめに

間葉系幹細胞(MSC)の特質は，線維芽細胞と比較して，骨，軟骨，脂肪，筋，腱，歯周靱帯など各種の間葉系組織への顕著な分化能をもつことである。血管内皮細胞[1]，糸球体内皮細胞へも分化するとの報告もある。さらに肝細胞，肺上皮細胞[2]，アストロサイト，神経細胞，膵臓ベータ細胞への分化転換も報告されている。興味深いことに，Lee-RHによるとMSCはマウス糖尿病モデルでのインスリン分泌と腎臓傷害を回復させた。ただし分化転換と考えられた現象の一部は細胞融合の結果であるとも指摘されている。これについては議論があるが，細胞融合はまれであり分化転換を否定できないとの意見が多い[2]。一方，MSCの間葉系への分化能は確実であり，これに基づいて，骨，軟骨疾患(www.mesoblast.com)，歯周病，心筋梗塞に対するMSC移植治療がすでに臨床応用されている[3~7]。また神経変成疾患(www.brainstorm-cell.com)や肝硬変への臨床応用も計画されている。しかもMSCには免疫抑制作用があり，臓器移植による拒絶反応を抑制することから[8]，自家のみならず同種(他家)MSCも臨床ですでに使用されている。また骨形成不全症[9]，ALS(amyotrophic lateral sclerosis)，リソソーム酵素蓄積症(Hurler症候群)などの遺伝性疾患を正常な同種MSCの移植で治療する試みがある[3]。あるいは正常遺伝子をウイルスベクターなどで導入した自家MSCで遺伝性疾患を治療する試みもある[2]。いずれにせよMSCには炎症抑制作用もあるので[10]，再生治療にMSCを用いることは有利である。

3.2　MSCの臨床応用は安全か？

自家MSCの移植は，日本だけでもすでに100人以上行われているが，副作用の報告はない(表1)。文献的にもMSC移植は安全であるとされている。骨髄の機能低下した患者へ，兄弟あるいは父親由来，あるいはまったくの他人のMSC(10^6-10^7細胞/kg)の移植後，副作用はなく拒絶反応はみられないか少なかった[11]。これらの観察は，自家MSC移植の安全性を示すとともに同種(他家)MSC移植が再生医療に利用できるかもしれないことを示唆している。

＊1　Yukio Kato　広島大学大学院　医歯薬学総合研究科　創生医科学専攻　探索医科学講座　教授

＊2　Akira Igarashi　㈱ツーセル

＊3　Masakazu Shimizu　㈱日本シグマックス

＊4　Hiroshi Kubo　㈱ツーセル

表1　すでに病院で実施されているMSCを用いた細胞治療

関節症	…60人以上
歯周病	…15人以上
歯科用インプラントの支持骨形成	…30人以上
口蓋裂での骨欠損	…2人
骨欠損	…10人
心筋梗塞	…10人
難治性皮膚潰瘍	
造血支持（抗がん剤投与後）	
臓器移植での拒絶反応	
骨形成不全症	
腎疾患，腹膜透析，血管障害	

人数は日本での患者数，日本での患者数が不明の場合と海外のみでの実施の場合は人数を表記していない。

3.3　MSCの問題点—生理的役割は不明—

MSCは臨床に用いられているにも関わらず，MSCの分子レベルでの特徴，生体内分布および生理的役割はいまだ不明である。骨髄中に骨，軟骨再生能をもつ所謂MSCが存在することは間違いないものの，生体内での働きや意義を示す証拠はない。このことが，MSC移植療法の普及を妨げている一つの要因である。骨髄MSCが生理的に骨の成長に関与している証拠があれば，培養MSCを骨疾患に用いることは合理的であると説明できる。

3.4　病気とMSC

関節症の滑膜では，活発な細胞増殖と炎症反応があり，病態を悪化させている。ところが最近の研究で，正常および関節症の滑膜細胞は，骨，軟骨，脂肪分化能をもつMSCであることが判明した。通常MSCは基質分解酵素を大量には産生しないが，滑膜由来MSCはMMPを放出して基質破壊を誘導する。関節症では，MSCニッチェが病的に変化したり，サイトカインが増加するために，MSCの性質が非可逆的に変化していると推察される。したがって，関節症由来の滑膜線維芽細胞（MSC様細胞）は，継代培養しても高レベルの基質分解活性／炎症増幅活性を維持している。滑膜MSCは他のMSCと異なり元来基質分解活性が高いが，正常ではその作用が制限されているのかもしれない。いずれにせよ今後は，変形性関節症や関節リウマチの診断と治療にMSCからの観点を加える必要がある。

さらにMSCは，当然ながら骨，軟骨，筋肉，脂肪の病気や老化に関わると推察されるが，この方面の研究はまだほとんどない。

またMSC由来である骨肉腫，脂肪肉腫，軟骨肉腫，筋肉腫，線維肉腫などでの癌遺伝子およ