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Establishment and Characterization of a Simian Virus 40-Immortalized Osteoblastic Cell Line from Normal Human Bone

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We established a human osteoblastic cell line immortalized by simian virus 40 (SV40) *in vitro*, and designated it SV-HFO. Immunocytochemically, the cells were positive for SV40 large T-antigen, vimentin and osteocalcin, but negative for keratin and epithelial membrane antigen. The cells had characteristic morphologic and ultrastructural features of osteoblasts, produced alkaline phosphatase, and synthesized osteocalcin, the levels of which were elevated by treatment of the cells with 1α,25-dihydroxyvitamin D_3. The cells proliferated and showed such osteoblastic properties even under serum-free conditions. The cells grew in soft agar, but did not form tumors when transplanted into athymic nude mice. Karyotypic analysis by the Q-banding technique showed that these cells were of human origin. The SV-HFO cell line is expected to serve as a suitable model for studying metabolism and carcinogenesis in human bone.

Key words: Osteoblast — Human — Simian virus 40 — Cell line — Osteocalcin

For studying bone metabolism, osteoblastic cells in culture have been extensively employed. Primary culture of normal bone cells isolated by explant or enzymatic digestion procedures is generally used.[1] Several cell lines derived from normal bones of mice[2,3] and rats,[4–7] and from osteosarcomas of rats[8–10] and humans[11–14] are also available.

However, normal human bone cells in culture often show alteration of phenotypic properties with increasing passage number, and finally lose their proliferative activity. On the other hand, rodent bone cells frequently transform spontaneously *in vitro*. Thus, immortalized cell lines from normal human bone have long been desired for studying human bone metabolism and carcinogenesis.

In this study we obtained a propagatable osteoblastic cell population from nomal human fetal calvaria, transformed the cells with SV40,[5] and established a cell line.

MATERIALS AND METHODS

Cell culture and SV40 infection The HFO cells for primary culture were obtained at autopsy from fetal tissues aborted at the 26th week of gestation and prepared by the following procedure. Parietal bones were minced with scissors after peeling off the periosteum, washed with DMEM (Nissui Pharmaceutical, Tokyo), and placed on 100-mm culture dishes in DMEM supplemented with 10% FBS (JRH Bioscience, Lenexa, KS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco Laboratories, Grant Island, NY). The cells that migrated from the explants were harvested by trypsinization (0.05% trypsin and 0.02% EDTA; Gibco), and passaged once a week at a split ratio of 1:3. These cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was changed two or three times a week.

For SV40 infection, the HFO cells at P4 were plated at a cell density of 3×10^5 cells/75-cm^2 flask. One day after subculture, the cells were treated with wild-type SV40 in serum-free DMEM, kindly provided by Dr. Nobuo Yamaguchi (The Institute of Medical Science, The University of Tokyo, Tokyo), at multiplicities of 400 plaque-forming units per cell. After a 2-h incubation period, the medium containing the virus was drawn off, and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4) and re-fed DMEM containing 10% FBS. One day after SV40 treatment, the SV40-infected and non-infected HFO cells were harvested by trypsinization and passaged at cell densities of 1×10^3 or 1×10^4 cells/60-mm culture dish. After transformed foci had appeared (about 2 weeks later), the SV40-infected cells were subcultured and fed every 2 or 3 days. The cells through P55 were subcultured every week at a split ratio of 1:4 or 1:8 and the cells after 55 passages were split once a week at a ratio of 1:16. The transformed cell strain thus established was designated as SV-HFO cells.

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[5] Abbreviations: SV40, simian virus 40: HFO, human fetal osteoblastic cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; P, passage; SV-HFO, SV40-transformed human fetal osteoblastic cells; ALP, alkaline phosphatase; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; mAb, monoclonal antibody; EMA, epithelial membrane antigen; PDs, population doublings.

All experiments reported here were performed using SV-HFO cells recovered from frozen stock. For examining growth properties, the cells at P15 or P30 were seeded at a cell density of 5×10^3 cells/cm^2 on 35-mm culture dishes in DMEM containing 10% FBS, or on dishes coated with 20 μg/dish type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) in serum-free medium supplemented with 0.5% bovine serum albumin (Albumax^{TM}; Gibco) and ITS (containing 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenious acid; Collaborative Research, Inc., Bedford, MA). For examining ALP activity and osteocalcin synthesis, the cells at P15 or P30 were plated at a cell density of 2×10^4 cells/cm^2 on 12-well tissue culture plates in serum-supplemented or serum-free medium as described above. The culture medium was changed every 3 days. To determine the effects of $1,25(OH)_2D_3$ on the synthesis of ALP and osteocalcin, cells cultured in serum-free medium for 6 days were treated with either $10^{-7}M$ $1,25(OH)_2D_3$ (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) or ethanol for 3 consecutive days. Morphological analysis was carried out with a phase-contrast microscope.

Growth properties At the 1st, 3rd, 5th, 7th, 9th, 11th and 13th days after plating, the cultured cells were dispersed by trypsinization, and the cell number was counted in a hemocytometer using 0.15% trypan blue.

Immunocytochemical staining The SV-HFO cells were cultured on cover-slips in DMEM supplemented with 10% FBS, or cover-slips coated with 2 μg/cm^2 type I collagen in serum-free medium. After reaching subconfluence, these cells were rinsed with PBS, and fixed in ice-cold acetone for 10 min at -20°C.

For immunocytochemical detection of SV40 large T-antigen, the cells were stained with mouse mAb Pab 419 which was provided by the Japanese Cancer Research Resources Bank (Tsukuba Science City). The presence of osteocalcin was determined by the immunoperoxidase technique using mouse mAb 10E8 against human osteocalcin.[15] The cells were also stained by the immunoperoxidase technique using mouse mAb against human vimentin (Dako Corp., Carpinteria, CA) and human EMA (Nichirei, Tokyo) and rabbit polyclonal antibody against human keratin (Nichirei). Briefly, fixed cells were incubated for 1 h at room temperature with the primary antibodies, washed three times with PBS (pH 7.4), and subsequently incubated for 30 min with biotinylated horse anti-mouse immunoglobulin G (IgG) or biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA). For immunofluorescent observation, the cells were incubated with fluorescein-labeled streptoavidin (Vector) after being rinsed with PBS, washed with PBS, and mounted with glycerol. For the peroxidase technique, the cells were incubated with avidin-biotin-peroxidase complexes (Vector), washed with PBS, and treated with 0.03% 3,3′-diaminobenzidine in 5 mM Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2 for 3 min, followed by counterstaining with hematoxylin. Primary antibodies and biotinylated horse anti-mouse IgG were diluted with PBS containing 1% BSA and 0.1% sodium azide, and fluorescein-labeled streptoavidin was diluted to 1:50 with a solution containing 10 mM N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, MO), 0.15 M NaCl and 0.08% sodium azide (pH 8.5). Cells incubated without primary antibody or with non-immunized serum served as a control.

Measurement of ALP activity Nine days after subculture, the cells were rinsed twice with PBS, scraped into 0.3 ml of a solution containing 0.1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 0.02% NaN_3 at 37°C, and sonicated for 30 s. The cell extracts were centrifuged for 5 min at 10,000g at 4°C, and kept frozen at -20°C until use.

ALP activity was spectrophotometrically measured using p-nitrophenyl phosphate as a substrate according to the method of Lowry.[16] The content of cellular protein was measured by using a Pierce BCA protein assay kit (Pierce Chemical Co., Rockford, IL) with BSA as a standard. ALP activity was expressed as nanomoles/minute/milligram protein.

Measurement of osteocalcin To avoid serum osteocalcin contamination from FBS, the cells employed for this experiment were cultured under serum-free conditions. The amount of osteocalcin secreted into the culture medium was determined by radioimmunoassay using a commercially available kit (CIS Biointernational, Gif-Sur-Yvette, France). Results were expressed as nanograms/10^6 cells.

Ultrastructural analysis For transmission electron microscopy, the SV-HFO cells at confluent density were fixed in a mixture of 1% glutaraldehyde and 4% paraformaldehyde adjusted to pH 7.4 with cacodylate buffer for 30 min at 4°C. They were then postfixed with 1% OsO_4, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 1200-Ex transmission electron microscope.

Colony formation in soft agar The colony-forming ability of the SV-HFO cells was determined by the procedure described by Macpherson and Montagnier[17] with slight modifications. Subconfluent cultures of the SV-HFO cells at P12 or P30 and the HFO cells at P8 were dispersed and suspended in DMEM containing 0.33% agar and 10% FBS. The cell suspensions were plated at 1×10^3, 1×10^4 or 1×10^5 cells per 35-mm dish on the bottom layer of 1 ml of DMEM containing 0.5% agar and 20% FBS. Eight dishes were used for each cell density, and dishes

were examined immediately after seeding to make sure that there were no clumps resulting from plating. After 14 days, colonies greater that 100 μm in diameter were counted. Colony formation was expressed as the number of colonies in relation to the number of cells seeded.

Tumorigenicity The tumorigenicity of the SV-HFO cells was determined by subcutaneously inoculating the cells into the backs of 5- to 6-week-old female athymic nude mice (BALB/c). Sixteen mice were used, and each of them was given $1-2\times10^7$ cells at P7 or P30 suspended in 0.5 ml of medium. All the mice were examined weekly for tumor development for a period of at least 6 months.

Chromosome analysis Chromosome preparations were obtained from the cultured cells at P15 by the ordinary air-drying method. The slides were stained with quinacrine mustard-33258 Hoechst by the method of Yoshida *et al.*[18]

RESULTS

Morphology and growth properties The SV-HFO cells were easily distinguished from the HFO cells by phase-contrast microscopy (Fig. 1). The HFO cells showed a spindle-shaped appearance and were larger than the SV-HFO cells in their longer axis. In contrast, the SV-HFO cells were predominantly polygonal or cuboidal in shape and intermingled with a few fibroblast-like cells. The SV-HFO cells proliferated in a sheet-like pattern occasionally accompanied by focus formation of piled-up cells.

The SV-HFO cells grew stably for 46 PDs by P19, after which they reached a stage of little or no proliferation, termed "crisis." During the crisis phase which lasted for about 50 days, the great majority of the cells became bizarre or multinucleated, and detached from the surface of the culture flask. The remaining cells, from which we established a cell line, appeared to recover proliferative potential.

The growth of SV-HFO cells before and after crisis (P15, P30) is shown in Table I. These cells underwent cell division even in the serum-free medium supplemented with 0.5% BSA and ITS. The doubling time of cells at P30 was shorter than that at P15. The saturation density of cells at P15 grown in serum-supplemented medium was approximately two times higher than that of cells cultured in serum-free medium. In contrast, the

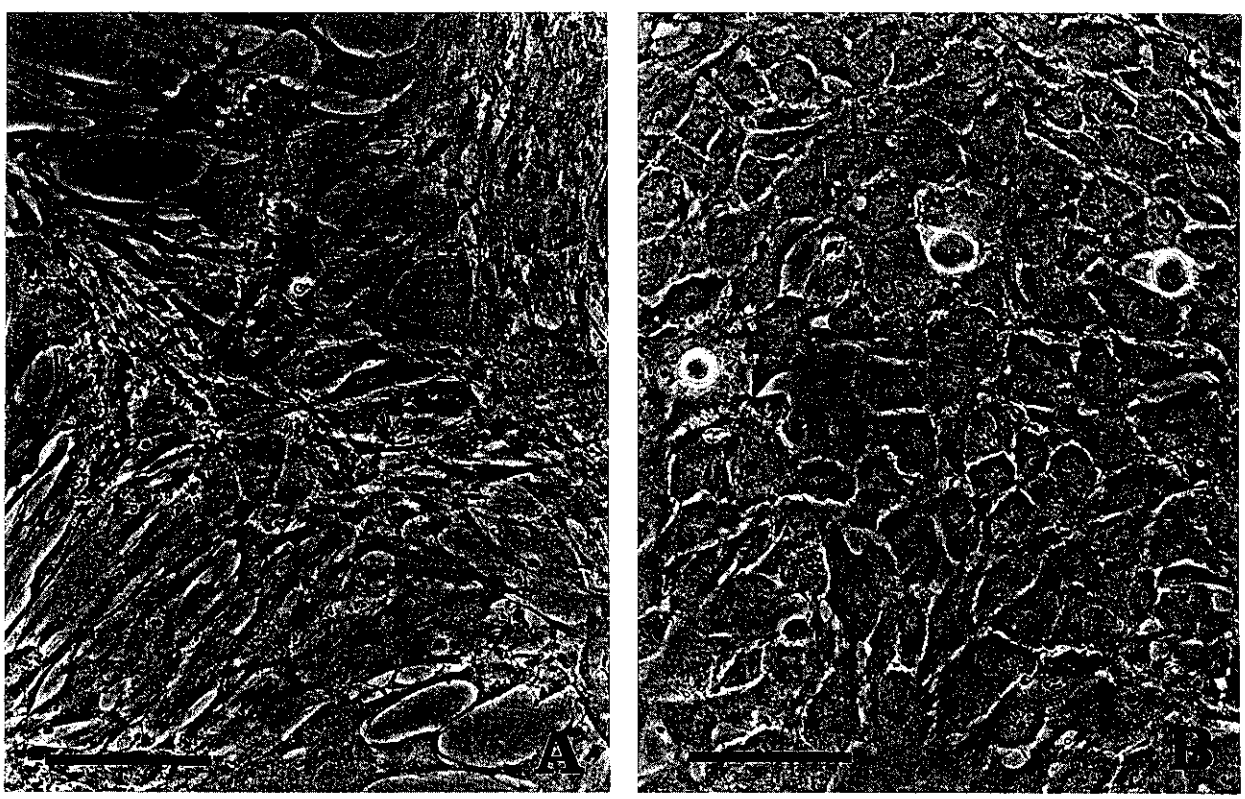

Fig. 1. Phase-contrast micrographs of (A) the HFO cells at passage 8 showing fibroblast-like morphology and (B) the SV-HFO cells at passage 30 showing polygonal or cuboidal morphology. Bar, 100 μm.

Table I. Growth of the SV-HFO Cells at Passage 15 and Passage 30

Culture conditions	Doubling time (h)		Saturation density (10^5 cells/cm^2)	
	Passage 15 (before crisis)	Passage 30 (after crisis)	Passage 15 (before crisis)	Passage 30 (after crisis)
Serum-supplemented (10%FBS)	37.7	25.9	1.62	5.35
Serum-free	34.1	25.6	0.82	5.00

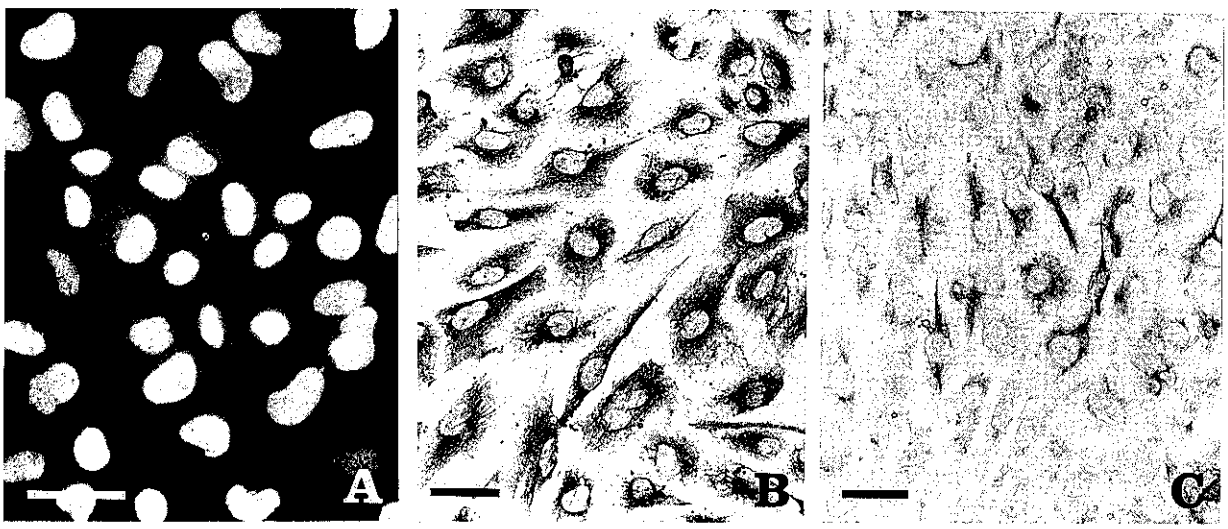

Fig. 2. Immunocytochemical staining of (A) SV40 large T-antigen, (B) vimentin, and (C) osteocalcin in the SV-HFO cells. Bar, 50 μm.

saturation densities of cells at P30 cultured under serum-supplemented and serum-free conditions were similar, the levels of both being higher than at P15. At present, the cells have reached P65 (approximately 150 PDs). In contrast, the primary HFO cells did not proliferate after P10 (approximately 16 PDs).

Immunocytochemical properties SV40 large T-antigen was immunocytochemically detected in all of the nuclei of the SV-HFO cells (Fig. 2A), but not in the HFO cells. The SV-HFO cells were positive for vimentin (Fig. 2B) and negative for keratin and EMA. Osteocalcin was also demonstrated in a small number of SV-HFO cells (Fig. 2C).

ALP activity Table II shows ALP activity in the SV-HFO cells 9 days after plating at a cell density of 2×10^4 cells/cm^2. The cells before crisis (P15) showed a considerable level of ALP activity under serum-supplemented and serum-free conditions. Treating the cells at P15 with 10^{-7} *M* $1,25(OH)_2D_3$ for 3 days induced 3.3 times the amount of ALP found at the basal level of the cells cultured in serum-free medium. ALP activity and the responsiveness of the enzyme to $1,25(OH)_2D_3$ were also retained in the cells after crisis (P30), though they were lower than those in the cells before crisis.

Osteocalcin synthesis To avoid contamination with bovine osteocalcin from FBS, the cells were cultured in serum-free medium, as described in "Materials and Methods." As shown in Table III, the SV-HFO cells before crisis (P15) cultured for 9 days in serum-free medium without addition of $1,25(OH)_2D_3$ secreted 0.27 ng/10^6 cells of osteocalcin into the medium. Treatment of the cells with 10^{-7} *M* $1,25(OH)_2D_3$ for 3 days induced 30 times the amount of osteocalcin found at the basal level. The cells after crisis (P30) also secreted osteocalcin into the medium, and this was significantly enhanced by treatment of the cells with $1,25(OH)_2D_3$.

Ultrastructural analysis Transmission electron microscopy was performed on the postconfluent SV-HFO cells at P15 (Fig. 3) and P30. These cells had large nuclei, which often showed clefting and were located eccentrically in the cytoplasm. The cytoplasm contained arrays of short rough endoplasmic reticulum (r-ER), Golgi apparatus, small mitochondria and abundant free ribosomes. The cells were surrounded by collagen fibrils, but no

Table II. Alkaline Phosphatase Activity in the SV-HFO Cells at Passage 15 and Passage 30

Culture conditions	Alkaline phosphatase activity (nmol/min/mg protein)	
	Passage 15 (before crisis)	Passage 30 (after crisis)
Serum-supplemented (10%FBS)	22.62±3.36	4.66±0.55
Serum-free	39.96±3.37[a)]	8.43±1.01[c)]
Serum-free+1,25(OH)$_2$D$_3$ (10^{-7} M)	129.70±10.24[b)]	14.33±2.52[d)]

The figures are mean values of four different dishes with duplicate determinations.
a) Significantly different from the value of the cells cultured in medium containing 10% FBS ($P<0.001$).
b) Significantly different from the value of the cells cultured in medium containing 10% FBS ($P<0.001$) and that of cells cultured under serum-free conditions ($P<0.001$).
c) Significantly different from the value of the cells cultured in medium containing 10% FBS ($P<0.01$).
d) Significantly different from the value of the cells cultured in medium containing 10% FBS ($P<0.01$) and that of cells cultured under serum-free conditions ($P<0.01$).

Table III. Osteocalcin Synthesis in the SV-HFO Cells at Passage 15 and Passage 30

Culture conditions	Osteocalcin (ng/10^6 cells)	
	Passage 15 (before crisis)	Passage 30 (after crisis)
Serum-free	0.27±0.21	0.14±0.05
Serum-free+1,25(OH)$_2$D$_3$ (10^{-7} M)	8.00±0.72[a)]	0.49±0.04[a)]

The figures are mean values of four different dishes with duplicate determinations.
a) Significantly different from the value of the cells cultured under serume-free conditions ($P<0.001$).

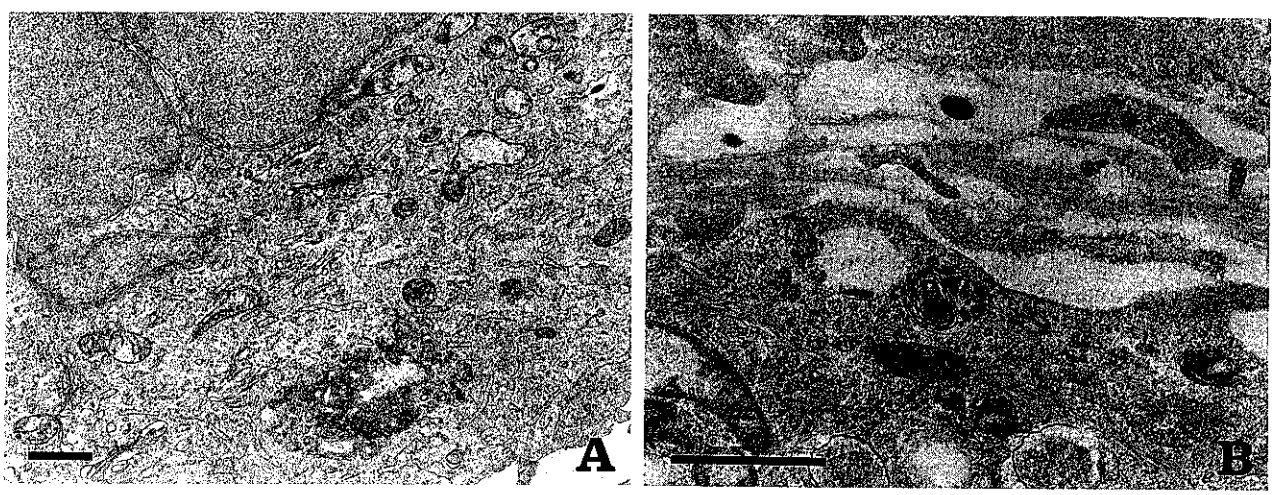

Fig. 3. Transmission electron micrographs of SV-HFO cells at passage 15: (A) array of short rough endoplasmic reticulum, small Golgi apparatus, small mitochondria, and free ribosomes, which are located eccentrically to the large nucleus, and (B) the presence of collagen fibrils around the cells. Bar, 1 μm.

junctional apparatus was found at the cell boundary. Spindle-shaped fibroblastic cells, which contained well-developed anastomosing r-ER, were also observed.

Colony formation in soft agar and tumorigenicity The SV-HFO cells formed colonies in soft agar. The mean frequency of colony formation was approximately 4.3×10^{-4} at P12 and 2.2×10^{-3} at P30. However, no tumor was observed in nude mice during the period of 6 months after subcutaneous inoculation of the SV-HFO cells at P7 or P30 ($1–2\times10^7$ cells). The control HFO cells were

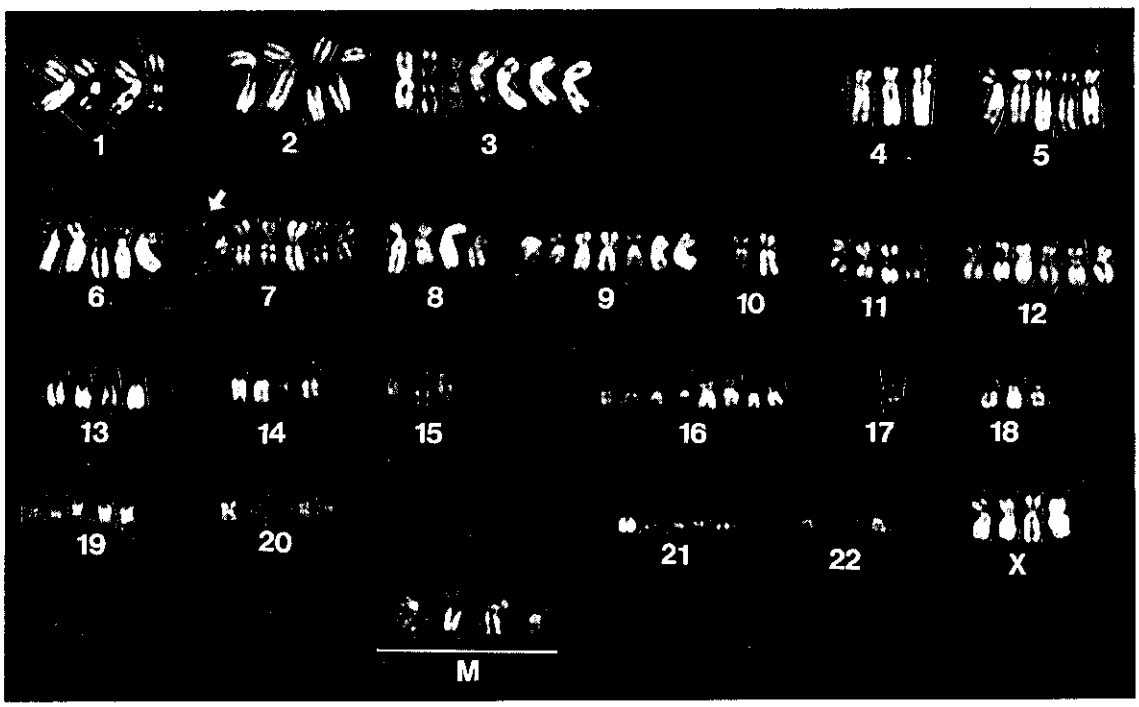

Fig. 4. QM-staining karyotype of the SV-HFO cells at passage 15. The arrow indicates an abnormal chromosome of no. 7. M indicates abnormal chromosomes of which the origin could not be determined.

capable of neither colony formation in soft agar nor tumor formation in nude mice.

Chromosome analysis The number of chromosomes of the SV-HFO cells at P15 varied from 46 to 111 with no clear modal number in 25 cells examined. Although the distribution of chromosome numbers was dispersed, in about 60% of the cells observed it ranged from 81 to 87. Q-Banding analysis showed that the numbers of each human chromosome appeared to be inconsistent from one cell to another. Several abnormal chromosomes normally not present in human metaphases were identified, as shown in Fig. 4.

DISCUSSION

In the present experiments, we established an SV40-immortalized osteoblastic cell line from normal human fetal calvaria.

SV40 has been widely used to transform a variety of human cell types as reviewed previously.[19, 20] In general, the SV40-transformed human cells have extended lifespans, although many of them reached a crisis phase. However, a small number of SV40-transformed human cells are immortalized without a crisis or with only a transient crisis.[21–24] In the present study, we were able to establish a cell line from SV-HFO cells that survived the crisis phase, although the great majority of the SV-HFO cells went through such a crisis phase.

The SV-HFO cells were immunocytochemically positive for vimentin but negative for keratin and EMA, which suggested that they were of mesenchymal origin. Phase-contrast microscopy revealed polygonal or cuboidal configuration, but electron microscopic observations supported the mesenchymal nature of these cells. Osteoblastic nature was indicated by evidence showing that the cells possessed a considerable level of ALP activity and osteocalcin. Osteocalcin has been shown to be expressed exclusively in the bone tissues.[25, 26] By immunocytochemistry, osteocalcin has been demonstrated only in osteoblasts and young osteocytes.[15, 27] In this sense, the fact that the SV-HFO cells synthesized osteocalcin confirmed the osteoblastic nature of these cells. Production of ALP and osteocalcin was further enhanced by the treatment of the SV-HFO cells with $1,25(OH)_2D_3$, a well known modulator of bone metabolism. These characteristics are consistent with those of several other osteoblastic cells.[12, 28–32] Furthermore, the SV-HFO cells proliferated and maintained such osteoblastic features under serum-free conditions as well as in serum-supplemented conditions. Thus, SV-HFO cells are expected to

serve as a promising model for elucidating the metabolism of human bone cells, including the modulating effects of hormones, cytokines and extracellular matrices on differentiation and proliferation of human osteoblasts.

The SV-HFO cells expressing large T-antigen did not form tumors in athymic nude mice, while the cells exhibited an extended lifespan and a potential for anchorage-independent growth. It is well-known that transformation of human cells by SV40 or the transgene of large T-antigen is insufficient for acquisition of tumorigenicity. For such acquisition *in vivo,* further genetic alterations, such as introduction of other oncogenes or mutations, are required.[20, 33] In this regard, the SV-HFO cells are considered to show intermediate properties between normal and neoplastic cells. Thus, the SV-HFO cell line should be a suitable model for the study of the multistep carcinogenesis of human osteogenic tumors.

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