

A Murine Osteosarcoma Cell Line with a Potential to Develop Ossification upon Transplantation

Tomomi Kusumi,^{1,4} Takashi Nishi,^{1,2} Masanori Tanaka,¹ Shigeki Tsuchida³ and Hajime Kudo¹

Second Departments of ¹Pathology, ²Surgery and ³Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562

An osteosarcoma cell line has been established from a soft tissue tumor that occurred spontaneously in a BALB/c mouse. This cell line showed ossification when transplanted into syngeneic mice. To examine the mechanism of bone formation, the expression of mRNAs for osteoblastic and chondroblastic markers and factors associated with ossification has been investigated. In culture, the cells exhibited a spindle shape in the growth phase, but had a polygonal shape in the stationary phase. Reverse transcription-polymerase chain reaction analysis showed that the cells expressed mRNAs for pro- $\alpha 1$ (I) chain of type I collagen, alkaline phosphatase, osteopontin, osteocalcin, and core binding factor $\alpha 1$, suggesting differentiation into the stage of osteoblasts during the stationary phase. After transplantation, histological examination revealed small foci of pale blue material and basophilic networks that were scattered in the tumor tissues at one week. The former stained positive with alcian blue, suggesting a chondroid matrix. Pro- $\alpha 1$ (II) chain of type II collagen mRNA was expressed at one week. A large part of tumors at two and three weeks consisted of basophilic networks, which stained positive via von Kossa's method, indicating a calcified woven bone. *In situ* hybridization analysis showed strong expression of osteopontin and osteocalcin mRNAs in tumor cells surrounding the bone matrix. Bone morphogenetic protein-6 and -7 mRNAs were detected in transplanted tumors, but not in cultured cells. These results suggest that the cell line has the properties of an osteoblastic lineage when cultured *in vitro* and has an ossifying ability through endochondral bone formation processes when transplanted *in vivo*.

Key words: Murine osteosarcoma — Cell line — Transplantation — Osteoblastic differentiation — Endochondral ossification

Ossification during development and during remodeling necessitates stringent control of osteoblastic proliferation and differentiation. Osteoblastic lineage cells, which arise from multipotential stem cells, are categorized in a linear sequence progressing from osteoprogenitor to preosteoblast, osteoblast, and finally osteocyte.¹⁾ During primary culture of osteoblastic lineage cells derived from fetal or neonatal animals, individual differentiation stages are characterized by expression of particular proteins. For example, preosteoblasts and osteoblasts have the ability to synthesize type I collagen, alkaline phosphatase (ALP), and osteopontin (OPN), while osteoblasts in later stages express osteocalcin (OC).¹⁾ Core binding factor $\alpha 1$ (Cbfa1) has recently been identified as an essential transcription factor that stimulates osteoblastic differentiation during embryonic development.^{2–5)} Induction of osteoblastic differentiation has been shown to be mediated by various factors, including hormones, growth factors, and transcription factors.^{6, 7)}

Bone formation occurs either by membranous or endochondral ossification. In membranous ossification, mesen-

chymal cells differentiate directly into bone-forming osteoblasts, while endochondral ossification entails the conversion of an initial cartilage template into bone. During the formation of a cartilage template, mesenchymal cells are condensed and then differentiate into chondrocytes, which subsequently undergo a process of hypertrophy, calcification, and cell death. Concurrent neovascularization occurs, and osteoclasts and osteoblasts are recruited to replace the cartilage scaffold with a bone matrix.⁸⁾ Since both osteoblastic and chondroblastic lineages share common functions in endochondral ossification, their individual differentiation processes are considered to influence each other, although the exact relationship between the two lineages remains unclear.¹⁾

Transplantable osteosarcoma cell lines with ossifying ability would be useful for study of the ossification process *in vivo* and to clarify factors derived from such cells and host cells that are involved in cell-to-matrix interactions.⁹⁾ There are several human and rat osteosarcoma cell lines with bone formation,^{10–13)} but they are only transplantable into athymic mice. Only a few murine cell lines have been reported.^{14–16)}

An osteosarcoma cell line (Nishi-Hirosaki Osteosarcoma, NHOS) has recently been established from a spon-

⁴To whom correspondence should be addressed.
E-mail: kusumito@cc.hirosaki-u.ac.jp

taneous soft tissue tumor in a BALB/c mouse in this laboratory. In the present study, we demonstrated that this cell line has ossifying ability when transplanted *in vivo*. To examine the mechanism of bone formation, the expression of mRNAs encoding osteoblastic and chondroblastic markers and factors associated with ossification has been investigated.

MATERIALS AND METHODS

Cell culture The NHOS cell line had been established from a soft tissue tumor that occurred spontaneously in a female BALB/c mouse, purchased from CLEA Japan, Inc. (Tokyo) and bred under specific pathogen-free conditions at the Institute for Animal Experiments, Hirosaki University School of Medicine. Half of the tumor was used for histological examination. The remainder was minced aseptically and prepared for cultivation. The chopped tumor tissues were placed in 60-mm Petri dishes together with 3 ml of the growth medium. The cells were cloned by the colony formation technique using cloning rings. The resulting clone (NHOS cell line) was maintained over 40 passages in continuous *in vitro* culture, and stored in liquid nitrogen. The cells used in the present study were those at three passages after thawing. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂; 95% air. A growth medium consisting of RPMI-1640 (Nissui Pharmaceutical, Tokyo) was supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 units/ml of Penicillin G Potassium (Banyu Pharmaceutical, Tokyo), 100 µg/ml of Streptomycin Sulfate (Meiji Seika Kaisha, Tokyo), and 2.5 µg/ml of Amphotericin B (Life Technologies, Grand Island, NY). Contamination with mycoplasma was checked by the polymerase chain reaction (PCR) method using a PCR Mycoplasma Detection Set (TaKaRa Shuzo, Otsu) according to the manufacturer's instructions. Growth experiments were conducted by plating 2.0×10⁵ cells after trypsinization in 35-mm Petri dishes with a base area of 9 cm², using 2 ml of growth medium. The medium was changed every other day during the experiment. Three dishes were trypsinized each day, and the viable cells were counted by the trypan blue exclusion method using a hemocytometer.

Transplantation to mice and examination for ossification in tumors A cell suspension (4.0×10⁶ cells) was inoculated into the thigh muscle of 6- to 10-week-old female BALB/c mice. Five mice were analyzed individually at one, two, and three weeks after transplantation. All animal experiments in this paper followed the Guidelines for Animal Experimentation, Hirosaki University. The tumor-bearing mice were radiologically examined using a soft X-ray apparatus (Type EMB, Softex, Ebina) under anesthesia with intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, IL). Then, they were

sacrificed by cervical dislocation, and the tumors were quickly excised, weighed, and stored in liquid nitrogen. Calcium content of a transplanted tumor at three weeks or of control muscle was analyzed with a flame atomic absorption spectrophotometer (Type Z-8100, Hitachi Ltd., Hitachi). For histological examination, the tumors were fixed with 20% formalin without decalcification and embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin and eosin. Alcian blue stain at pH 2.5 and von Kossa's method were also employed to detect cartilage proteoglycan and calcium deposition, respectively.

Preparation of probes for *in situ* hybridization Complementary DNAs (cDNAs) of mouse used as probes for *in situ* hybridization (ISH) were pro-α1(I) chain of type I collagen (COL1A1), ALP, OPN, OC, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH). RNA was extracted from MC3T3-E1 cells of a newborn mouse calvaria cell line¹⁷ purchased from RIKEN Cell Bank (Tsukuba, http://www.rtc.riken.go.jp/CELL/HTML/RIKEN_Cell_Bank.html), and the cells were cultured under the conditions recommended by the cell bank. The cDNAs for individual genes were obtained from the RNA by reverse transcription (RT)-PCR using the specific primers shown in Table I. The PCR products were subcloned into plasmids using a TOPO TA Cloning Kit Dual Promoter with TOP10 Cells (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions. In these plasmids, the inserts were checked by PCR, and the sequences were confirmed with an automatic DNA sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA). The plasmids were linearized with *Spe*I and transcribed with T7 RNA polymerase to generate the digoxigenin (DIG)-labeled single strand antisense RNA probes using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and similarly linearized with *Eco*RV and transcribed with SP6 RNA polymerase to generate the sense probes. After ethanol precipitation, the probes were resuspended in distilled water with 50% deionized formamide added, and stored at -20°C.

***In situ* hybridization** Tumors dissected from mice at three weeks after transplantation were fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4, at 4°C overnight. The specimens were dehydrated and embedded in paraffin. Sections were prepared on slides under ribonuclease-free conditions, and stored at 4°C before analysis. ISH was carried out essentially as described by Hara *et al.*¹⁸ The paraffin sections were rehydrated, digested with 5 µg/ml proteinase K at 37°C for 10 min, and treated with 0.2 N HCl for 10 min. They were subsequently acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min, dehydrated and air-dried. The composition of the hybridization solution was as follows: 50% deionized formamide; 10 mM Tris-

Table I. Oligonucleotide Primers Used in RT-PCR

Gene ^{a)}	Primers	Product size (bp)	Nucleotides in accession number ^{c)}	Annealing temperature (°C)
COL1A1 ^{b)}	5'-CTGCCTGCTTCGTGTAAA 3'-ACGTTTCAGTTGGTCAAAGGTA	213	23-235 X57981	53
ALP ^{b)}	5'-ACTGCCACTGCCTACTTGTGT 3'-CAGCCCTGGCTCAGAGCCTC	266	519-784 J02980	58
OPN ¹⁹⁾	5'-CTGCACCCAGATCCTATAGC 3'-GGTCTAGGACTAGCTTGTCC	706	169-874 J04806	58
OC ²⁰⁾	5'-CAAGTCCCACACAGCAGCTT 3'-AAAGCCGAGCTGCCAGAGTT	371	8-378 X04142	68 ^{d)}
Cbfa1 ²¹⁾	5'-CCGCACGACAACCGCACCAT 3'-CGCTCCGGCCACAAATCTC	289	511-799 AF010284	58
COL2A1 ^{b)}	5'-AACACTTTCCAACCGCAGTCA 3'-TGGGCTGGGAACAGTCACTC	250	69-318 X57982	55
PTH/PTHrPR ^{b)}	5'-CTTGCCGTACACCGAGGTCTC 3'-GGCCACATTGGTCACACTC	251	1334-1584 X78936	55
BMP-2 ²²⁾	5'-GGTCTTTGCACCAAGATGAAC 3'-CAGCCCTCCACAACCATGTC	436	8490-8925 L25602	58
BMP-4 ²²⁾	5'-CCCAGAGAATGAGGTGATCTCC 3'-TGGCAGTAGAAGGCCTGGTAG	570	800-1369 X56848	58
BMP-6 ²²⁾	5'-GTGGCCTTCTTCAAGGTGAG 3'-TCATAAGGTGGACCAAGGTCTG	328	868-1195 J04566	55
BMP-7 ²²⁾	5'-AGGGCTTCTCCTACCCCTACAA 3'-CGCTCCCGGATGTAGTCCTT	250	402-651 X56906	55
BMPR-IA ^{b)}	5'-GCTACGCAGGACAATAGAATG 3'-CCGAACCATCTGAATCTGTTT	364	492-855 D16250	53
BMPR-IB ^{b)}	5'-TGGCGCTGAGCTATGACAAGA 3'-GGACAGTGGTGGTGGCATTTA	199	104-302 Z23143	55
BMPR-II ^{b)}	5'-CATGTATGCGCTTGGACTCA 3'-ATTCTGCATAGCAGTTGACAT	367	1589-1955 AF003942	53
G3PDH ²²⁾	5'-CCATCACCATCTTCCAGGAG 3'-GCATGGACTGTGGTCATGAG	322	258-579 M32599	55

a) The abbreviations of genes used: COL1A1, pro- α 1(I) chain of type I collagen; ALP, alkaline phosphatase; OPN, osteopontin; OC, osteocalcin; Cbfa1, core binding factor α 1; COL2A1, pro- α 1(II) chain of type II collagen; PTH/PTHrPR, parathyroid hormone/parathyroid hormone-related peptide receptor; BMP, bone morphogenetic protein; BMPR, BMP receptor; and G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

b) Designed with OLIGO Primer Analysis Software (National Biosciences).

c) Nucleotides numbered as in DDBJ/GenBank/EMBL accession number.

d) Annealing/extension temperature.

HCl, pH 7.6; 200 μ g/ml yeast tRNA; 1 \times Denhardt's solution; 10% dextran sulfate; 600 mM NaCl; 0.25% SDS; and 1 mM EDTA. Then, hybridization solution with DIG-labeled RNA probes, 1:10–100 diluted, was placed on each slide, and the slides were incubated in a moist chamber at 50°C for 16 h in an atmosphere saturated with 50% formamide. After hybridization, the slides were washed in 2 \times SSC/50% formamide, then digested with 10 μ g/ml ribonuclease A at 37°C for 10 min and successively washed in 2 \times SSC and 0.2 \times SSC at 50°C. Hybridized DIG-labeled probes were detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics). The slides were

counter-stained with Nuclear Fast Red (Merck KGaA, Darmstadt, Germany) and mounted with Pristin Mount (Research Genetics, Huntsville, AL).

RT-PCR Total RNAs were extracted from both cultured cells and frozen specimens of the transplanted tumors using an RNeasy Kit (Qiagen, Hilden, Germany). A one- μ g individual portion of total RNA was digested by deoxyribonuclease I and converted to first-strand cDNA by reverse transcription using a SuperScript Preamplification System (Life Technologies) with oligo(dT) priming methods in a volume of 20 μ l. The resulting cDNA (0.5 μ l) was subjected to PCR with the primers shown in Table I.

Eight of the primer sequences were derived from previously published ones,¹⁹⁻²²⁾ while the others were designed from DDBJ sequences (DNA Database of Japan, Mishima, <http://www.ddbj.nig.ac.jp/>) with OLIGO Primer Analysis Software (National Biosciences, Plymouth, MN). The templates without reverse transcription were also prepared to evaluate contamination with genomic DNA. PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems) using a GeneAmp PCR System 9600 (Applied Biosystems). The amplification conditions except for OC were as follows: initial denaturing at 95°C for 10 min, 35 cycles of denaturing at 94°C for 30 s, annealing at adequate temperatures shown in Table I for 30 s, and extension at 72°C for 30 s. The conditions for OC were as follows: initial denaturing at 95°C for 10 min, 35 cycles of denaturing at 94°C for 30 s, and annealing/extension at 68°C for 1 min. After the completion of 35 cycles, the reaction mixtures were incubated at 72°C for 7 min. Ten microliters of each PCR product were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide. Under UV light illumination, gels were photographed and recorded digitally using a charge-coupled device camera (FAS-II system, Toyobo, Osaka). For semi-quantitative assessments of RT-PCR products, the digital images of gels were densitometrically analyzed with normalization in the G3PDH band, using NIH Image, version 1.62, according to the methods described by Ide *et al.*²³⁾ and Kojima²⁴⁾ with some modification. Furthermore, the PCR products were also analyzed by direct sequencing in order to confirm their sequences. They were prepared using Centricon-100 columns (Amicon, Beverly, MA) and Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ); sequencing was performed by an automatic DNA sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

RESULTS

Characterization of cultured cells The cells seeded onto dishes began to proliferate without a noticeable lag phase. They had a doubling time of 20 h and reached the stationary phase with a saturation level of 6.5×10^5 cells/cm² after four days. In the growth phase, NHOS formed a monolayer consisting of spindle cells (Fig. 1A). While becoming confluent, the cells exhibited polygonal shapes (Fig. 1B). Neither extracellular matrix formation nor mineralization was apparent for an observation period of a month.

Ossification in the original and transplanted tumors The histological examination revealed that the original tumor had features of osteosarcoma, which is defined as a malignant tumor characterized by the direct formation of bone and/or osteoid by proliferating tumor cells (Fig. 2A).²⁵⁾ Because it originated in the soft tissue, the tumor is regarded as a murine extraskeletal osteosarcoma.²⁶⁾

After transplantation of the suspended cells, tumor masses were detected as swelling of the inoculated limb in all of the mice within five days after transplantation. Their weights were 1.7 ± 0.5 , 7.1 ± 2.5 , and 9.2 ± 1.8 g (mean \pm SD), at one, two, and three weeks, respectively. After three weeks, the tumors occupied almost all of the thigh muscle. Over four weeks, necrotic and cystic changes became prominent in the masses, and most of the transplanted mice died.

Histologically, the transplanted tumor consisted of short spindle and pleomorphic cells, exhibiting an occasional fascicular pattern. Necrosis was often found in the center of the tumors. Small foci of pale blue and homogeneous material (Fig. 2B) and basophilic networks were scattered throughout the tumor tissues at one week after transplantation. The former stained positive with alcian blue, suggesting that it was a chondroid matrix (Fig. 2C). The chondroid matrix was gradually replaced by basophilic

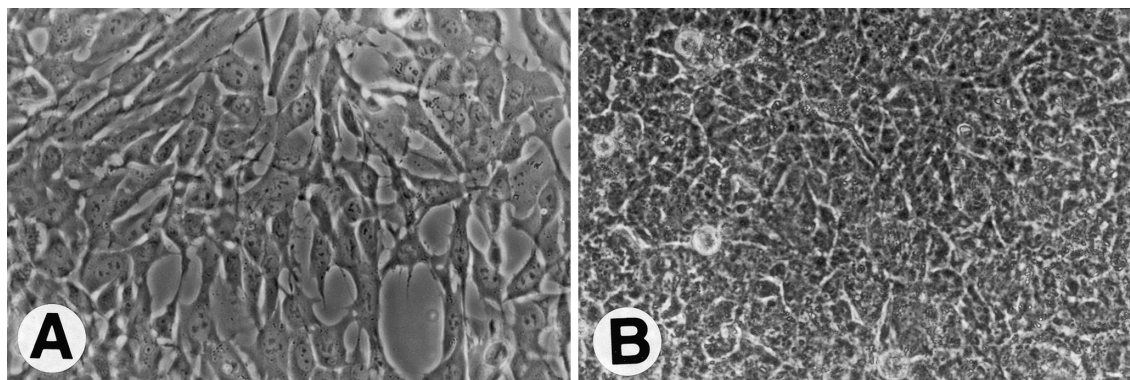


Fig. 1. Morphological features of NHOS cells in culture during the growth phase (A) and upon reaching confluency (B). Magnification $\times 170$.

networks, which occupied a large part of the tumors at two weeks as well as three weeks (Fig. 2D). These histological features were similar to those of the original tumor (Fig. 2A). The networks of transplanted tumors stained positive in von Kossa's method (Fig. 2E), indicating that they were calcified woven bones. The cells surrounding chondroid matrix showed similar pleomorphism of nuclei to those surrounding bone matrix (Fig. 2, B and D).

The calcified bones showed a cloudy texture on soft X-ray photographs. Calcification was slight in small areas of tumors at one week after transplantation (Fig. 3A), while marked calcification extended over most areas of the tumors at two and three weeks (Fig. 3B). Calcium content in a tumor at three weeks was 36 times higher than that in

muscle (8700 ppm/g versus 240 ppm/g). All transplanted mice had ossification in tumors.

Expression of genes involved in ossification in transplanted tumors Type I collagen, ALP, OPN, and OC are considered markers for osteoblastic differentiation and ossification.¹⁾ ISH using antisense RNA probes was performed to examine the expression and localization of mRNAs encoding these proteins and G3PDH in the transplanted tumor at three weeks. The mRNAs for COL1A1 (Fig. 4A), ALP (Fig. 4B), and G3PDH (data not shown) were detected extensively in almost all of the tumor cells. Strong signals of OPN mRNA were shown particularly in tumor cells surrounding the bone matrix (Fig. 4C). OC mRNA was detected in tumor cells at the ossification area,

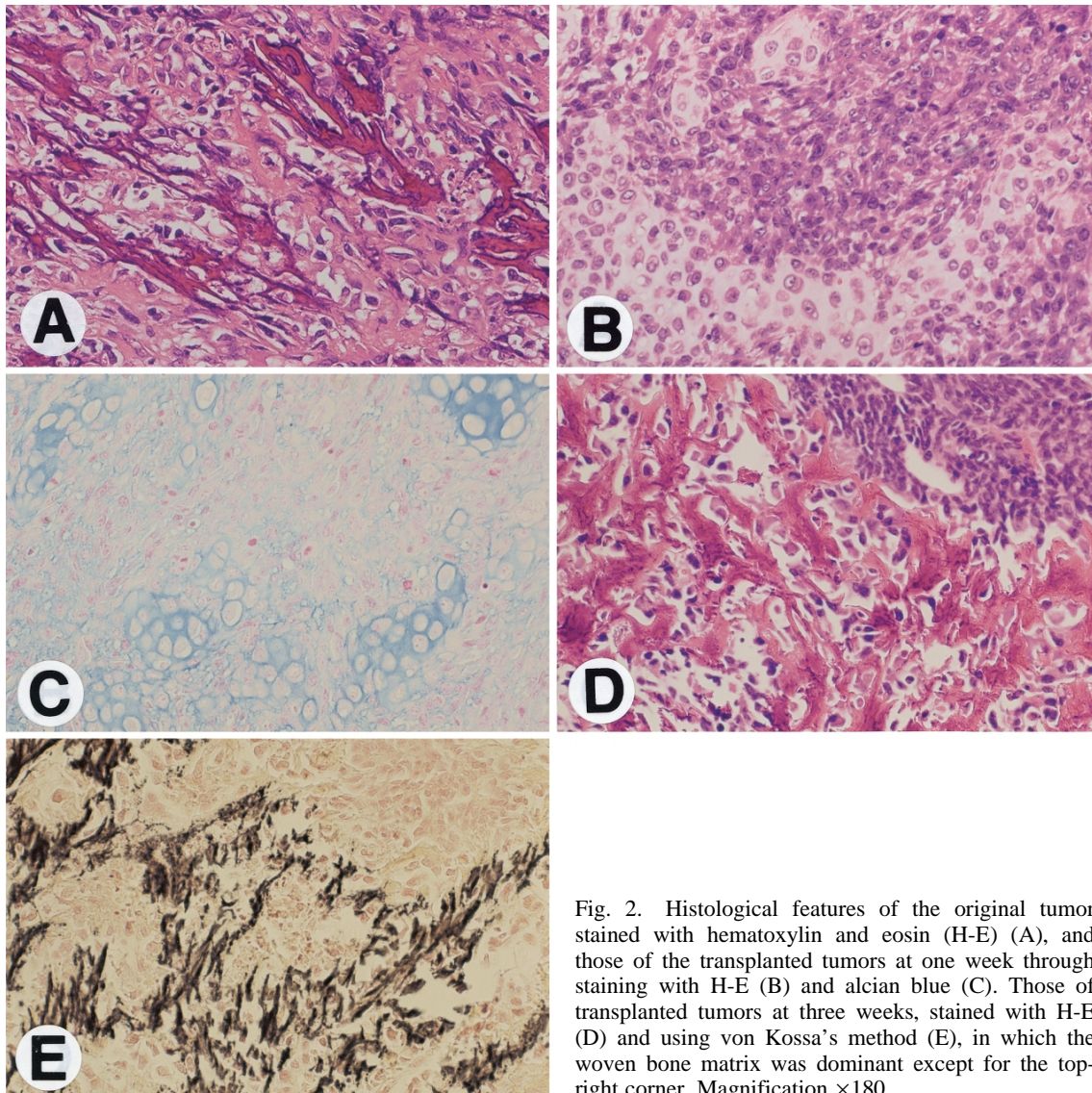


Fig. 2. Histological features of the original tumor stained with hematoxylin and eosin (H-E) (A), and those of the transplanted tumors at one week through staining with H-E (B) and alcian blue (C). Those of transplanted tumors at three weeks, stained with H-E (D) and using von Kossa's method (E), in which the woven bone matrix was dominant except for the top-right corner. Magnification $\times 180$.

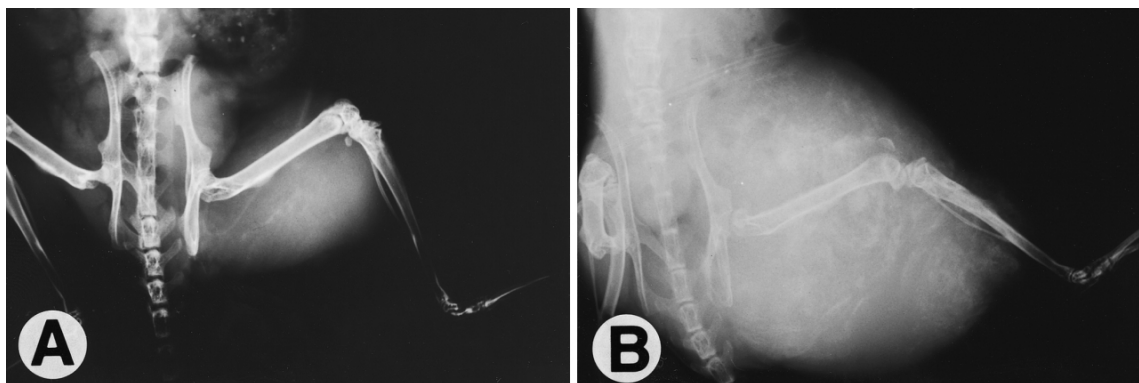


Fig. 3. Soft X-ray photographs of tumor-bearing mouse at one week (A) and three weeks (B) after transplantation. The tumor was located at the right hind limb in each mouse.

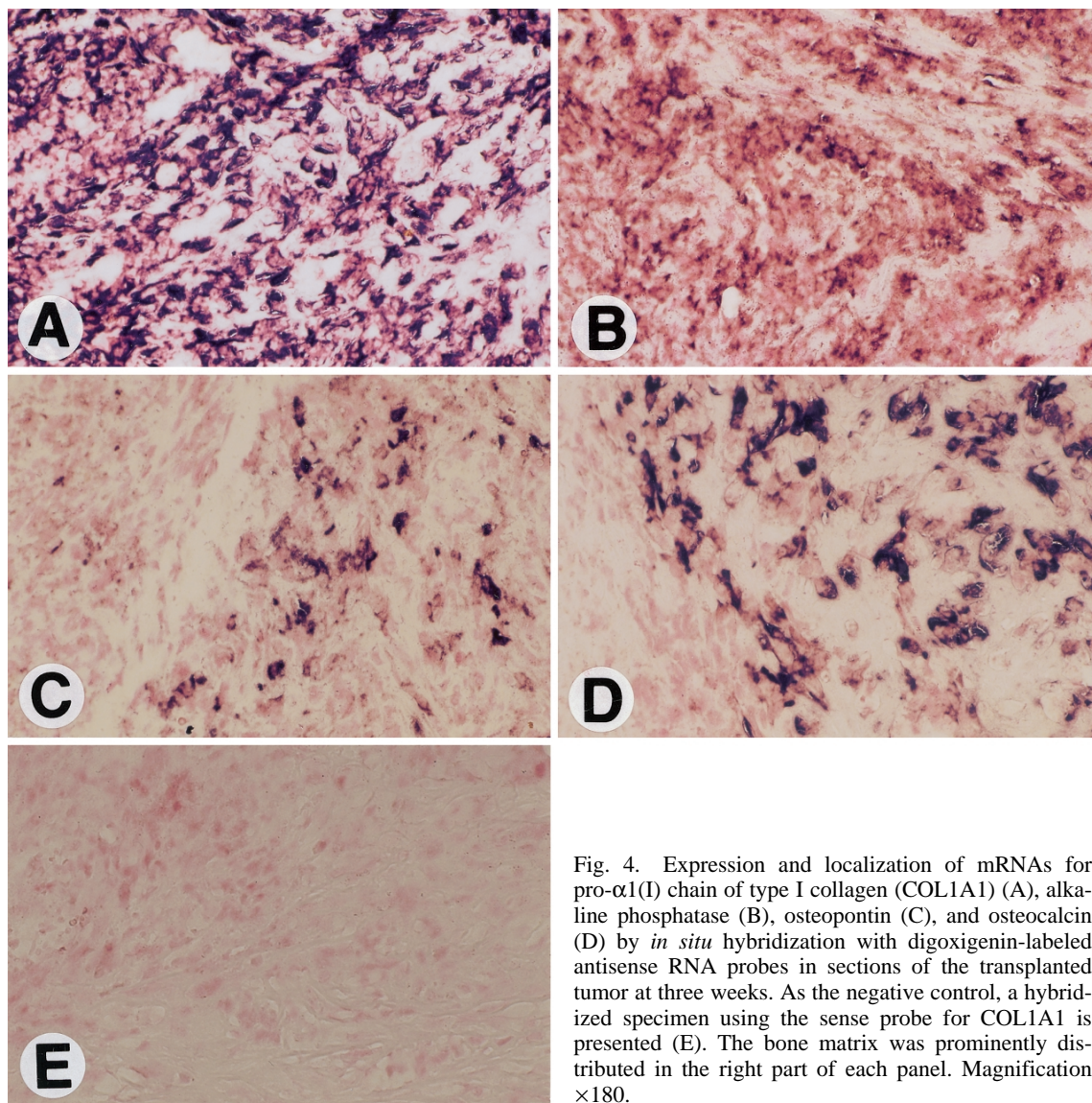


Fig. 4. Expression and localization of mRNAs for pro- α 1(I) chain of type I collagen (COL1A1) (A), alkaline phosphatase (B), osteopontin (C), and osteocalcin (D) by *in situ* hybridization with digoxigenin-labeled antisense RNA probes in sections of the transplanted tumor at three weeks. As the negative control, a hybridized specimen using the sense probe for COL1A1 is presented (E). The bone matrix was prominently distributed in the right part of each panel. Magnification $\times 180$.

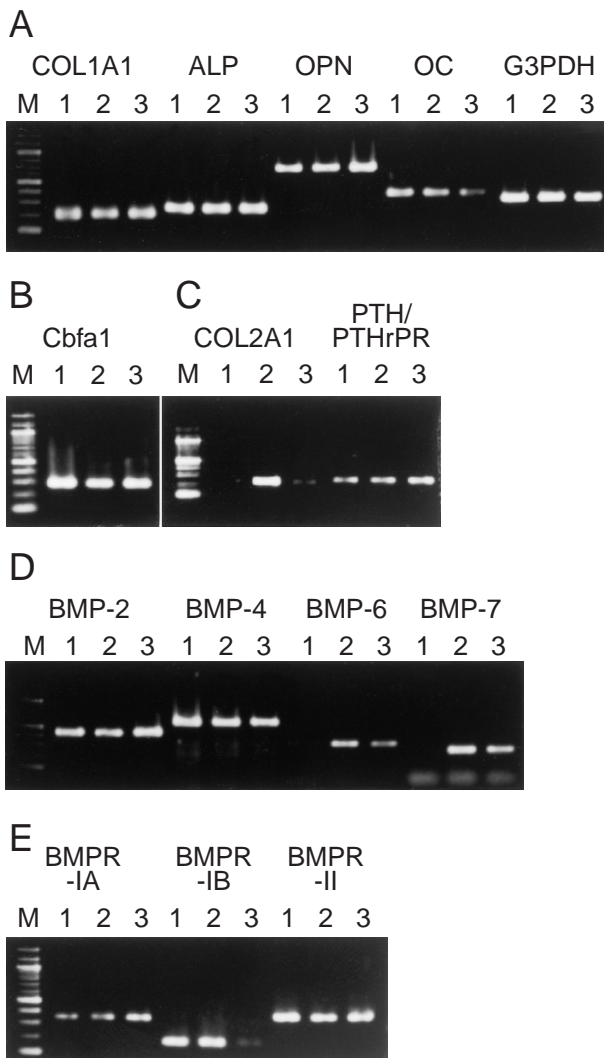


Fig. 5. Expression of mRNAs in NHOS by RT-PCR. (A) Pro- α 1(I) chain of type I collagen (COL1A1; product size of 213-bp), alkaline phosphatase (ALP; 266-bp), osteopontin (OPN; 706-bp), osteocalcin (OC; 371-bp) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH; 322-bp). (B) Core binding factor α 1 (Cbfa1; 289-bp). (C) Pro- α 1(II) chain of type II collagen (COL2A1; 250-bp) and parathyroid hormone/parathyroid hormone-related peptide receptor (PTH/PTHrPR; 251-bp). (D) Bone morphogenetic protein (BMP)-2 (436-bp), BMP-4 (570-bp), BMP-6 (328-bp) and BMP-7 (250-bp). (E) BMP receptor (BMPR)-IA (364-bp), BMPR-IB (199-bp) and BMPR-II (367-bp). Lane 1, RNA sample from cultured cells; lane 2, that from the transplanted tumors at one week; lane 3, transplanted tumors at three weeks; M, molecular weight marker (100-bp DNA ladder, New England BioLabs, Beverly, MA).

but not in the non-ossification area (Fig. 4D). ISH using sense probes did not detect these mRNAs, and the case of COL1A1 mRNA is shown in Fig. 4E.

Difference between cultured cells and transplanted tumors in expression of genes involved in ossification

RT-PCR revealed that mRNAs for COL1A1, ALP, OPN, OC, Cbfa1, and G3PDH were expressed equally among samples derived from cultured cells in the stationary phase (lane 1) and transplanted tumors at one week (lane 2) and three weeks (lane 3) (Fig. 5, A and B). Pro- α 1(II) chain of type II collagen (COL2A1) mRNA was expressed to a greater extent in the transplanted tumors at one week than at three weeks (Fig. 5C), while the parathyroid hormone/parathyroid hormone-related peptide receptor (PTH/PTHrPR) mRNA was detected equally among the three samples (Fig. 5C). No obvious differences among the samples were observed in expression levels of bone morphogenetic protein (BMP)-2, BMP-4 (Fig. 5D), BMP receptor (BMPR)-IA, and BMPR-II (Fig. 5E). BMP-6 and -7 were evidently detected in the transplanted tumors, but not in the cultured cells (Fig. 5D). BMPR-IB was expressed in cultured cells and transplanted tumors at one week, but only to a slight extent in transplanted tumors at three weeks (Fig. 5E). Semi-quantitative evaluation of RT-PCR supported differences in expression levels of COL2A1, BMP-6, BMP-7, and BMPR-IB among the samples (data not shown). The PCR products were not observed in templates without reverse transcription, and the sequences of the individual products were confirmed by direct sequencing (data not shown).

DISCUSSION

In culture, the NHOS cells changed their morphological features, when proliferation was repressed. These cells exhibited a spindle shape in the growth phase and a polygonal shape in the stationary phase. The results of RT-PCR indicated that the cultured cells expressed mRNAs encoding COL1A1, ALP, OPN, OC, and Cbfa1. It is therefore reasonable to assume that NHOS cells have the properties of the osteoblastic lineage and differentiate into stages of osteoblasts in the stationary phase. Although ossification was obscured *in vitro*, it occurred after transplantation. RT-PCR analysis revealed that the transplanted tumors at one and three weeks expressed the same levels of mRNAs for osteoblast markers as the cultured cells. However, alcian blue-positive chondroid matrix and expression of COL2A1 mRNA, a marker for chondroblastic lineage cells,²⁷⁾ were observed in transplanted tumors at one week. The chondroid matrix was replaced by calcified bone matrix and marked calcification extended over most areas of the tumors at three weeks. COL2A1 mRNA was barely detected at three weeks. ISH analysis of the transplanted tumor at three weeks showed that OPN and OC mRNAs were strongly expressed in cells around the bone matrix, suggesting that these matrix proteins are derived from transplanted tumor cells. In fact, chondroid and bone

matrices were surrounded by tumor cells with similar morphologic properties and were not detected in host tissues or at the interface between the tumor and host tissues.

Since BMPs, members of the transforming growth factor- β superfamily, are known to initiate the biological events of endochondral and membranous ossification,^{28–33} expression of BMPs and their possible receptors was examined. In NHOS, mRNAs for BMP-2, BMP-4, BMP-IA, and BMP-IB were expressed equally *in vitro* and *in vivo*, whereas BMP-6 and -7 mRNAs were expressed only in transplanted tumors, and BMP-IB mRNA decreased in transplanted tumors at three weeks. BMP-2 and -4 induce an osteoblast-like and chondroblast-like phenotype in undifferentiated mesenchymal cells of embryonic and adult origin.³⁴ These proteins are expressed in many osteosarcoma cell lines and are suggested to be associated with the survival of osteoblastic lineage cells.³⁵ BMP-6 is expressed in chondrocytes undergoing hypertrophic maturation, suggesting that BMP-6 expression may represent a signal for chondrocyte maturation and the subsequent replacement of cartilage with bone.^{36–38} The expression of BMP-6 in an osteoblastic cell line is reported to be enhanced by estrogen³⁹ or BMP-7.⁴⁰ Although the receptor for BMP-6 has not been identified, BMP-2, -4, and -7 bind to BMPRs,^{41,42} which belong to a family of serine/threonine kinase receptors.²⁸ Of the two types of BMPR-I, type-IB is necessary for the initial steps of chondrogenesis,⁴³ and type-IA is expressed in an osteoblastic cell line, MC3T3-E1.⁴⁴ In NHOS cells after transplantation, it is therefore presumed that the expression of BMP-6 and -7 may be involved in chondrocyte maturation and that BMP-IB might be involved in the signaling of BMPs at an early stage of cartilage and bone formation that is subsequently downregulated.

NHOS expressed PTH/PTHrPR mRNA in both cultured cells and transplanted tumors. Although PTH/PTHrPR is known to be associated with the differentiation of chondroblastic lineage cells,²⁷ it is reported to be expressed in osteoblasts as well as chondroblasts.⁴⁵ Moreover, following treatment with BMP-2, murine cell lines derived from embryonal limb bud have been reported to produce transiently type II collagen and subsequently to differentiate

into osteoblasts.⁴⁶ This finding is similar to a series of biological events in the transplanted tumors of NHOS in the present study. Since NHOS cells share the phenotypes of both chondroblasts and osteoblasts, at least at one week after transplantation, bone formation might reflect the process of endochondral ossification. The occurrence of bone formation only in transplanted tumors suggests that some host-derived factors may be involved in osteoblastic or chondroblastic differentiation and ossification. Expression of BMP-6 and -7 may be regulated by such factors. A number of studies have suggested that chondroblastic lineage cells may transdifferentiate into osteoblastic lineage.^{47–49} To the authors' knowledge, however, there have been no reports on transdifferentiation from osteoblastic lineage cells to chondroblasts. Since the NHOS cell line has the same nature as osteosarcoma, the regulatory mechanisms of differentiation may be disturbed, in contrast to the situation in osteoblastic lineage cells derived from fetal or neonatal animals.

In conclusion, the NHOS cell line has the properties of the osteoblastic lineage, differentiating to osteoblasts in the stationary phase *in vitro*, and exhibits an ossifying ability following transplantation. The expression of members of the BMP family of growth factors and receptors was observed in this cell line. The cells transiently manifest chondroblastic phenotype during the ossification processes. Thus, this cell line may be a useful model for studies of not only osteosarcoma, but also the ossification process, especially with *in vivo* experiments.

ACKNOWLEDGMENTS

The authors thank Dr. Akinori Kusumi, Department of Dentistry and Oral Surgery, and Professor Seiko Harata, Department of Orthopedic Surgery, Hirosaki University School of Medicine, for helpful suggestions and advice. We also thank Professor Tatsusuke Sato, Department of Medical Technology, School of Allied Medical Sciences, Hirosaki University, for technical advice.

(Received December 28, 2000/Revised March 10, 2001/Accepted March 19, 2001)

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