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Original Article

G-protein Stimulatory α Subunit is Involved in Osteogenic Activity in Osteoblastic Cell Line SaOS-2 Cells

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Abstract. In an attempt to study the roles of G-protein stimulatory subunit α ($G_s\alpha$) in osteoblasts, we introduced an expression vector encoding $G_s\alpha$ into human osteoblastic cell line SaOS-2, and established the clones stably overexpressing $G_s\alpha$ (SaOS-2-$G_s\alpha$). In SaOS-2-$G_s\alpha$, the intracellular content of cyclic AMP (cAMP) was increased compared with the parental SaOS-2 cells. In addition, when treated with PTH[1-34], SaOS-2-$G_s\alpha$ exhibited more accumulation of intracellular cAMP compared with the parental cells, suggesting an increased responsiveness to PTH. We evaluated the proliferation rates of SaOS-2-$G_s\alpha$ and the parental SaOS-2 cells, and found that the proliferation was accelerated in SaOS-2-$G_s\alpha$ cells. Reverse transcription-polymerase chain reaction (RT-PCR) analyses exhibited the increased expression of Runx2, a transcription factor involved in osteoblast differentiation, in SaOS-2-$G_s\alpha$ cells. Finally, to examine the osteoblastic function *in vivo*, we inoculated SaOS-2-$G_s\alpha$ or parental SaOS-2 cells subcutaneously to immunocompromised nude mice. Although tumors in nude mice were not formed after inoculation of parental SaOS-2 cells, SaOS-2-$G_s\alpha$ cells proliferated in host animals leading to the formation of tumors with mineralized bone-like tissues. Taken together, these results suggest that the signals via $G_s\alpha$ play critical roles in the proliferation and osteogenic functions of osteoblasts.

Key words: osteoblast, $G_s\alpha$, SaOS-2, Runx2, osteogenic

Introduction

G-proteins are a family of guanine-nucleotide binding proteins coupled to seven transmembrane domain receptors, and are involved in signal transductions of several extracellular stimuli including hormones and peptides (1). G_s activates and G_i inhibits adenylyl cyclase in response to hormonal stimuli; transducin (G_t) regulates cyclic GMP phosphodiesterase activity, and G_o is another G-protein expressed predominantly in brain (1). G-proteins function as heterotrimers composed of α, *β*, and *γ* subunits, and $G_s\alpha$ is one of the subunits inducing the stimulation of adenylyl cyclase, which leads to the production of cyclic AMP (cAMP) as a second messenger (2).

GNAS1 is the gene encoding $G_s\alpha$ protein, and mutations in this gene are associated with heterogeneous clinical manifestations. Deficiency or loss of function mutations of $G_s\alpha$ lead to

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resistance to PTH [pseudohypoparathyroidism (PHP)] and other hormones (TSH, gonadotropins, glucagon, epinephrine) as well as Albright's hereditary osteodystrophy (AHO) (OMIM: 103580) (3–7). On the other hand, somatic gain of function mutation in *GNAS1* results in McCune-Albright syndrome (OMIM: #174800), which is characterized by polyostotic fibrous dysplasia, pigment patches of the skin and endocrinologic abnormalities, including precocious puberty, thyrotoxicosis, pituitary gigantism, and Cushing syndrome (8). The fact that the conditions caused by the mutations of $G_s\alpha$ are often associated with bone phenotype suggests that $G_s\alpha$ plays a role in skeletal development. In addition, it is known that intermittent administration of PTH leads to increased bone formation, which also indicates the critical role of PTH signaling mediated by $G_s\alpha$ in osteoblast function (9). However, the molecular mechanisms underlying the regulation of osteoblast function by $G_s\alpha$ are still not fully understood.

In the current study, to investigate the role of $G_s\alpha$ in osteoblast function, we established an osteoblastic cell line overexpressing $G_s\alpha$. These cells exhibited increased intracellular accumulation of cAMP, accelerated proliferation and enhanced expression of Runx2, a transcription factor involved in osteoblast differentiation. When subcutaneously inoculated into nude mice, the cells overexpressing $G_s\alpha$ formed ossified tumors. These findings provide evidence for the critical roles of $G_s\alpha$ in the proliferation and function of osteoblasts.

Materials and Methods

Cell culture and establishment of $G_s\alpha$-overexpressing cells

The human osteoblastic cell line SaOS-2 was obtained from the American Type Culture Collection, and was maintained in α-MEM (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, ICN Biomedicals, Inc., Aurora, OH, USA) and antibiotics under a 5% CO_2 atmosphere. To establish SaOS-2 cells overexpressing Gsα, the expression vector pcDNA3.1-Gsα was constructed and introduced into SaOS-2 cells using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), and the stable transfectants were selected for resistance to neomycin (Promega, Madison, WI, USA).

Western blot analysis

Whole cell extracts were harvested in RIPA buffer [1% Triton, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 5 mM EDTA, and protease inhibitor cocktail (Complete™; Roche Diagnostics GmbH, Mannheim, Germany)]. The cell lysates containing 10 μg of each protein were then subjected to SDS-PAGE, and were transferred to PVDF membrane (Biorad, Helcules, CA, USA). After blocking with Block Ace reagent (Dainippon Pharmaceuticals, Osaka, Japan), the membranes were incubated with anti-Gsα antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the corresponding secondary antibody, the proteins were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA).

Measurement of intracellular cAMP content

Cells were plated onto 96-well culture plates at the density of 1×10^4/well. On the next day, the cells were incubated in 0–5 μM PTH [1-34] (Sigma, St. Louis, MO, USA) for 25 min. Then, the intracellular cAMP contents were determined using cAMP EIA system (Amersham) following the manufacturer's protocols.

Cell proliferation assay

The proliferation rate of the parental SaOS-2 cells and SaOS-2-$G_s\alpha$ cells were evaluated using CellTiter 96® Non-radioactive Cell Proliferation Assay kit (Promega), which is based on the cellular conversion of a tetrazolium salt into a

formazan product.

Characterization of osteoblastic phenotype by RT-PCR analysis

Total RNA was extracted from SaOS-2-$G_s\alpha$ and the parental SaOS-2 cells using TRIZOL reagent (Invitrogen). Then, total RNA (2.5 μg) was reverse transcribed using a random hexamer (Promega) and SuperScript II (Invitrogen), and PCR was performed using the following sets of human-specific primers: alkaline phosphatase (ALP): sense, 5'-GGATGGGTGTCTCCACAGT-3' and antisense, 5'-TTCCACCAGCAAGAAGAAGCC-3'; osteocalcin: sense, 5'-ATGAGAGCCC-TCACACTCCTC-3' and antisense, 5'-CTAGACCGGGCCGTAGAAGCG-3'; bone morphogenetic protein (BMP)-2: sense, 5'-TCATAAAACCTGCAACAGCCAACTCG-3' and antisense, 5'-CACCCACAGCGATCATGTCG-3'; BMP-4: sense, 5'-GACCTATGGAGCCATTCCGTA-3' and antisense, 5'-TCAGGGATGC-TGCTGAGGTT-3'; Runx2: sense, 5'-ATTTAGGGCGCATTCCTCATC-3' and antisense, 5'-GTGGTGGAGTGGATGGATGG-3'; β-actin: sense, 5'-GTGGGGCGCCCCAGGCACCA-3' and antisense, 5'-CTCCTTAATGTCACGCACGATTTC-3'. Amplification of the expected fragments was confirmed by sequencing using an automated sequencer (377A model; Perkin-Elmer Corp., Norwalk, CT, USA).

Cell inoculation into nude mice

Animal protocols were approved by the Institutional Animal Care and Use Committee of Osaka Medical Center and Research Institute for Maternal and Child Health. Four-week-old female nude mice (BALB/cA Jcl-nu, nu/nu) were supplied by Clea Japan (Tokyo, Japan) and kept under pathogen-free conditions. Parental SaOS-2 or SaOS-2-$G_s\alpha$ cells [2.5×10^7 cells in 100 μl of phosphate-buffered saline (PBS)] were mixed with the same volume of Matrigel™ (Becton Dickinson Collaborative Biomedical Products, Bedford, MA, USA) and subcutaneously injected into the back of nude mice.

X-ray of mice

Animals in a prone position against film were X-rayed using SOFTEX CBM-2 (SOFTEX Co. LTD., Tokyo, Japan) under anesthesia.

Analysis of tumors

Four weeks after the cell inoculation, the mice were sacrificed, and the tumors that had formed at the inoculation sites were excised from subcutaneous tissue. After fixation in 10% buffered formalin and decalcification in a 14% EDTA solution (pH 7.4), the tumor specimens were embedded in paraffin and cut into 5-μm thick sections. Slides were then subjected to hematoxylin and eosin (H&E) staining and *in situ* hybridization as described below.

Preparation of RNA probes for in situ hybridization

To identify the origin of the cells found in the tumors, we performed *in situ* hybridization using human- or mouse-specific *Alu* sequences as probes. The probes were generated by polymerase chain reaction (PCR) using specific primers (human *Alu*: sense, 5'-GTGGCTCACGCCTGTAATCC-3' and antisense, 5'-TTTTTTGAGACGGAGTCTCGC-3'; mouse *Alu*: sense, 5'-GAGTCTATTAGTTACCTTTT-3' and antisense, 5'-GCAGCCAGTTCTCTTAACC-3') to amplify the *Alu* sequences specific to the human and mouse genomes, respectively, followed by cloning of the PCR products into pGEM3zf (+) vector (Promega). The sequences of the fragments were confirmed using a model 377 sequencer (Perkin-Elmer). Digoxigenin (DIG)-labeled sense and antisense probes were prepared with SP6 and T7 RNA polymerase using DIG RNA Labeling Mix (Roche).

In situ hybridization

The deparaffinized sections were immersed in 0.2 N HCl for 20 min, washed in PBS, and

then treated with 50 μg/ml of proteinase K at room temperature for 10 min. Then the sections were fixed again in 4% paraformaldehyde in PBS for 5 min, treated with 0.25% acetic anhydride containing 0.1 M triethanolamine (pH 8.0) for 10 min, and dehydrated in a series of increasing concentrations of ethanol. The dried sections were incubated in 50% formamide containing 2 × SSC (pH 4.5) at 45°C for 60 min. The sections were then heated at 95°C for 5 min to denature the genomic DNA, and hybridized at 45°C overnight with denatured DIG-labeled antisense or sense RNA probes in hybridization solution [1 mg/ml yeast tRNA, 20 mM Tris buffer (pH 8.0), 2.5 mM EDTA (pH 8.0), 1 × Denhardt's, 300 mM NaCl, 50% deionized formamide, and 10% dextran sulfate]. After being washed in 50% formamide containing 2 × SSC and 50% formamide containing 1 × SSC, the sections were treated with 10 μg/ml of RNase A at 37°C for 30 min. The sections were washed again in 50% formamide in 2 × SSC, 50% formamide in 1 × SSC, incubated in blocking solution (Roche), and reacted with anti-DIG antibody-coupled alkaline phosphatase at 4°C overnight. The probed molecules were detected using NBT/BCIP for the anti-DIG antibody-coupled alkaline phosphatase (Roche).

Results

Establishment of SaOS-2 cells overexpressing $G_s\alpha$

As described in "Materials and Methods", we introduced the expression vector pcDNA-$G_s\alpha$ into osteoblastic SaOS-2 cells, and selected the clones for resistance to neomycin. Then, the selected clones were subjected to Western blot analyses using antibody against $G_s\alpha$. As a result, the overexpression of $G_s\alpha$ in the clone #2 (SaOS-2-$G_s\alpha$ #2) was confirmed, but not in the clone #1 (Fig. 1A). Therefore, clone #2 was subjected to further analyses. The intracellular content of cAMP in the steady state was increased in SaOS-2-$G_s\alpha$ #2 compared with the parental cells (59.2 vs. 26.4 fmol/well). The intracellular accumulation of cAMP in response to PTH was

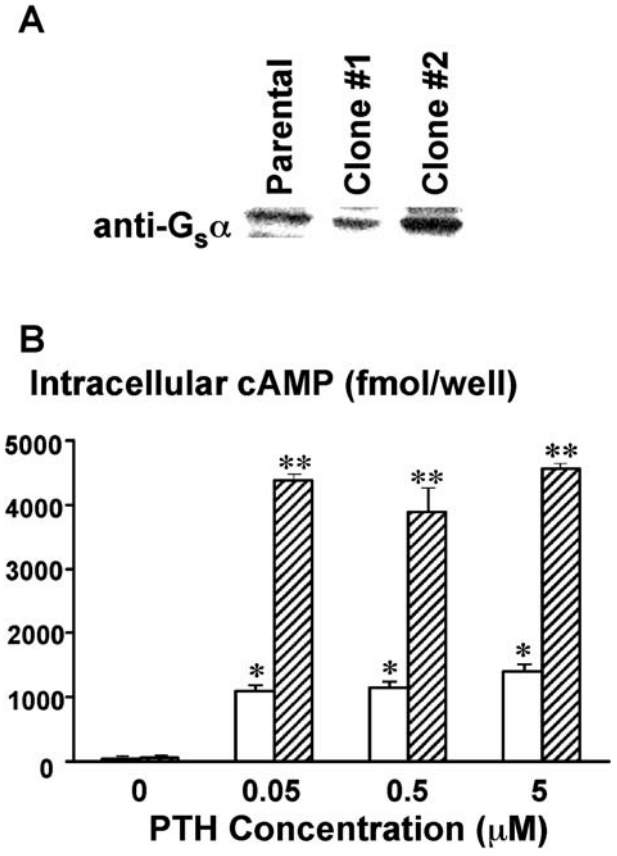

Fig. 1 Establishment of SaOS-2 cells overexpressing $G_s\alpha$. (A) Overexpression of $G_s\alpha$ in the clone SaOS-2-$G_s\alpha$ #2. Cell lysates (10 μg each) harvested from the parental SaOS-2 cells or the neomycin-resistant clones were subjected to Western blot analysis using an antibody against $G_s\alpha$. (B) Increased intracellular accumulation of cAMP in SaOS-2-$G_s\alpha$ #2 cells. The parental SaOS-2 cells (open columns) or the SaOS-2-$G_s\alpha$ #2 cells (hatched columns) were incubated in various concentrations of PTH (0–5 μM) for 25 min, and the intracellular cAMP contents were determined. The data are expressed as mean ± SE (n=3). *Significantly different from the values in untreated cells (p<0.001). **Significantly different from the values in parental cells (p<0.001).

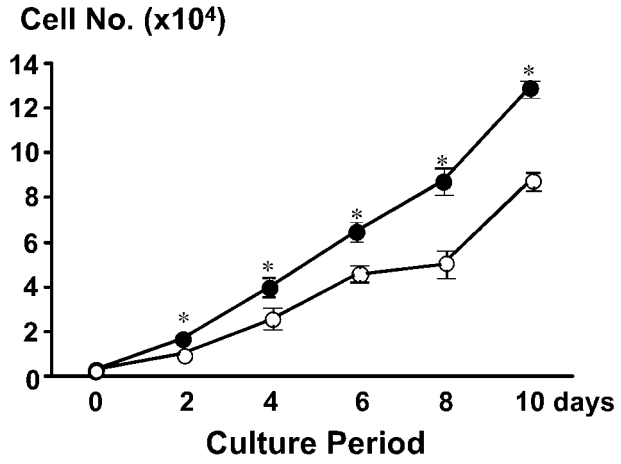

Fig. 2 Proliferation of SaOS-2-$G_s\alpha$ #2 (closed circles) was accelerated compared with the parental SaOS-2 cells (open circles). The proliferation rate of the cells was evaluated using a non-radioactive proliferation assay kit based on cellular conversion of a tetrazolium salt into a formazan product. The data are expressed as mean ± SE (n=3). *Significantly different from the values in parental cells ($p<0.001$).

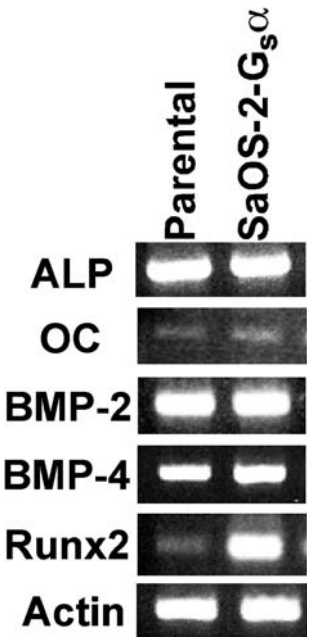

Fig. 3 Characterization of SaOS-2-$G_s\alpha$ #2 by RT-PCR analyses. The expressions of alkaline phosphatase (ALP), osteocalcin (OC), BMP-2, BMP-4 and Runx2 were examined. The expression of β-actin as an internal control is also depicted.

also increased in SaOS-2-$G_s\alpha$ #2 (Fig. 1B).

Characterization of SaOS-2-$G_s\alpha$ #2 cells *in vitro*

The proliferation rate determined by CellTiter 96® Non-radioactive Cell Proliferation Assay (Promega) was increased in SaOS-2-$G_s\alpha$ #2 cells compared with the parental SaOS-2 cells (Fig. 2).

The expression of osteoblastic marker genes in SaOS-2-$G_s\alpha$ #2 and the parental SaOS-2 cells were examined by RT-PCR analyses. Among the molecules examined, we found that the expression of Runx2, which is a critical transcription factor in osteoblast differentiation, was markedly enhanced in SaOS-2-$G_s\alpha$ #2 cells (Fig. 3). The expression of ALP, osteocalcin, BMP-2 and -4 were comparable between SaOS-2-$G_s\alpha$ #2 and the parental SaOS-2 cells.

Mineralized tumor formation by SaOS-2-$G_s\alpha$ #2 cells in nude mice

As others have reported, SaOS-2 parental cells do not form tumors in nude mice, although they can mineralize *in vitro* (10). However, we found that with SaOS-2-$G_s\alpha$ #2, mineralized tumors were formed 4 weeks after subcutaneous inoculation into nude mice as observed by X-ray (Fig. 4A, B). We performed the inoculation 48 times (4 sites/mouse, 12 mice), and obtained 48 mineralized tumors. Histological examination revealed the tumors contained lamellar bone matrix with osteoblastic cells, osteoclasts and bone marrow (Fig. 4C). There was no cartilaginous tissue in the tumor at 4 weeks after inoculation. We confirmed that all of the xenografts originated from SaOS-2-$G_s\alpha$ #2 cells contained bone-like tissue.

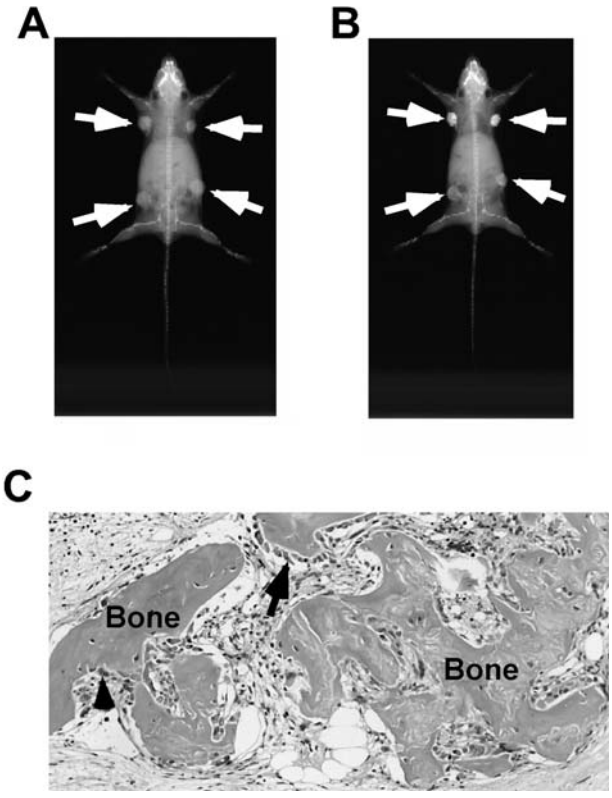

Fig. 4 Mineralized tumors formed by SaOS-2-$G_s\alpha$ #2 cells. SaOS-2-$G_s\alpha$ #2 cells (2.5×10^7/site) were inoculated subcutaneously at 4 sites into the back of 4-week-old nude mice. The mice were X-rayed 2 weeks (A) and 4 weeks (B) after the inoculation. The white arrows indicate the mineralized tumors. (C) H&E-stained section of tumor excised 4 weeks after the cell inoculation. Lamellar bone matrix with bone marrow, cuboidal active osteoblasts attached to the bone surface (arrow) and multinucleated osteoclasts (arrowhead) are observed.

Origin of the bone cells in the tumors

It was reported that SaOS-2 cells expressed various kinds of BMPs and induced the formation of ectopic bone when defatted, freeze-dried cells were implanted into nude mice (10). Therefore, to test the possibility that SaOS-2-$G_s\alpha$ #2 cells formed bone by promoting the differentiation of osteogenic cells of host mice, we investigated the origin of the cells in ectopic bones. *In situ* hybridization using the human-specific Alu sequence as a probe revealed the human origins of some of the osteoblastic cells located on the bone surface (Fig. 5B). The absence of signals in osteoclasts and bone marrow cells suggested a murine origin for these cells. *In situ* hybridization using a mouse-specific Alu sequence as a probe revealed that the formed bone also included cells of host origin (Fig. 5C); osteocytes, some of the osteoblastic cells adherent to bone, osteoclasts and bone marrow cells were of murine origin. RT-PCR using total RNA extracted from the ectopic bone followed by sequencing revealed the expression of human Runx2/cbfa-1, which is further evidence of the existence of human osteoblasts in the ossified tumors (data not shown). These results suggest that ossified bone formed by SaOS-2-$G_s\alpha$ #2 cells contains both human and mouse cells.

Discussion

In the current study, to investigate the roles of $G_s\alpha$ in osteoblast function, we established $G_s\alpha$-overexpressing osteoblastic cells by introducing an expression vector coding $G_s\alpha$ into an osteoblastic cell line, SaOS-2. The obtained clone SaOS-2-$G_s\alpha$ #2 exhibited increased accumulation of cAMP both in the steady state and in response to PTH treatment, indicating that the downstream signaling was enhanced by overexpression of $G_s\alpha$. In SaOS-2-$G_s\alpha$ cells, the proliferation *in vitro* was accelerated compared with the parental cells. In addition, SaOS-2-$G_s\alpha$ reproducibly formed tumors after subcutaneous inoculation into nude mice, while the parental SaOS-2 failed to induce tumor formation. These observations support the notion that the overexpression of $G_s\alpha$ results in accelerated proliferation *in vivo* as well as *in vitro*. In parallel with our findings, it was reported that osteoblastic cells expressing constitutively active $G_s\alpha$ isolated from displastic bone lesions in patients with McCune-Albright syndrome

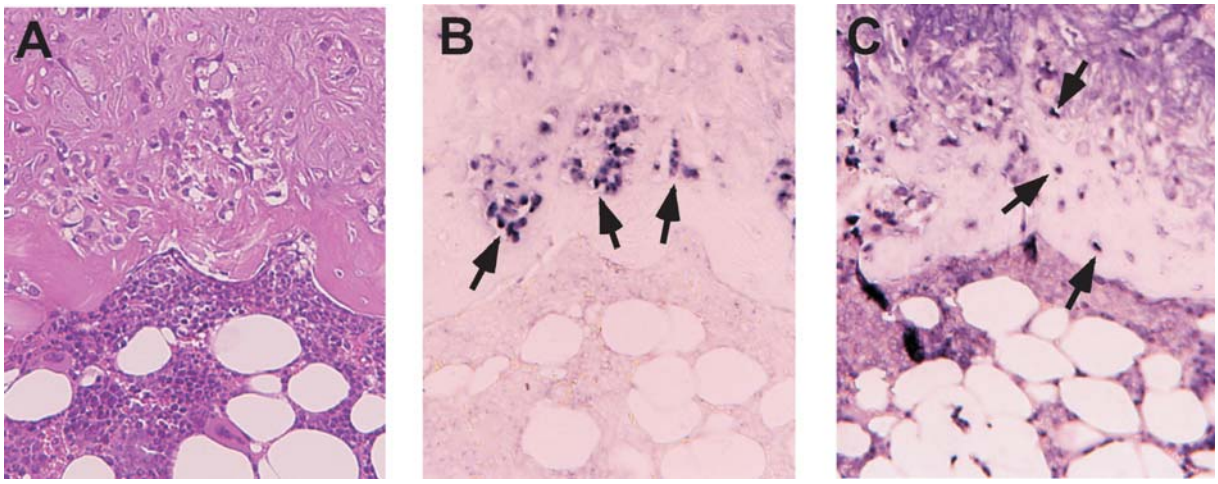

Fig. 5 Determination of the origin of the bone cells in the tumor formed by SaOS-2-$G_s\alpha$ #2 cells. Serial sections were prepared from the tumor, and were subjected to H&E staining (A) and *in situ* hybridization using human (B) or mouse (C) genome-specific Alu sequences as probes. Note that some of the osteoblastic cells attached to the bone surface are of human origin. Osteocytes and bone marrow cells were of murine origin. Representative signals are shown by arrows.

exhibited increased cell proliferation compared with normal cells from the same patient (11). Therefore, we speculate that $G_s\alpha$ is involved in the regulation of proliferation of osteoblastic cells.

Interestingly, the tumors formed after inoculation of SaOS-2-$G_s\alpha$ cells contained bone-like tissue. SaOS-2 cells are known to express several kinds of BMPs and have bone-inducing activity when a defatted cell pellet is inoculated into the muscle of immunocompromized mice, although they themselves do not form tumors (10). In that experiment, however, mesenchymal progenitor cells of host mice differentiated into bone via the formation of cartilaginous tissue. In contrast, living SaOS-2-$G_s\alpha$ cells, at least partly, but not exclusively, contributed to the bone formation. *In situ* hybridization using the human or murine genome-specific *Alu* sequences as probes revealed the existence of osteoblastic cells of both human and murine origin in the tumor (Fig. 5). Osteoclasts and bone marrow cells in the tumor were originated from host cells. BMPs produced by inoculated SaOS-2-$G_s\alpha$ cells might be responsible for the recruitment of osteoprogenitors of host origin to the inoculation site.

In SaOS-2-$G_s\alpha$ cells, the expression of Runx2 was increased compared with the parental SaOS-2 cells. Runx2, also called Cbfa1, is a transcription factor playing a critical role in osteoblast differentiation (12, 13). It has been reported that Runx2 regulates the expression of some osteoblast-specific genes including the osteocalcin gene (12). In addition, targeted disruption of Runx2 results in a complete lack of bone formation, and heterozygous mutation of the gene causes cleidocranial dysplasia (13). These results indicate the indispensable role of Runx2 in skeletal development. Therefore, we assume that the increased expression of Runx2 in SaOS-2-$G_s\alpha$ might be due to its involvement in the osteogenic activity of these cells, although the mechanisms for up-regulation of Runx2 remain to be elucidated.

In conclusion, using SaOS-2 cells overexpressing $G_s\alpha$ as a cell model, we obtained the results suggesting the involvement of $G_s\alpha$ in the proliferation and function of osteoblasts.

Particularly, the *in vivo* data obtained by the inoculation of the cells into nude mice demonstrated that the introduction of $G_s\alpha$ leads to increased osteogenic activity. Based on these findings, we assume that $G_s\alpha$ might be a molecular target for the treatment of various bone diseases, especially osteoporosis.

Acknowledgements

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