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In vitro transformation of mesenchymal stem cells by oncogenic H-*ras*^{Val12}

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Abstract

Tissue stem cells may serve as progenitors for malignant tumors derived from the same tissue. Here, we report the establishment of immortalized human mesenchymal stem cells (ihMSC) and tested the feasibility of using ihMSC as presarcomatous cells. Immortalization was achieved by introducing the genes for human telomerase reverse transcriptase and Bmi1. ihMSC retained the potential for multidirectional differentiation of the original MSC. To transform ihMSC, we introduced an oncogenic H-*ras*^{Val12} gene, and established the cell line ihMSC-*ras*. ihMSC-*ras* had the phenotype of fully transformed cells and retained adipogenic and chondrogenic, but not osteogenic, potential. Interestingly, ihMSC-*ras* demonstrated morphological features of autophagy, and inhibition of the ERK pathway suppressed the production of autophagosomes, indicating that ras/ERK signaling is responsible for the induction of autophagy. Thus ihMSC will serve as a material with which to analyze the tumorigenic and differentiation-modifying effects of candidate oncogenes involved in the development of sarcomas.

Keywords: Mesenchymal stem cell; TERT; Bmi1; *ras*; Transformation; Autophagy; Differentiation

Sarcomas are defined as malignant tumors derived from non-epithelial tissues, but the precursors of sarcoma cells are equivocal in most cases. For example, osteosarcomas, the most common malignant bone tumor, are defined as tumors producing immature bone tissue called osteoid. This definition, however, does not mean that the precursors of osteosarcomas are cells committed to the osteoblastic lineage. One major histological subtype of osteosarcoma is the chondroblastic osteosarcoma, in which cartilaginous tissues are formed directly by tumor cells expressing a number of cartilage-related genes [1], suggesting that cells of this type of osteosarcoma have the potential to differentiate in at least two directions. Most osteosarcomas develop from bone marrow, and therefore bone marrow stromal cells are reasonable candidates for the precursors of osteosarcomas, and the most plausible candidate cells are mesenchymal stem cells (MSCs). MSCs are defined as cells with the potential to differentiate into a variety of mesenchymal cell lineages [2], and therefore have the potential to be the precursor of a variety of mesenchymal tumors, although there have been no reports showing the development of a particular type of sarcoma from MSC. Considering that transformation requires the immortalization step, immortalized

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human MSC as presarcomatous cells would be a suitable material with which to test the oncogenic potential of candidate genes.

In this study, we tried to immortalize hMSC by inactivating the $p16^{INK4A}$ gene using the Bmi1 gene. Bmi1 was first identified as an oncogene that cooperates with c-myc in the generation of mouse lymphomas [3]. Bmi1 is a nuclear protein which makes a complex with other polycomb group proteins and binds to polycomb response elements to inhibit the transcription of target genes including the $p16^{INK4A}$ gene [4]. A recent report stated that Bmi1 is required for the self-renewal of stem cells in the peripheral and central nervous systems [5]. The Bmi1 gene is also thought to regulate the replicative life span of human fibroblasts [6] and recent reports described the immortalization of human ovarian surface epithelial cells [7] and mesenchymal stem cells [8].

Here, we established immortalized human MSC (ihMSC), which retained the potential for multi-directional differentiation of the original cells, and tested the feasibility of using ihMSC as presarcomatous cells.

Materials and methods

Cell culture and reagents. Adult hMSC were purchased from Bio Whittaker, and cultured in MSC growing medium (Bio Whittaker). EJ and Saos2 were obtained from American Type Culture Collection, and Ampho293 was obtained from Clontech and cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma–Aldrich) with 10% fetal bovine serum (FBS, Hyclone).

Antibodies against phospho-p44/42 MAPK (p-ERK1/2), pan-ERK, phospho-p38 (p-p38), pan-p38, and $p16^{INK4A}$ were purchased from BD Biosciences. The antibody against human LC3 [9] was kindly provided by Dr. Mizushima (National Institute for Basic Biology, Japan). The MEK inhibitor U0126 was obtained from Promega.

Reverse transcription (RT)-PCR. Total RNA was extracted from cells and pellets using an RNAse Easy Mini Kit (Qiagen), and reverse-transcribed with the Superscript III first strand system (Invitrogen). The synthesized cDNA was used as a template for each PCR, and the products were electrophoresed on 1.5–2% agarose gels and visualized by ethidium bromide staining. Information on the primer pairs used in the RT-PCR is available upon request.

Retroviral vectors and infections. Retroviral vectors expressing hTERT and the Bmi1 gene were previously described [10]. A retroviral vector expressing oncogenic H-ras^{Val12} was constructed using RNA derived from the bladder carcinoma cell line EJ, which contained an oncogenic H-ras^{Val12} gene [11]. After the reverse transcription, the entire coding region of the H-ras^{Val12} gene was amplified and first cloned into a TA vector (Invitrogen), plasmids containing the correct sequence were selected, and the subcloned into a retroviral vector pQCXIP-IRES-PURO (Clontech).

Amphotropic retroviruses were produced by transfection of the ampho293 producer cell lines with each retroviral vector using lipofectamine 2000 (Invitrogen). Cells were infected with amphotropic retroviruses in 6 μg/ml of polybrane, and purified by selection with 100 μg/ml of hygromycin B (Sigma–Aldrich) or 100 μg/ml of G418 (Sigma–Aldrich).

Western blotting. SDS-sample buffer was added directly to the dishes, and cells were stripped with a cell scraper, and the cell lysate was then sonicated. Total protein (20 μg) was electrophoresed on SDS polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). The membranes were blocked using a 5% dried milk powder, and incubated with the antibody against each protein. They were then washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) and HRP-conjugated swine anti rabbit IgG (DAKO) and detected using enhanced chemiluminescence plus reagents (Amersham Pharmacia Biotech).

Northern blot analysis. Total RNA was isolated from cells using Sepasol reagent (Nacalai tesque). Ten micrograms of total RNA was separated on a 1% agarose–formaldehyde gel, and blotted on to a nylon membrane, and hybridized with ^{32}P-labeled probes. A part of the cDNA of the human H-*ras* gene (NM005343) was amplified by RT-PCR and used as a probe.

Telomere length and telomerase assay. Telomere length was measured as described previously [12]. The telomerase activity in cells was detected with the telomeric repeat amplification protocol (TRAP) as described previously [12] using a Telo Chaser Kit (Toyobo).

Transmission electron microscopy. Cells were fixed with ice-cold 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0), and thin sections (70–80 nm) were prepared and stained with uranyl acetate and lead citrate and observed using a transmission electron microscope.

G-banding karyotypic analysis. Metaphase spreads were prepared from cells treated with colcemid, and 50 metaphases were analyzed by the standard G-banding method.

Phenotype analyses

Proliferation assay. Cells (1×10^4) were seeded in 60-mm culture dishes in triplicate, and cultured in DMEM with 1% or 10% FBS. The number of cells in each dish was counted by hemocytometer at each time point.

Soft agar assay. Cells (1×10^4) were suspended in 0.35% low-melting point agarose dissolved in DMEM with 10% FBS, and seeded in 60-mm culture dishes precoated with 0.7% agarose. After 2 weeks, colonies in three random fields of view at 200× magnification were scored. Experiments were performed in triplicate.

Matrigel invasion assay. Cell suspensions (2.5×10^4/0.5 ml DMEM) were added into the upper wells of 24-well chambers (BD Biosciences). After incubation for 22 h, invading cells were fixed with 100% methanol and stained with 1% Toluidine blue. Cells in five random fields of view at 200× magnification were counted and expressed as the average number of cells per field of view. Data were expressed as the percent invasion through the matrigel membrane relative to the migration through the control membrane.

In vivo tumor formation. Cell suspensions containing 5×10^6 cells in 100 μl of PBS were injected subcutaneously into immunodeficient mice (BALB/c nu/nu). Three mice were used for each cell type. Tumor volume was calculated with the formula $4/3\pi\ r^3$.

Differentiation assay. Adipogenic, osteogenic, and chondrogenic differentiation were performed as previously described [12], and Oil-Red-O staining, Alizarin red staining, and Alcian blue staining were used to evaluate the differentiation as previously described [12].

Results

Establishment of Bmi1-hTERT-immortalized hMSC

After the sequential introduction of the hTERT and Bmi1 genes into hMSC, drug-resistant cells were selected and propagated. This polyclonal cell line, designated hMSC-Bmi1-hT, grew much faster than the original hMSC with a doubling time of 48 h, and showed no signs of entering senescence after more than 24 months. The telomerase activity of hMSC-Bmi1-hT was comparable with that of Hela (Fig. 1A), and the telomere length was maintained even in late passaged cells (Fig. 1B). Expression of the exogenous Bmi1 gene was confirmed by Northern blotting (Fig. 1C), and the expression of the $p16^{INK4A}$ gene was markedly downregulated at both the mRNA and protein level (Fig. 1D). This immortalized line may consist of cells

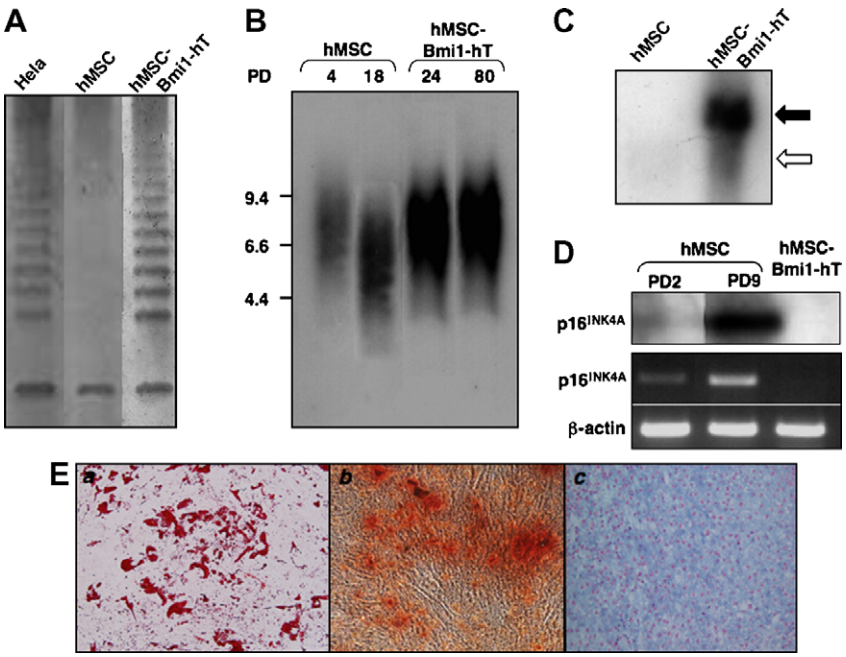

Fig. 1. Establishment of immortalized hMSC. (A) Telomerase activity of immortalized hMSC (hMSC-Bmi1-hT). (B) Telomere length of hMSC-Bmi1-hT in early and late passaged cells. (C) Expression of the exogenous Bmi1 gene. Closed and open arrows indicate the exogenous and endogenous Bmi1 gene, respectively. (D) Downregulation of $p16^{INK4A}$ expression at the mRNA and protein level in hMSC-Bmi-hT. (E) Potential of ihMSC to differentiate. Oil-Red-O, Alizarin red, and Alcian blue staining were performed after adipogenic, osteogenic, and chondrogenic induction, respectively.

differing in the potential to differentiate, as we previously found in a study of hMSC immortalized by the HPVE6/E7 genes [12]. Single cell cloning by the limited dilution method using 96-well culture plates was performed, and a total of 100 clones were isolated, and the potential of each clone to differentiate was analyzed. Five clones showed adipogenic, osteogenic, and chondrogenic differentiation potential, one of which was randomly selected for further study. The differentiation potential of this clone, hereafter called ihMSC, was stable even 24 months after cloning (Fig. 1E).

In vitro transformation of ihMSC by oncogenic H-ras^{Val12}

To test the potential of ihMSC to serve as cells for the screening of candidate oncogenes, the H-*ras*^{Val12} gene was introduced by a retroviral vector. The transduction of retroviral vectors expressing the oncogenic H-*ras*^{Val12} gene into ihMSC was independently performed in two experiments. Drug selection was started 48 h after the transduction, and cells were cultured and propagated until they grew stably. Polyclonal cell lines for each experiment were established, and designated ihMSC-*ras*-1 and ihMSC-*ras*-2. As a control, Mock-transfected cells were also established (ihMSC-Mock-1 and ihMSC-Mock-2). Expression of the exogenous H-*ras*^{Val12} gene was confirmed by Northern blotting (Supplementary Fig. 1). The karyotypic analysis of ihMSC-*ras*-1 showed that the dominant mode (39/50 metaphase) was (46, XY) with a marker chromosome (Supplementary Fig. 2). The doubling-time of the ihMSC-*ras* under standard (10% FBS) and serum-starved (1% FBS) conditions was 18.3 and 30.0 h, respectively, shorter than that of ihMSC (26.2 and 38.6 h). The anchorage-independent growth potential of ihMSC-*ras* was confirmed by performing a colony formation assay in soft agar (Fig. 2A). The motility of ihMSC-*ras* was significantly increased compared to that of ihMSC and ihMSC-Mock (Fig. 2B). Although the number of cells capable of invading the matrix was small, there was a significant difference between ihMSC-*ras* and ihMSC-Mock (Fig. 2C). The innoculation of ihMSC-*ras* into subcutaneous tissue in nude mice produced rapidly growing tumors with a frequency of 100% (Fig. 2D), the histological diagnosis being undifferentiated spindle cell tumors with no particular features (Fig. 2E). These results indicated that the introduction of the H-*ras*^{Val12} gene was able to completely transform ihMSC into undifferentiated sarcoma cells.

Induction of autophagy by oncogenic H-ras^{Val12}

There was a clear morphological difference val12 between the ihMSC and ihMSC-*ras* lines. Both ihMSC-*ras*-1 (Fig. 3A, b) and *ras*-2 (data not shown) had an abundance of cytosolic vacuoli, which were not found in ihMSC (Fig. 3A, a), ihMSC-Mock-1 or -2 (data not shown).

Transmission electron microscopic analysis revealed these vacuoli to be secondary lysosomes containing residual bodies (Fig. 3B), a feature of autophagy [13]. LC3 (microtubule-associated protein 1 light chain 3) is a human homologue of Atg8, which is a key molecule in the

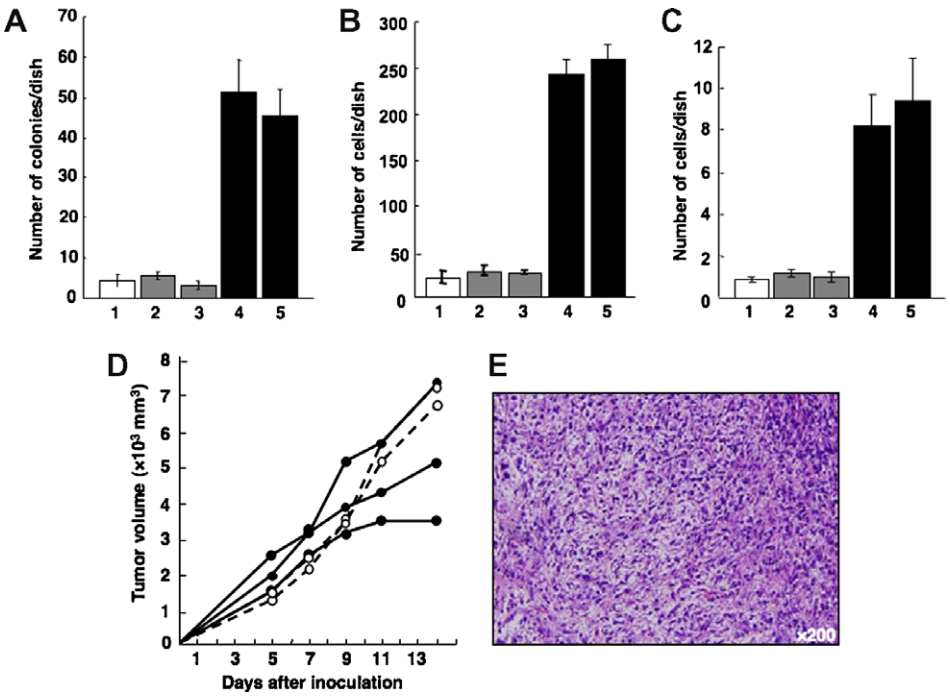

Fig. 2. Oncogenic property of ihMSC-*ras*. (A) Anchorage-independent growth. (B) Cell motility assay. (C) Cell invasion assay. Samples in each lane were: lane 1, ihMSC; lane 2, ihMSC-Mock-1; lane 3, ihMSC-Mock-2; lane 4, ihMSC-*ras*-1; and lane 5, ihMSC-*ras*-2. (D) Growth curve of *in vivo* tumors. Closed circles, ihMSC-*ras*-1 (three mice); open circles, ihMSC-*ras*-2 (two mice). (E) Histology of *in vivo* tumors produced by ihMSC-*ras*-1.

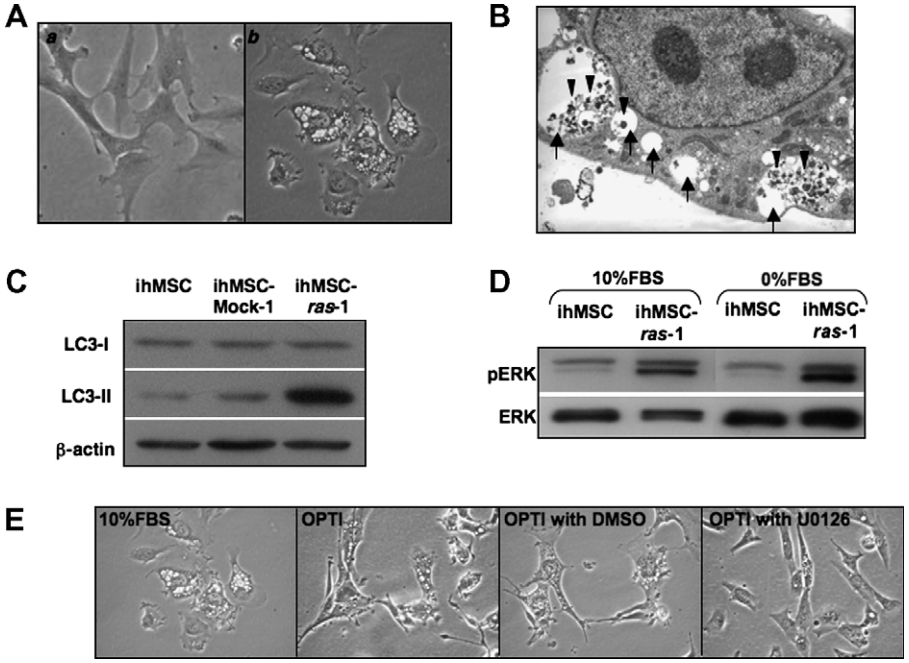

Fig. 3. Induction of autophagy by oncogenic H-*ras*^{Val12} in ihMSC. (A) Phase contrast micrographs of ihMSC (a), and ihMSC-*ras*-1 (b). (B) Transmission electron micrographs of ihMSC-*ras*-1. Arrows and arrowheads indicated secondary lysomes and residual bodies, respectively. (C) Western blot for LC3. Cell lysate was prepared from each cell line. (D) Expression of ERK1/2 in ihMSC lines. (E) Phase-contrast micrographs of ihMSC-*ras*-1 with or without treatment with U0126.

development of autophagosomes in yeast [14]. A C-terminal fragment of LC3 is cleaved immediately after synthesis, producing a cytosolic form known as LC3-I, and a subpopulation of LC3-I is further converted to an autophagosome-associating form, LC3-II [14]. Western blot analysis using the antibody against LC3 clearly showed the induction of LC3-II in ihMSC-*ras*-1 (Fig. 3C), and ihMSC-*ras*-2 (data not shown).

The mitogen-activated protein kinase (MAPK) pathway is the major pathway transferring the *ras* signal, in which three different routes are known; the ERK, p38, and c-jun N-terminal kinase (JNK) pathways [15]. The activity of these MAP kinases was examined under standard or low serum conditions (Fig. 3D). The amount of phosphorylated ERK (pERK) was much greater in ihMSC-*ras* than in ihMSC under both standard and serum-free conditions (Fig. 3D). The amount of phosphorylated p38 (pp38) showed no significant difference between ihMSC and ihMSC-*ras* (Supplementary Fig. 3), and hardly any phosphorylated JNK was observed in any cell line (data not shown). Therefore, the expression of the oncogenic *ras* gene in ihMSC induced the phosphorylation of ERK in a serum-independent manner. U0126, a specific inhibitor for MEK, a kinase of ERK, was used to inhibit the ERK activity in the hMSC-*ras* lines. All three cell lines showed a reduction in the expression of pERK after the treatment with U0126 (Supplementary Fig. 4). The morphology of ihMSC and ihMSC-*ras* was compared before and after the treatment. ihMSC showed no remarkable morphological change after the treatment with U0126 (data not shown), whereas the number of ihMSC-*ras* cells with autophagosomes was significantly reduced (Fig. 3E) These results indicated the induction of autophagy by oncogenic H-ras^{Val12}.

Differentiation potential of ihMSC-ras

Adipogenic differentiation

After the induction, ihMSC-*ras*-1 showed Oil-Red-O-positive droplets (Fig. 4A, a) as did ihMSC (Fig. 1E, a). In the case of ihMSC, the expression the PPARγ gene was silent in the non-induced state, and became positive after the adipogenic induction, whereas ihMSC-*ras* expressed the PPARγ gene without the induction in association with the AP2 gene, which was one of the genes downstream of the PPARγ gene (Fig. 4B). In contrast, the expression of the adipsin gene was not induced in ihMSC-*ras* (Fig. 4B).

Osteogenic differentiation

The osteogenic differentiation of ihMSC-*ras*-1 was significantly inhibited. ihMSC ras-1 formed almost no alizarin-red-positive calcified nodules (Fig. 4A, b), which were abundantly observed in ihMSC (Fig. 1E, b). As for the mRNA expression, the most striking difference between ihMSC-Mock-1 and ihMSC-*ras*-1 was in the gene expression of osteocalcin (OC) and its regulator, OSF2/RUNX2 (Fig. 4B). Under standard culture conditions, ihMSC-Mock-1 expressed the OSF2/RUNX2 gene weakly, which was evoked by the osteogenic induction (Fig. 4B). No expression of the OSF2/RUNX2 gene, however, was observed in ihMSC-*ras*-1 and the expression of the OC gene also remained suppressed (Fig. 4B).

Chondrogenic differentiation

After 3 weeks of chondrogenic induction, the ihMSC-*ras*-1 produced a solid pellet in the three-dimensional culture, which contained matrices positive for Alcian blue staining (Fig. 4A, c). The gene expression of cartilage oligomeric matrix protein (COMP), aggrecan (AGC), and to a lesser extent, α1 chain of type 2 collagen (COL2A1) was upregulated in hMSC-*ras* (Fig. 4B). These results suggested

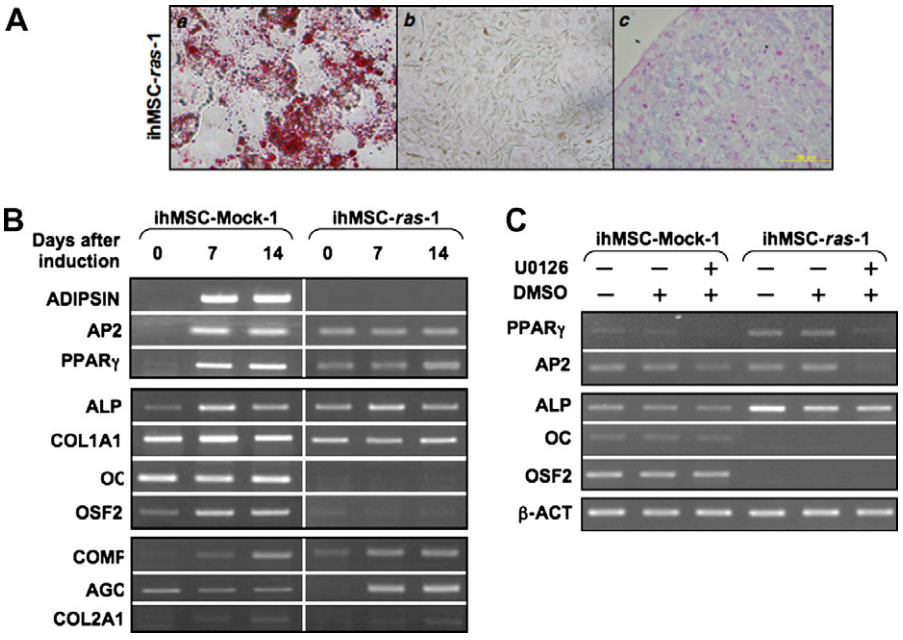

Fig. 4. Potential of ihMSC-*ras* to differentiate. (A) Lineage-related histochemical staining after the induction of each lineage. (B) mRNA expression of lineage-related genes after the induction of each lineage in ihMSC-Mock and ihMSC-*ras*. (C) mRNA expression of adipogenic and osteogenic lineage-related genes in ihMSC-Mock and ihMSC-*ras* with or without treatment with U0126.

that the oncogenic H-ras^{Val12} protein had no significant effect on the chondrogenic differentiation.

The mRNA expression of adipo- and osteo-related genes in cells treated with U0126 was analyzed by RT-PCR (Fig. 4C). The expression of the PPARγ and AP2 genes in ihMSC-*ras* was reduced when the cells were treated with U0126 (Fig. 4C), whereas no restoration of the expression of the OSF2/RUNX2 and OC genes was observed (Fig. 4C). These results suggested that the acceleration of adipogenic differentiation was mediated through the ERK pathway, but the loss of osteogenic potential was caused by other pathways. Identical results were obtained in ihMSC-ras-2 (data not shown), suggesting the constant effect of H-ras^{Val12} for the differentiation potential of ihMSC.

Discussion

As far as we know, this is the first report of the transformation of hMSC *in vitro*. The result was compatible with a previous spontaneous transformation of hTERT-introduced hMSC, in which p16^{INK4A} was inactivated by a deletion and the K-*ras* gene was mutated [16].

The transformation of ihMSC by the H-*ras*^{Val12} gene revealed two interesting findings. First, the oncogenic H-*ras*^{Val12} induced, to our surprise, autophagy in ihMSC. Autophagy is a critical process responsible for the degradation of intracellular material by a membrane of uncertain origin to form an autophagosome that sequesters such material and subsequently fuses with the lysosome [13], and involved in physiological processes as well as pathological conditions such as neurodegenerative disease, cardiomyopathies, and cancer [17]. Because autophagy is suppressed in most cancer cells, autophagy-related genes may work as tumor suppressors [18]. In some types of cancer cells, however, autophagy was activated as a defense mechanism to protect against poor nutrition in tumor tissues [18]. Therefore, it is not clear whether the autophagy observed in ihMSC-*ras* reflected the process of transformation or not. However, because this feature was constantly observed in two independently established cell lines, it might be used as a morphological feature to detect potentially transformed cells *in vitro*. The relationship between the *ras* signal and autophagy is controversial. Raf-1 is an effector for MAPK signaling pathways, which constitute three routes; ERK, p38, and JNK [19]. Activation of raf-1 induced autophagy in colon cancer HT-29 cells through the activation of ERK [19], indicating that the *ras* signal stimulates autophagy. On the other hand, the introduction of the H-*ras*^{Val12} gene into NIH3T3 reduced the autophagy stimulated by nutrient starvation through the activation of class I PI3-K [20]. Thus the PI3-K pathway and MAPK pathway exert opposite effects on autophagy, and the effect of the activation of ras may depend on cell specificity. We have shown that the introduction of H-*ras*^{Val12} into ihMSC induced autophagy through the activation of the ERK pathway without growth inhibition.

Second, the H-*ras*^{Val12}-mediated transformation completely abolished the osteogenic potential of ihMSC. OSF2, an osteoblast-specific form of the RUNX2 transcription factor, is shown to play a central role in determining osteogenic and adipogenic differentiation. OSF2/RUNX2−/− chondrocytes differentiate spontaneously *in vitro* into adipogenic cells expressing the PPARγ gene, and introduction of the OSF2/RUNX2 gene inhibited the expression of the PPARγ gene [21]. On the other hand, PPARγ activated by its ligand inhibited the DNA-binding activity of OSF2/RUNX2, and therefore the gene expression of its targets such as OC [22]. In this report, activated *ras* induced the expression of the PPARγ gene and accelerated the adipogenic differentiation, which mimicked the effect of knocking out OSF2/RUNX2. However, inhibition of the ERK pathway by U0126 inhibited the expression of PPARγ without restoring the expression of the OSF2/RUNX2 gene, suggesting that the induction of PPARγ expression by H-*ras*^{Val12} was independent of the inactivation of OSF2/RUNX2. Because activated mutations of *ras* genes are rarely found in osteosarcomas [23], there must be molecules fulfilling the role of the ras signal in osteosarcomas. The ihMSC described here can serve as a material to investigate the oncogenic properties and effects on differentiation of candidate molecules.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.137.

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