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

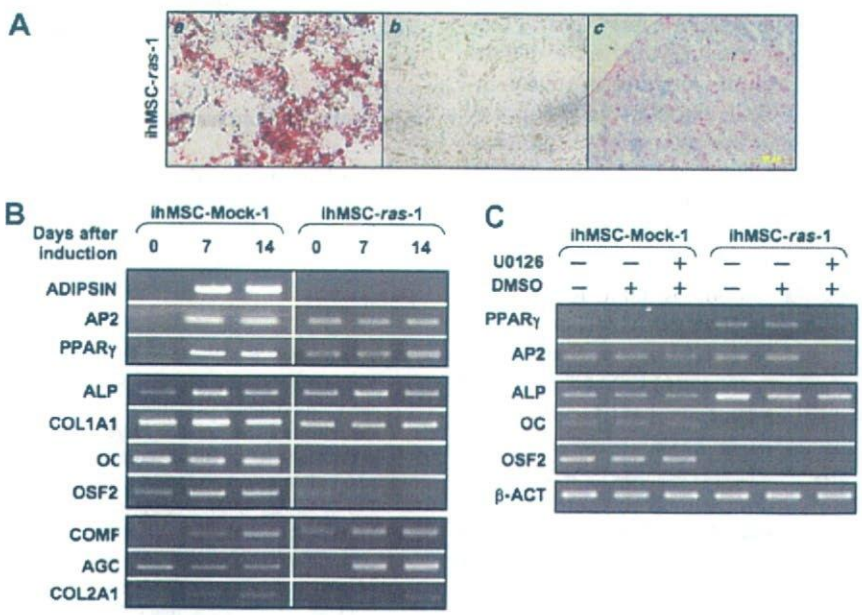

Fig. 4. Potential of ihMSC-*ras* to differentiate. (A) Lineage-related histochemical staining after the induction of each lineage. (B) mRNA expression of lineage-related genes after the induction of each lineage in ihMSC-Mock and ihMSC-*ras*. (C) mRNA expression of adipogenic and osteogenic lineage-related 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.

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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.

References

[1] T. Aoyama, T. Okamoto, S. Nagayama, K. Nishijo, T. Ishibe, K. Yasura, T. Nakayama, T. Nakamura, J. Toguchida, Methylation in the core-promoter region of the chondromodulin-I gene determines the cell-specific expression by regulating the binding of transcriptional activator Sp3, J. Biol. Chem. 279 (2004) 28789–28797.

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

[3] M. van Lohuizen, S. Verbeek, B. Scheljen, E. Wientjens, H. van der Guiden, A. Berns, Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging, Cell 65 (1991) 737–752.

[4] J.J. Jacobs, B. Scheijen, J.W. Voncken, K. Kieboom, A. Berns, M. van Lohuizen, Bmi-1 collaborates with c-myc in tumorigenesis by

inhibiting c-myc-induced apoptosis via ink4a/ARF, Genes Dev. 13 (1999) 2678–2690.
[5] A.V. Molofsky, R. Pardal, T. Iwashita, I.K. Park, M.F. Clarke, S.J. Morrison, Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation, Nature 425 (2003) 962–967.
[6] K. Itahana, Y. Zou, Y. Itahana, J.L. Martinez, C. Beausejour, J.J. Jacobs, M. van Lohuizen, V. Band, J. Campisi, G.P. Dimri, Control of the replicative life span of human fibroblasts by p16 and the polycomb protein bmi-1, Mol. Cell. Biol. 23 (2003) 389–401.
[7] T. Maeda, H. Tashiro, M. Begum, H. Ohtake, T. Kiyono, H. Okamura, Establishment of an immortalized human ovarian surface epithelial cell line without chromosomal instability, Br. J. Cancer. 93 (2005) 116–123.
[8] Y. Takeda, T. Mori, H. Imabayashi, T. Kiyono, S. Gojo, S. Miyoshi, N. Hida, M. Ita, K. Segawa, S. Ogawa, M. Sakamoto, S. Nakamura, A. Umezawa, Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation? J. Gene. Med. 6 (2004) 833–845.
[9] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[10] T. Kiyono, S.A. Foster, J.I. Koop, J.K. McDougall, D.A. Galloway, A.J. Klingelhutz, Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalize human epithelial cells, Nature 396 (1998) 84–88.
[11] L.F. Parada, C.J. Tabin, C. Shih, R.A. Weinberg, Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene, Nature 297 (1982) 474–478.
[12] T. Okamoto, T. Aoyama, T. Nakayama, T. Nakamata, T. Hosaka, K. Nishijo, T. Nakamura, T. Kiyono, J. Toguchida, Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells, Biochem. Biophys. Res. Commun. 295 (2002) 354–361.
[13] W.A. Dunn Jr., Autophagy and related mechanisms of lysosome-mediated protein degradation, Trends Cell. Biol. 4 (1994) 139–143.
[14] I. Tanida, T. Ueno, E. Kominami, LC3 conjugation system in mammalian autophagy, Int. J. Biochem. Cell. Biol. 36 (2004) 2503–2518.
[15] T. Tanoue, E. Nishida, Molecular recognitions in the MAP kinase cascades, Cell. Signal. 15 (2003) 455–462.
[16] N. Serakinci, P. Guldberg, J.S. Burns, B. Abdallah, H. Schrodder, T. Jensen, M. Kassem, Adult human mesenchymal stem cell as a target for neoplastic transformation, Oncogene 23 (2004) 5095–5098.
[17] T. Shintani, D.J. Klionsky, Autophagy in health and disease: a double-edged sword, Science 306 (2004) 990–995.
[18] E. Ogier-Denis, P. Codogno, Autophagy: a barrier or an adaptive response to cancer, Biochim. Biophys. Acta 1603 (2003) 113–128.
[19] S. Pattingre, C. Bauvy, P. Codogno, Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells, J. Biol. Chem. 278 (2003) 16667–16674.
[20] S. Furuta, E. Hidaka, A. Ogata, S. Yokota, T. Murata, Ras is involved in the negative control of autophagy through the class I PI3-kinase, Oncogene 23 (2004) 3898–3904.
[21] H. Enomoto, T. Furuichi, A. Zanma, K. Yamana, C. Yoshida, S. Sumitani, H. Yamamoto, M. Enomoto-Iwamoto, M. Iwamoto, T. Komori, Runx2 deficiency in chondrocytes causes adipogenic changes in vitro, J. Cell. Sci. 117 (2004) 417–425.
[22] B. Lecka-Czernik, I. Gubrij, E.J. Moerman, O. Kajkenova, D.A. Lipschitz, S.C. Manolagas, R.L. Jilka, Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARγ2, J. Cell. Biochem. 74 (1999) 357–371.
[23] F. Antillón-Klüssmann, M. García-Delgado, I. Villa-Elízag, L. Sierrasesúmaga, Mutational activation of ras genes is absent in pediatric osteosarcoma, Cancer Genet. Cytgenet. 79 (1995) 49–53.