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Effect of Forced Expression of Basic Fibroblast Growth Factor in Human Bone Marrow-Derived Mesenchymal Stromal Cells

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Mesenchymal stromal cells (MSCs) derived from human bone marrow are expected to be utilized for the purpose of tissue engineering, because of their extensive self-renewal or proliferation capability. The capability decreases after several passages, however. Basic fibroblast growth factor (bFGF) is commonly used for culture of various cells including bone marrow-derived MSCs. With the aim of conferring higher capability on human bone marrow MSCs, we introduced the bFGF gene into the passaged cells by retroviral system. The bFGF-expressing MSCs, even at 7 to 8 passages after the infection, showed consistent proliferation capability. The capability was not detected in control cells even in culture media containing the bFGF protein. Thus, we could not mimic the effect of forced expression of bFGF by exogenously adding the bFGF protein in culture media. Although we expressed the shortest isoform of bFGF, which was considered to be mostly cytosolic, we found the protein mostly in the nucleus. Our observations demonstrate not only an effective way to maintain proliferation potentials of MSCs, but also a possibility that there may be mechanistic and functional differences in the signal transduction events between endogenously expressed and exogenously added bFGF protein in MSCs.

Key words: basic FGF, bone marrow, forced expression, mesenchymal cells, retrovirus.

Abbreviations: bFGF, basic fibroblast growth factor; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; IRES, internal ribosome entry site; MSC, mesenchymal stromal cell; PBS, phosphate-buffered saline.

Mesenchymal stromal cells (MSCs) derived from human bone marrow have capability to differentiate into cells of mesenchymal lineage, and have already been applied in various clinical situations (*1–5*). We have demonstrated that *in vitro* culture of bone marrow-derived MSCs on various ceramics showed osteogenic differentiation resulting in the formation of bone matrix, which serves as the basis for clinical use of tissue engineered ceramic for the treatment of osteoarthritic patients (*6*). Although bone marrow MSCs are presently considered to be one of the most promising stem cells for cellular therapy because of their expansion and differentiation potentials, the cells lose their potentials relatively quickly after several passages.

Basic fibroblast growth factor (bFGF) is a potent mitogen that stimulates growth and differentiation of a broad spectrum of mesodermal and neuro-ectodermal cell types. bFGF belongs to a large family of small peptides (17–34 kDa) that mediates cell proliferation, differentiation and migration (*7*). A single bFGF mRNA encodes five protein isoforms. Differential initiation of translation from upstream CUG codons yields four higher molecular weight isoforms of bFGF (22–34 kDa) (*8*). Those high isoforms of bFGF have been found to localize in the nucleus and trigger an active intracrine signaling pathway, whereas the shortest (18 kDa) bFGF isoform is translated from the AUG codon and secreted from plasma membranes (*9*). These different molecular forms of bFGF may act through distinct but convergent pathways (*10*). The 18 kDa bFGF binds and activates cell surface FGF receptors. Activation of FGF receptors triggers downstream signal transduction and regulates gene expression. Although the 18 kDa bFGF is mostly cytosolic, this isoform was also found in the nucleus in various cell types, suggesting an involvement in an active intracrine signalling pathway (*11–13*). The functional significance of the presence of the bFGF protein in the nucleus is not understood very well (*14*).

bFGF is commonly used for culture of various cells including bone marrow-derived MSCs. For example, bFGF is considered to be critical for self-renewal of neural stem cells and human embryonic stem cells (*15–19*). It is known, however, bFGF signalling plays diverse developmental pathways and effects on various cells. For example, it has been reported that bFGF supports proliferation potential of bone marrow stromal cells (*20*). bFGF was shown, however, to mediate osteogenesis and neural differentiation of MSCs as well (*20–22*).

In this study, we introduced the bFGF gene into human bone marrow MSCs by retroviral system. We observed significant effects of forced expression of bFGF

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in MSCs on cell proliferation. The effects are clearly distinct from those of exogenously added bFGF protein in culture media.

MATERIALS AND METHODS

Cell Culture—In this study, we used cryopreserved human bone marrow MSCs, which we had previously established from aspirated human bone marrow of donors under sufficient informed consent in accordance with the Ethics Committee of the National Institute of Advanced Industrial Science and Technology. We used four MSC lines from different individual donors (No. 0306, 0402, 0202 and 0305). No. 0306, 0402, 0202 and 0305 are from 83-year-old male, 30-year-old male, 30-year-old male and 8-year-old male donors, respectively. For maintenance, the cells were cultured in Eagle's minimum essential medium (Invitrogen) containing 10% FBS and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO_2 at 37°C. Culture media were changed two or three times a week. For cryopreservation, the cells were trypsinized and suspended at a concentration of 1×10^6 cells/ml in a Cell Banker (Juji Field). We added, in some cases, human bFGF (PeproTech or Sigma-Aldrich) in culture media. To evaluate proliferation potential of a cell population, we counted the number of cells by using either a haemocytometer or a particle analyzer (CDA-500) (Sysmex). We also evaluated cell viability and proliferation potential by measuring the activity of a mitochondrial dehydrogenase by using Premix WST-1 proliferation assay system (Takara) according to the manufacture's instructions.

Retrovirus Infection—Forced expression of genes of interest in human bone marrow MSCs was done by using MSCV Retroviral expression system (BD Biosciences Clontech). Retroviral producing mouse cells, PT67, were transfected with a plasmid using Fugene 6 transfection reagent (Roche Diagnostics) according to the manufacture's instructions. The PT67 cells were passaged the next day in the presence of G418 (Geneticin) (Invitrogen) (400 μg/ml). After a couple of passages, almost all the PT67 cells became positive for fluorescent signal from the Venus protein. For infection to MSCs, the virus-containing supernatants derived from the PT67 cells were filtered through a 0.45 μm cellulose acetate filter. The supernatants were concentrated by centrifugation at 8,000*g* overnight at 4°C, and the pellet was resuspended with an appropriate amount of the culture medium. The final suspension was supplemented with 4 μg/ml polybrene (Chemicon). Target MSCs were incubated in the virus/polybrene containing supernatants for 4 h to overnight. In most cases, we used second passage cells (P2) for infection. Infection experiments were repeated twice with an interval of 1 day. The next day after the second infection, we added G418 at a final concentration of 400 μg/ml for selection. The cells were passaged for 2 to 3 times before expansion for analyses. We froze the infected cells either at P4 or P5 to make cryopreserved cell stock. For analyses of the cells, we used the infected cells directly (P4 or P5), or thawed the cryopreserved cells.

Plasmid Constructions—For the bFGF gene, we used the shortest (18 kDa) isoform of bFGF (GenBank accession no. NM_002006), which was translated from the AUG codon. The fragment containing the entire open reading frame was isolated by PCR from cDNA plasmid library prepared from fetal brain (Takara). The bFGF cDNA was subcloned into pCl-neo mammalian expression vector (Promega). The Venus gene, a variant of EGFP, was generously provided by Dr Miyawaki of RIKEN (*23*). The Venus cDNA was subcloned into pMSCVneo retroviral expression vector (BD Biosciences Clontech) to make Venus/pMSCV. Expression of target genes are driven by a LTR promoter that is from the murine stem cell PCMV virus. The Venus cDNA and the IRES sequence, which was excised from pIRES vector (BD Biosciences Clontech), were subcloned into pCl vector to generate both Venus-IRES/pCl and IRES-Venus/pCl. The IRES sequence was used to build constructs that allow concurrent expression of the gene of interest (bFGF) with the Venus gene from a single bicistronic mRNA. The bFGF cDNA and the Venus-IRES fragment were subcloned into pMSCV to make Venus-IRES-bFGF/pMSCV. In order to put the myc tag at the C-terminus of the bFGF protein, the bFGF cDNA was subcloned in-frame into pEF1/Myc-His vector (Invitrogen) (bFGF-myc/pEF1). The bFGF-myc fragment was subcloned into Venus-IRES/pCl to make Venus-IRES-bFGFmyc/pCl. The bFGF-myc and either Venus-IRES or IRES-Venus fragment were subcloned into pMSCV to make Venus-IRES-bFGFmyc/pMSCV and bFGFmyc-IRES-Venus/pMSCV, respectively. In order to generate the mutant bFGF protein that does not go to nucleus, in which both 118th Arg and 119th Lys were changed to Gly, we introduced mutations by using QuickChange II XL Site-Directed mutagenesis kit (Stratagene) according to the manufacture's instructions. The mutagenesis was done by using bFGF-myc/pEF1. The primers used were CAATACTTA CCGGTCAGGGGGGTACACCAGTTGG and CCAACTGG TGTACCCCCCTGACCGGTAAGTATTG. The mutagenesis resulted in bFGF(mut118119)-myc/pEF1. The bFGF(mut118119)-myc fragment was subcloned into Venus-IRES/pCl and IRES-Venus/pCl to make Venus-IRES-bFGF(mut)myc/pCl and bFGF(mut)myc-IRES-Venus/pCl, respectively. The bFGF(mut118119)-myc and Venus-IRES fragments were subcloned into pMSCV to make Venus-IRES-bFGF(mut)myc/pMSCV. The constructs were checked by sequencing using 3100 Genetic Analyzer (Applied Biosystems).

Immunohistochemistry—Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and incubated with PBT (0.4% Triton-X 100 and 0.5% bovine serum albumin in PBS) for more than 30 min. The cells were incubated with primary monoclonal antibodies at room temperature for 2 h. Primary antibody against the myc tag sequence (9E10)(Upstate) was used at a dilution of 1:200. The samples were then rinsed three times with PBS and incubated for 1 h at room temperature with either Alexa488 (Invitrogen) or Cy3 (Chemicon) conjugated secondary antibodies at a dilution of 1:250–1:500. After mounting in 90% glycerol, the samples were examined with an Olympus IX70 fluorescence microscope (Olympus).

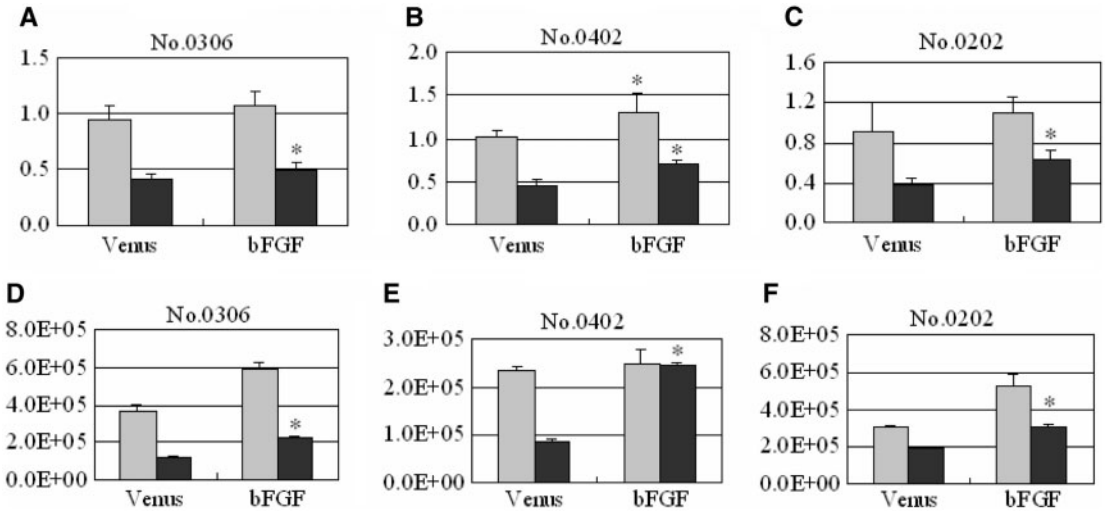

Fig. 1. **Proliferation activities of Venus- and bFGF-expressing MSCs.** Proliferation activities of human MSCs infected with either construct, Venus or Venus-IRES-bFGF. The cells were cultured in the presence of either 10% (light gray) or 1% (dark gray) FBS. The results of WST assay (OD450 nm) are shown in **A–C** ($n = 8$), and those of cell number count are in **D–F** ($n = 2$). The cells (No. 0306, 0402 and 0202, passage 5: P5) were seeded at 2×10^3 cells/well in an either 24- or 6-well plate. Measurement was done when the cells reached near confluence. Asterisk indicates a significant difference compared to the control (t-test: $P < 0.01$).

Statistical Analysis—Values are expressed as average and standard deviations. The unpaired Student's t-test was used for comparisons of the parameters between two groups. P values <0.01 were considered statistically significant.

RESULTS AND DISCUSSION

Forced Expression of bFGF in MSCs by Retroviral Infection—With the aim of conferring higher expansion potentials on human bone marrow MSCs, we introduced the bFGF gene into the cells. In order to achieve high efficiency of gene introduction and subsequent stable expression, we employed a retrovirus system. We used the construct in which IRES sequence was placed between the gene of interest and the Venus gene, a variant of EGFP, so that expression of the construct was easily detectable during the cell culture. We found, however, that translation efficiency of genes after IRES sequence was so low in bone marrow MSCs that fluorescent signal of the Venus protein was barely detectable when the Venus gene was placed after IRES sequence. We therefore mainly analysed the MSCs in which the gene of interest was placed after IRES sequence so that infection efficiency was monitored with ease. In most cases, we infected P2 bone marrow MSCs twice, and continued selection culture with G418 for two to three passages. We thereby obtained the gene-expressing cell population in which the majority of the whole cells expressed the Venus protein, which was confirmed by FACS analysis (data not shown).

Proliferation Activity of bFGF-Expressing Cells—To evaluate proliferation potential of the cell population, we either simply counted the number of cells or conducted WST assay. In most cases, we evaluated growth ability of a particular cell population in both high and low serum conditions. We found in both conditions that bFGF-expressing MSCs had higher proliferation activity than control cells, in which only Venus was expressed (Fig. 1A–F). The positive effect of forced expression of bFGF on cell growth was particularly significant in the case of a low serum condition.

Effect of Forced-Expression of bFGF is Distinct from that of Addition of bFGF in Culture Media—We next conducted cell-growth assay by counting cell number for several passages, and found that bFGF-expressing MSCs showed significantly higher cell growth than control cells, particularly in late passages and a low serum condition (Fig. 2A and B). In order to see if we were able to observe similar effects on cell proliferation, we added the bFGF protein in culture media. We found, however, that addition of bFGF (1 or 10 ng/ml) in culture media had negative effects on cell growth (Fig. 2A and B). Since the concentration of secreted bFGF protein from MSCs into media could be very low, we added the bFGF protein of less than 1 ng/ml for final concentration to see the effects of addition of bFGF in culture media on cell proliferation. We also added a high concentration of bFGF (50 ng/ml) in culture media, and conducted WST assay to examine the cell proliferation activity in each case. We failed, however, to observe similar positive effects on cell proliferation by adding the bFGF protein in culture media (Fig. 3A).

Effects of exogenously added bFGF protein on MSCs were conspicuous in terms of cell morphology. Characteristic morphology changes of MSCs in the presence of bFGF in culture media were consistently observed. MSCs become elongated shape with occasional narrow cell protrusions, and consequently take on more complex morphology with sharp contours after the addition of bFGF in culture media (Fig. 3B).

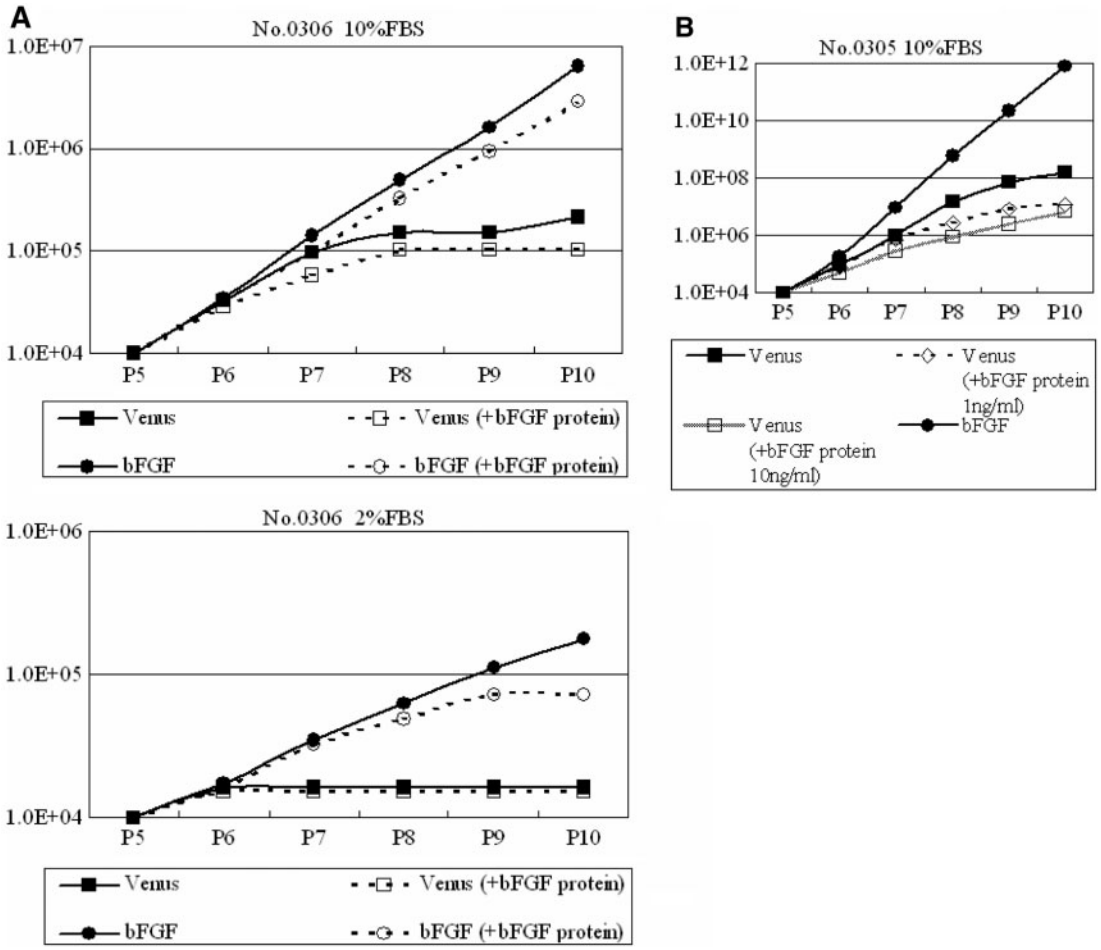

Fig. 2. **Proliferation activities of Venus- and bFGF-expressing MSCs with or without addition of the bFGF protein in culture media.** Proliferation activities of human MSCs infected with either construct, Venus or Venus-IRES-bFGF. (**A**) MSCs (No. 0306, P5) were cultured in the presence of either 10 or 2% FBS, with or without addition of bFGF (10 ng/ml). (**B**) MSCs (No. 0305, P5) were cultured in the presence of 10% FBS, with or without addition of bFGF (1 or 10 ng/ml). In both cases, the cells were seeded at 1×10^4 cells/well in a 6-well plate, and the cell number was counted at every passage (5 times until P10) ($n = 3$). The passage number of the cells is indicated.

Interestingly, bFGF-expressing cells never showed such morphology changes (Fig. 3Bg and i). However, the cells responded similarly to exogenously added bFGF by showing the characteristic morphology changes (Fig. 3Bh and j). Taken together, these observations demonstrated that the effects of forced-expression of bFGF in MSCs were clearly distinct from those of addition of the bFGF protein in culture media.

In order to check a possibility that the bFGF protein was modified post-translationally in MSCs, we tried the conditioned medium of bFGF-expressing cells for culture of non-infected MSCs. We failed, however, to observe any significant difference in terms of effects on growth of MSCs between conditioned medium of bFGF-expressing and non-infected MSCs (data not shown).

We also analysed differentiation potential of bFGF-expressing cells for osteoblasts. Although we did not observe consistent effects of forced expression of bFGF on osteogenic differentiation, some of the bFGF-expressing cells showed mineralization as well as high alkaline phosphatase activity in osteogenic differentiation media, indicating that the cells had intrinsic osteogenic differentiation capability (Supplementary Fig. 1).

Localization of the bFGF Proteins Expressed from Transgenes in MSCs—We next made the constructs, in which myc tag sequence was put at the C-terminus of the bFGF protein, so that we were able to analyse localization of the bFGF proteins expressed from the constructs. When we introduced the bFGF gene with myc tag sequence transiently in MSCs, we found the bFGF protein expressed from the transgene mainly in the nucleus (Fig. 4D). This observation was unexpected because we used the shortest isoform of bFGF that was considered to be mostly cytosolic. We did not consider this observation an artifact due to the forced over-expression of the transgene for the following reasons. First, we changed two amino acids in the region that were considered to be critical for nuclear localization of the bFGF protein (*13*), and did the same transfection experiment. We observed cytosolic staining as dots in the case of this mutant protein (Fig. 4E). Second, we also observed similar nuclear localization of the bFGF protein

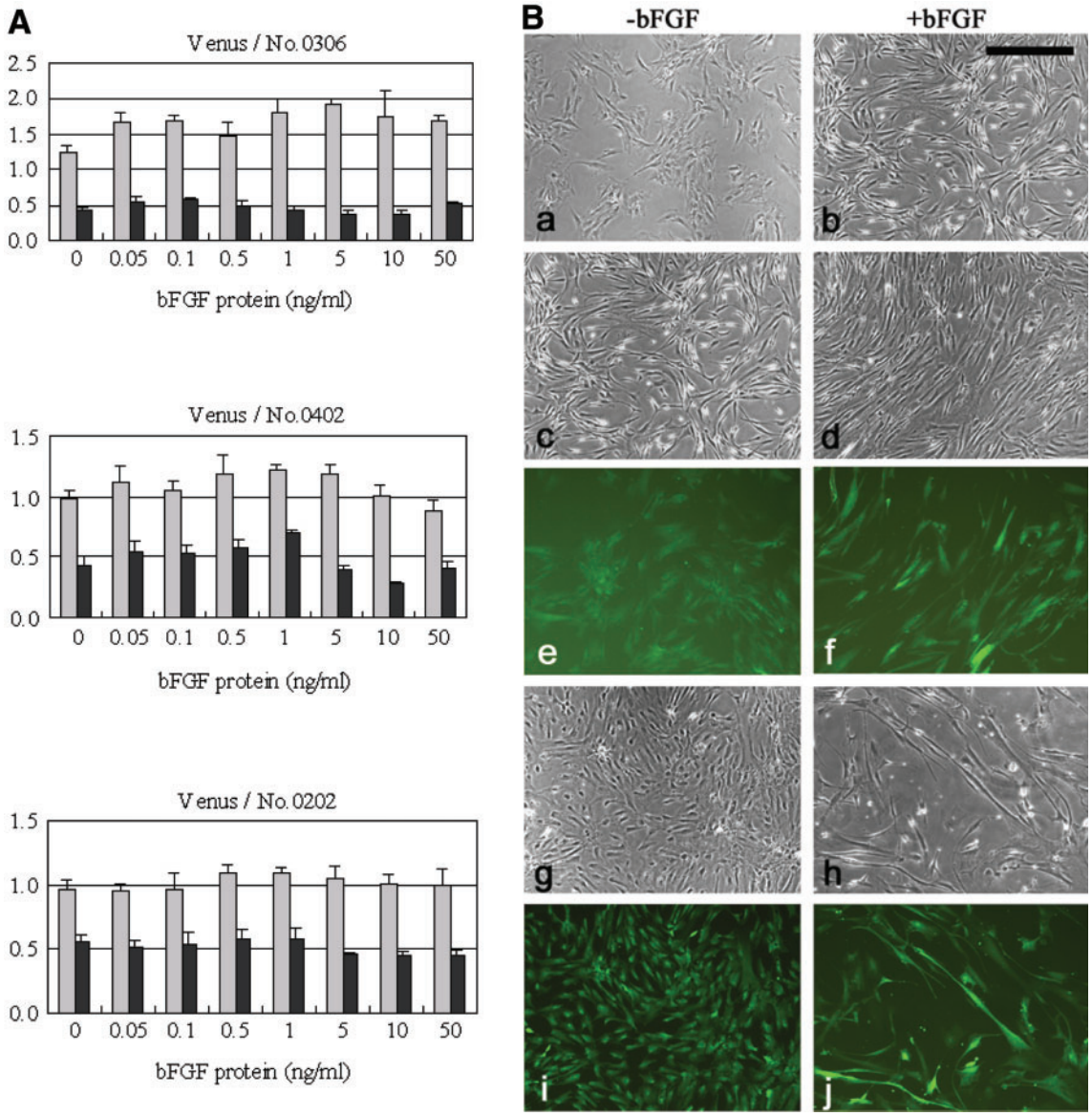

Fig. 3. **Effect of forced expression of bFGF in MSCs is distinct from that of addition of the bFGF protein in culture media.** (**A**) Proliferation activities of Venus-expressing MSCs with a wide range of concentration of the bFGF protein in culture medium. The results of WST assay (OD450 nm) are shown ($n = 8$). MSCs were cultured in the presence of either 10% (light gray) or 1% (dark gray) FBS. Absorbance was measured several days after the cells (No. 0306, 0402 and 0202, cryopreserved P5) were seeded at 2×10^3 cells/well in a 24-well plate. The final concentration of bFGF ranged from 0 to 50 ng/ml. (**B**) Morphology of Venus- and bFGF-expressing MSCs are shown. (**a**–**d**, **g**, **h**) phase-contrast image, (**e**, **f**, **i**, **j**) fluorescent image of the Venus protein. (**a**, **b**) non-infected MSC, (**c**–**f**) control MSCs (Venus), (**g**–**j**) bFGF-expressing MSCs (Venus-IRES-bFGF). bFGF was not added in left column (**a**, **c**, **e**, **g**, **i**), and bFGF(10 ng/ml) was added in culture media in right column (**b**, **d**, **f**, **h**, **j**). In every case, addition of bFGF in culture medium resulted in morphology changes of MSCs, where the cells became elongated shape with occasional narrow cell protrusions. Scale bar in $b = 200\ \mu m$.

in the case of stable expression by retrovirus (Fig. 4F). When the bFGF gene was placed after the IRES sequence, however, we could barely detect the protein expression by antibody staining (data not shown). Since we could clearly detect the protein in the nucleus in the case of transient expression even when the bFGF gene was placed after the IRES sequence (Fig. 4D), we consider the protein expression level very low in the case of stable expression when the bFGF gene is placed after the IRES sequence. We also note that we did not observe consistent difference in terms of effect on cell growth between two constructs, Venus-IRES-bFGF and bFGF-IRES-Venus (data not shown), suggesting that the protein expression level from transgenes was not critical for the effect.

It has already been reported that this 18 kDa bFGF is also found in the nucleus in various cells, raising a possibility that this shortest isoform is also involved in intracrine signalling events (*14*). It is not clear at present whether the bFGF protein was once secreted from the

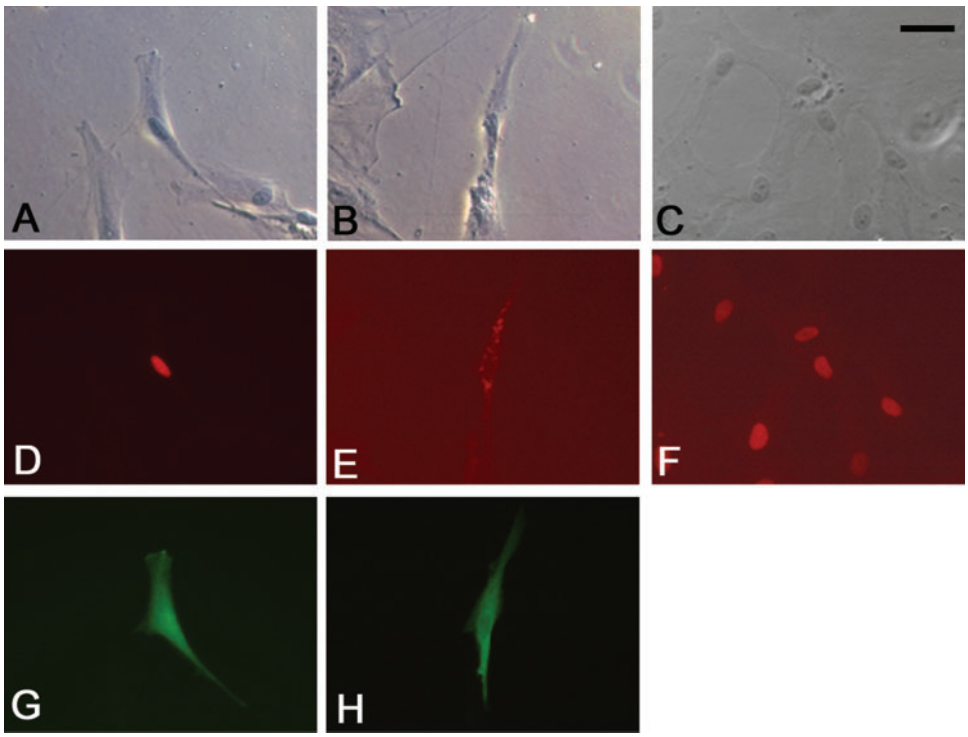

Fig. 4. **Localization of the bFGF proteins expressed from the transgenes in MSCs.** Localization of the wild-type and mutant bFGF proteins in human MSCs. The wild-type bFGF gene was introduced by either transfection with an expression vector (**A**, **D**, **G**) or infection with a retroviral vector (**C**, **F**). The mutant bFGF gene was introduced by transfection with an expression vector (**B**, **E**, **H**). The C-terminus of the bFGF genes was tagged with a myc sequence so that the proteins expressed from the constructs could be detected unambiguously. (**A**–**C**) phase-contrast image, (**D**–**F**) antibody staining to the myc tag, (**G**, **H**) antibody staining to the Venus protein. The results of wild-type bFGF are shown in left and right columns (**A**, **C**, **D**, **F**, **G**), those of mutant bFGF are in middle column (**B**, **E**, **H**). Scale bar in $C = 100\,\mu m$.

membrane or translocated to the nucleus directly in our experiments.

Proliferation Activity of the Mutant bFGF-Expressing Cells—We conducted cell-growth assay to see effect of forced expression of the mutant bFGF protein on cell proliferation of MSCs. We found that proliferation potential of MSCs expressing the mutant bFGF protein was as high as that of MSCs expressing the wild-type bFGF protein (Fig. 5). These results indicated that the nuclear localization itself was not essential for the effect of bFGF expression on cell proliferation. We also found that there was essentially no difference between the wild-type and mutant bFGF-expressing MSCs in terms of response to exogenously added bFGF (data not shown), indicating that forced-expression of the mutant bFGF in MSCs also did not mimic the effects of exogenously added bFGF in culture media.

It has also been previously shown that bFGF mitogenic and differentiation activities can be dissociated by a single-point mutation that impairs the interaction with casein kinase 2, which is a ubiquitous serine/threonine kinase in the nucleus (*24*, *25*). Our results indicate, however, a different possibility that localization of the bFGF proteins in the nucleus is not essential for the function of bFGF in terms of cell proliferation. There are at least two families of receptors that can bind bFGF and mediate its response: [1] tyrosine kinase-containing FGF receptors, designated FGFR-1 to FGFR-4, and [2] heparan sulfate proteoglycans that bind bFGF through their heparan sulfate chains. It has been shown that the bFGF proteins that bound to each receptor or both receptors can be internalized via different pathways and undergo different fates (*26*). Our results demonstrate not only an effective way to maintain proliferation potentials of MSCs, but also a possibility that there may be mechanistic and functional difference in the receptor activation and subsequent signal transduction events between endogenously expressed and exogenously added bFGF protein for MSCs.

SUPPLEMENTARY MATERIAL

Supplementary material is available at JBIOC online.

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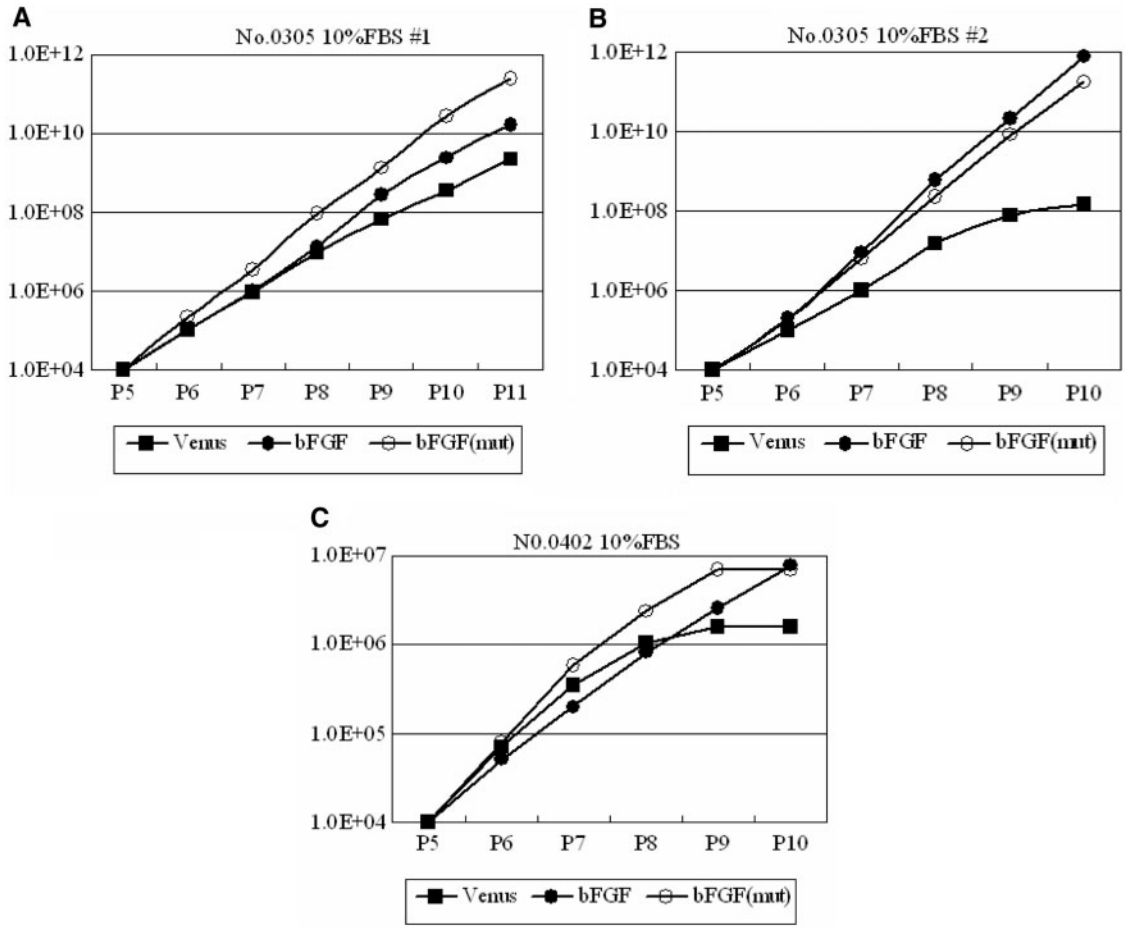

Fig. 5. **Proliferation activities of wild-type or mutant bFGF-expressing MSCs.** Proliferation activities of human MSCs infected with one of the following constructs, Venus, Venus-IRES-bFGF, Venus-IRES-bFGF(mut). MSCs were cultured in the presence of 10% FBS. The cells (No. 0305 and 0402, P5) were seeded at 1×10^4 cells/well in a 6-well plate, and the cell number was counted at every passage (5 times until P10 in A, 6 times until P11 in B, 5 times until P10 in C) ($n = 3$). The passage number of the cells is indicated. In all cases, proliferation activity of the mutant bFGF-expressing cells was as high as that of the wild-type bFGF-expressing cells. For No. 0305, the results in A and B are from different experiments, in which the MSCs were infected independently.

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