FULL PAPER *Pathology*

Establishment and Characterization of a Cell Line, MCO-Y4, Derived from Canine Mammary Gland Osteosarcoma

Atsushi KAWABATA[1,2], Kaori YAMAMOTO[2], Nguyen Thi LAN[1,2], Kazuyuki UCHIDA[2], Ryoji YAMAGUCHI[2], Tosiharu HAYASHI[3] and Susumu TATEYAMA[2]*

[1]*Departments of Pathogenetic and Preventive Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753–8515,* [2]*Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889–2199 and* [3]*Department of Veterinary Pathology, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753–8515, Japan*

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ABSTRACT. A cell line, MCO-Y4, was established from a mammary gland osteosarcoma of a 16-year-old female mongrel dog. Histopathologically the tumor was composed of osteoblastic cells with an osteoid meshwork and chondroid matrix. The mean doubling time of the cells at the 93rd passage was 32.39 ± 4.66 hr. Immunohistochemically, the osteoblastic and chondroblastic cells were positive for bone morphogenetic protein (BMP)-2/4 and BMP receptor (BMPR) II. The cultured cells were spindle in shape during the growth and the confluent phases. No tumor matrix was detected in the culture dish by alcian blue staining or von-Kossa silver impregnation. MCO-Y4 cells on the chamber slides showed intense immunoreactivity for BMP-2/4 and BMPR II. Noggin, an antagonist for BMP-2/4, showed the growth inhibition on MCO-Y4 cells. In addition, fibronectin might be potential for stimulating growth of MCO-Y4 cells. When transplanted into severe combined immunodeficiency mice, the cells formed tumors consisting of solid proliferation of osteoblastic and fibroblastic cells with woven-bone trabeculae. These tumor cells were intensely positive for BMP-2/4 and BMPR II. Our results suggested that the cell line might be useful for studying the role of BMPs in canine osteosarcoma and the mechanism of ossification.
KEY WORDS: bone morphogenetic protein, canine mammary gland, cell line, noggin, osteosarcoma.

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Extraskeletal osteosarcoma (EOS) is a rare malignant mesenchymal tumor characterized by osteoid production without bone or perosteal involvement. In dogs, EOS occurs mainly in older animals and is observed in several locations [23, 30], especially the mammary glands [30]. Mammary gland osteosarcoma (MGO) has aggressive biological behavior, and commonly shows pulmonary metastasis [23].

Bone morphogenetic proteins (BMPs), a subgroup of the transforming growth factor-β family [19], were originally identified as an important factor involved in endochondral ossification [33, 34, 38, 39]. BMPs also play a role in intramembranous ossification [35], and signal by binding to serine-threonine kinase receptors [40] and BMP receptor (BMPR) types I and II. The heterodimer of BMPR II with BMPR IA or IB is critical for signal transduction through Smads. The activated BMPR phosphorylates Smad 1/5/8. The Smad complex was then translocated to the nucleus and binds specific sites in DNA and associated with other regulatory proteins. Noggin, originally cloned based on its dorsaling activity in *Zenopus* embryo, has been shown to be an antagonist which has high affinity to BMPs and decreases its bioactivites [36]. Several studies suggested that BMPs might be involved in the progression or metastasis of human osteosarcoma [10, 42].

Osteosarcoma cell lines have been established from human, dog and rat, and these cell lines have the ability to undergo ossification after xenotransplantation [13, 16, 21, 29, 31]. Thus the cell lines are useful for studies of the mechanism of ossification as well as the relationship between BMPs and metastasis of osteosarcoma. A cell line, D-17, which is derived from canine osteosarcoma and used widely, however, does not have the ability to form xenotransplanted tumor in mice.

In the present study, we established a cell line from a canine MGO that showed ossification following xenotransplantation, and examined the expression of BMP-2/4 and BMPR II in both the original and xenotransplanted tumors and the cultured cells. In addition, the effect of noggin on the proliferative activity of MCO-Y4 cells was investigated, and we demonstrated that fibronectin might be potential for growth stimulation.

MATERIALS AND METHODS

Case history: A 16-year-old female mongrel dog was admitted to the Teaching Animal Hospital at University of Miyazaki with a mass in the right mammary gland. Primary bone tumors were not observed by the gloss evaluation. The tumor, measuring approximately 4 × 5 × 2 cm, was removed surgically. Grossly, the white mass was firm and divided into multiple lobules. Fresh tissue samples from the neoplastic mass were used for primary culture, and the remaining tissues were fixed for histopathology. The pathological diagnosis was established from these samples.

Histology: Most of the tissue samples were fixed in 10% formalin. For immunohistochemistry, small pieces of tissue were also fixed in methanol Carnoy's solution for 12–14 hr. All of these fixed tissues were embedded in paraffin, then sections 4 μm thick were cut and stained with hematoxylin and eosin (HE). Some selected sections were also stained with alcian blue (pH 2.5) and von-Kossa silver impregna-

tion. The neoplastic mass was diagnosed as MGO according to the World Health Organization (WHO) [24].

Establishment of the cell line: The tissue was dissected and digested at 37°C for 2 hr in a humidified atmosphere of 5% carbon dioxide in air with 4 mg/m*l* collagenase (232 U/mg Wako) in Dulbecco's Modified Eagle Medium (DMEM) and Ham's Mixture F-12 (Sigma) containing 10% fetal calf serum (FCS), 100 IU /m*l* penicillin and 100 μg/m*l* streptomycin. The tissue was cultured in 60-mm-diameter plastic dishes (Corning Coaster, Corning NY, U. S. A.) in DME/F-12 medium containing 10% FCS, 100 IU/m*l* penicillin and 100 μg/m*l* streptomycin. The culture dishes were maintained in 5% carbon dioxide in air at 37°C and observed daily by a phase-contrast microscopy. The cells were subcultured by washing them with phosphate-buffered saline (PBS) containing 2 mM ethylendiaminetetraacetic acid (EDTA) and dispersed in 0.05% trypsin with PBS containing EDTA. Then the cells were placed in a new 60-mm dish at 60 × 10 cells/m*l*. The cells were stocked in DME/F-12 containing 10% FCS and 10% dimethylsulfoxide (DMSO).

Growth assay: To better understand, the effect of noggin on the proliferative activity in MCO-Y4 cells was examined. MCO-Y4 was incubated in DME and Ham's Mixture F-12 containing 5% FCS. After 12 hr for the adhesion of the cells, 1 or 10 μg/m*l* of mouse recombinant noggin (R&D systems, Minneapolis, U.S.A.) were added to the media. The cells were detached and counted using hematocytometer after 2 days. In addition, we examined the effect of fibronectin on the proliferation of the MCO-Y4 cells. MCO-Y4 were incubated in DME and Ham's Mixture F-12 containing 5% FCS on the non-coated chamber slides (LAB-TEK, Christchurch, New Zealand) or fibronectin-coated dishes (Wako, Osaka, Japan). The cells were detached and counted using hematocytometer after 3 days.

Immunohistochemistry: Deparaffinized sections were incubated with 0.05% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity, then incubated with 3% bovine serum albumin (BSA) at 37°C for 20 min. The sections were incubated with primary antibodies against goat polyclonal human BMP-2/4 (1:20, Santa Cruz, CA, U.S.A.) and BMPR II (1:20, Santa Cruz) at 37°C for 40 min, and then sections were incubated with biotinylated rabbit serum against goat immunogloblin (1:20, DAKO-Japan, Tokyo, Japan) at 37°C for 40 min, followed by reaction with avidin-biotin-peroxidase complex (ABC) reagents (PK4000, Vectastain, Burlingame, CA, U.S.A.) at 37°C for 30 min. The attached antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St.Louis, MO, U.S.A.) and counterstained with Mayer's hematoxylin. Immunocytochemistry for BMP-2/4 and BMPRII was performed on the cells plated on the non-coated chamber slides using 10% formalin fixation for 10 min at room temperature. In the growth assay, MCO-Y4 cells in the control and high dose groups were used for immnunostaing using Envision polymer reagents (Dako-Japan). The primary antibody was against mouse monoclonal proliferating cell nuclear antigen (PCNA, prediluted clone PC10, Dako-Japan). The results of immunohistochemistry for BMP-2/4 and BMPRII were quantified by assessing the labeled cells in 10 high-power fields (× 200) using semi-quantitative analysis as follows: – = 0%, ± = 0–5%, + = 5–10%, 2+ = 10–50%, and 3+ = > 50% positive cells.

Tumorigenicity: A suspension of 10^6 cells was inoculated subcutaneously into the back of three 6-week-old female severe combined immunodeficiency (SCID) mice. Two of the mice were killed 76 days after inoculation. The studies were approved by our institutional guidelines for animal care and use committee (admission number: 2004–062–3).

Chromosomal study: The cells were incubated with 0.01 μg/m*l* of colcemid (Sigma) for 3 hr. They were then trypsinized, washed and treated with a hypotonic solution of 0.075 M potassium chloride (Wako) and fixed in methanol-acetic acid. After drying, the cells were stained with Giemsa (Merck Japan, Tokyo, Japan) and photographed for counting.

Special staining of the cell line: At confluency, the cells were fixed with ethanol for 10 min at room temperature. Chondroid matrix and mineralization were visualized using alcian blue stain and von Kossa silver impregnation.

Statistical analysis: For statistical analysis of PCNA staining, analysis of variance (ANOVA) was carried out to evaluate the difference in positive cell number between the control and low or high dose group. In addition, ANOVA was carried out to evaluate the difference in cell number between the fibronectin-coated dishes and non-coated chamber slides. The minimum level of significance was set at $P<0.05$.

RESULTS

Histopathology of the original tumor: The nonencapsulated mammary mass was divided into multiple lobules by thin fibrous septa. Each neoplastic lobule was composed of a proliferation of chondroblastic cells and osteoblastic cells in the central and peripheral areas, respectively. Some lobules had large necrotic foci. The osteoblastic cells were surrounded by an extensive osteoid meshwork or chondroid matrix (Fig. 1a), and multinucleated osteoclast-like giant cells were scattered among them. The osteoblastic cells had an irregularly shaped nucleus of varying size with several conspicuous nucleoli, and eosinophilic cytoplasm. There were approximately 2–3 mitotic figures per high-power field. No calcification was observed by von Kossa silver impregnation. The results of immunohistochemistry are summarized in Table 1. The osteoblastic and chondroblastic cells showed intense immunoreactivity for BMP-2/4 (Fig. 1b) and BMPR II (Fig. 1c).

Characterization of the established cell line: When plated on the dish, the cells had a spindle-shaped morphology in both the growth and confluent phases (Fig. 2a). Nuclear abnormalities characterized by large pleomorphic nuclei with prominent nucleoli were observed. Formation of nei-

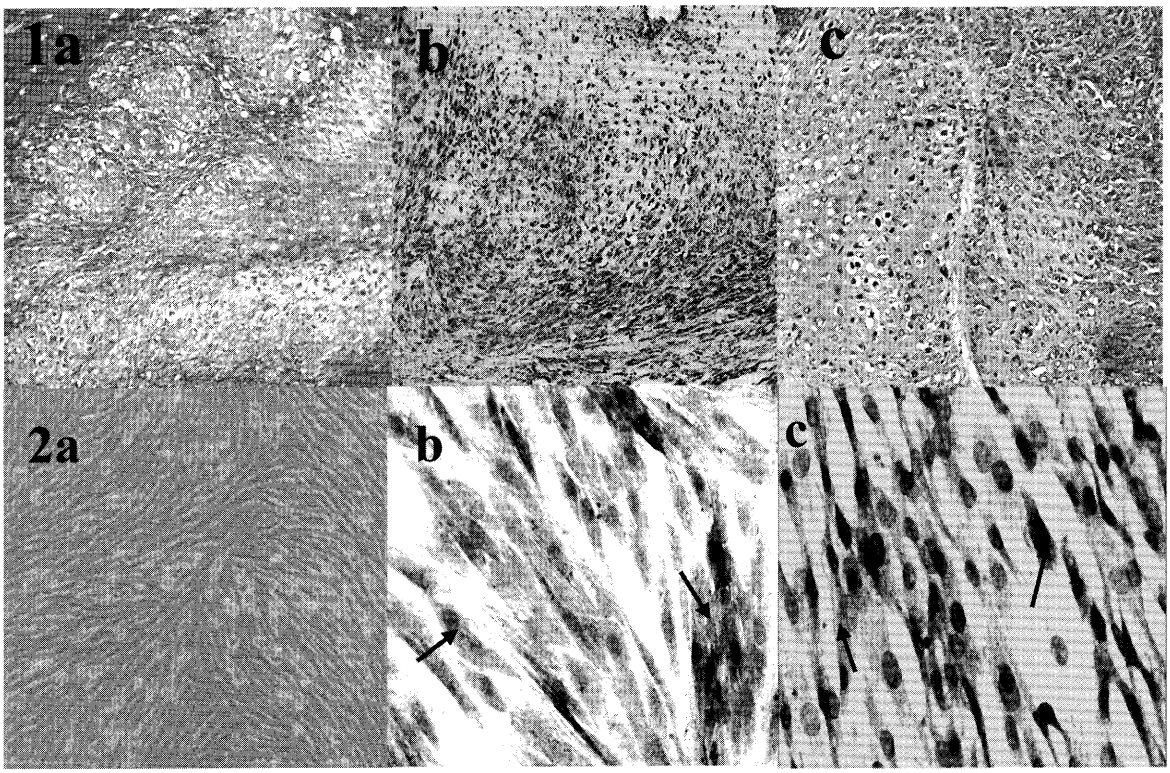

Fig. 1. Original tumor. a. The osteoblastic cells are surrounded by an extensive osteoid meshwork or chondroid matrix. HE stain. Original magnification, × 100. b. Immunostaining for BMP-2/4. The osteoblastic and chondroblastic cells are intensely positive for BMP-2/4. Original magnification, × 300. c. Immunostaining for BMPRII. The osteoblastic and chondroblastic cells are intensely positive for BMPRII. Original magnification, × 300.

Fig. 2. Cultured cells on the non-coated chamber slide. a. The cultured cells exhibit mixed cell type consisting of spindle and round cells. Original magnification, × 100. b. Immunostaining for BMP-2/4. The cultured cells are intensely positive for BMP-2/4 (arrows). Cultured cells on the non-coated chamber slide. Original magnification, × 200. c. Immunostaining for BMPR II. The cultured cells are intensely positive for BMPR II (arrows). Cultured cells on the non-coated chamber slide. Original magnification, × 200.

Table 1. Results of immunohistochemistry of original tumor and cultured cells

	Original		Cultured cells	
Antibodies	Osteoblastic cells	Chondroblastic cells	Non-coated chamber slide	Fibronectin-coated dish
BMP-2/4	3+	±	3+	3+
BMPR II	3+	±	3+	3+

– =0%, ± = 0–5%, + = 5–10%, 2+ = 10–50%, and 3+ = >50% positive cells.

ther extracellular matrix nor mineralization was observed by alcian blue staining and von Kossa silver impregnation. The mean doubling time of the cells at the 93rd passage was 32.39 ± 4.66 hr, and the cells reached a plateau on day 6 (Fig. 3). The mean number of chromosomes was 66 per cell, ranging from 46 to 99. In the chamber slides at 93rd passage, the neoplastic cells showed intense immunoreactivity for BMP-2/4 (Fig. 2b) and BMPR II (Fig. 2c).

Growth assay: Growth of MCO-Y4 cells was significantly decreased in 26.2% and 46.3% in low and high dose group, respectively (Fig. 4a). The mean number of PCNA-positive cells decreased to 11% in the high dose group compared to the control group (Fig. 4b). The mean number of MCO-Y4 cells on the non-coated dish and fibronectin-coated dish was 11 ± 0.84 and 14 ± 0.84, respectively. A significant difference between both dishes was observed (Fig. 5).

Tumorigenicity: Tumors were formed at the sites of injection of the cell line 8 weeks after inoculation in two SCID mice. These tumors measured 2 × 1 × 1.5 cm and 2 × 1.5 × 1 cm, respectively. Histologically, each tumor was composed of centrally located woven-bone trabeculae with proliferation of osteoblastic or fibroblastic cells. The osteoblastic cells were irregularly round in shape with mul-

Fig. 3

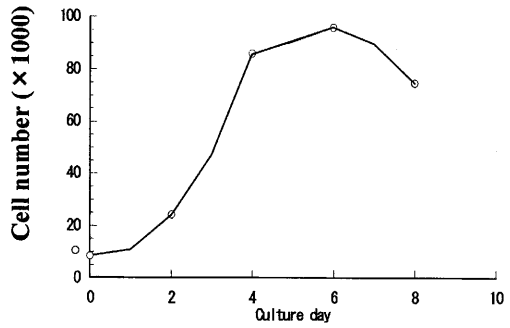

Fig. 4a

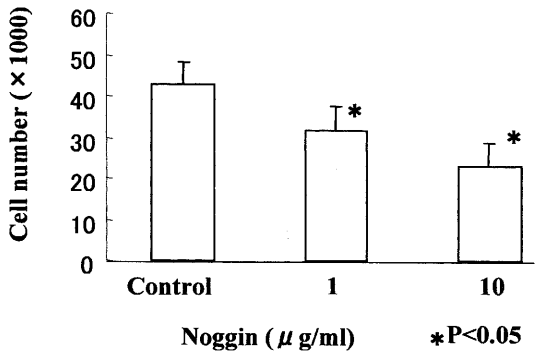

Fig. 4b

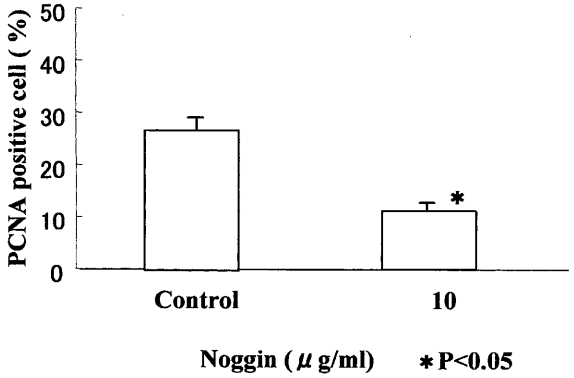

Fig. 5

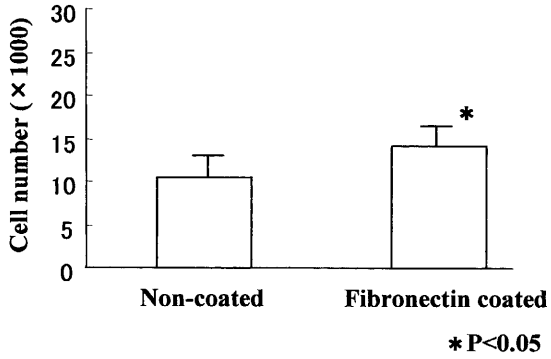

Fig. 3. Growth curve of MCO-Y4 at 93 rd passage.
Fig. 4. The effect of noggin on MCO-Y4 cells. a. Noggin inhibits growth of MCM-K1 cells. b. PCNA-positive cells are significantly decreased in 10 μg/ml group.
Fig. 5. The effect of fibronectin on MCO-Y4 cells. Fibronectin induces a slight growth stimulation in MCO-Y4 cells.

tiple nucleoli and had scanty eosinophilic cytoplasm. The fibroblastic cells showed a fascicular and/or interlacing growth pattern and were characterized by irregular nuclei with abundant eosinophilic cytoplasm. In the central area of the tumor, the intratrabecular stroma consisted of osteoblastic cells and collagen products (Fig. 6a). The results of immunohistochemistry are summarized in Table 2. The osteoblastic cells lining the trabeculae were positive for BMP-2/4 (Fig. 6b) and BMPR II (Fig. 6c). The fibroblastic cells showed mild immunoreactivity for BMP-2/4 (Fig. 6d) and weak immunoreactivity for BMPR II (Fig. 6e).

DISCUSSION

Histologically the original tumor was composed of osteoblastic cells with osteoid and a chondroid matrix, arranged in a fascicular and/or interlacing pattern. The origin of osteosarcoma in mammary gland is still unclear. In human osteosarcoma in breast, several researches revealed that the origin might be associated with connective tissue elements of pre-existing benign tumors such as fibroadenoma and papilloma [5, 9, 17, 27]. In the meantime, several reports suggested that derivation of osteosarcoma in mammary gland or salivary gland which has embryological similarities with mammary gland might be an anaplastic myoepithelial cells or pluripotential cells [4, 12]. Although immunohistochemical features of the xenotransplanted tumors were similar to those of the original tumor, the morphology in SCID mice was not representative of the original tumor. The results suggested that MCO-Y4 could form a well-differentiated tumor with bone formation after xenotransplantation. Some human osteosarcoma cells have no ability to form bone in xenotransplanted tumor [2, 25]. Mechanism of bone formation is still unclear. This unique feature of the MCO-Y4 cells could be a useful tool for the study of ossification as well.

Interactions between integrin receptors and fibronectin have been said to be important for osteoblast differentiation [25, 26]. Some studies suggested that integrin might mediate signal transduction associated with differentiation, proliferation and matrix remodeling [1, 6, 15]. In humans, Nissinen *et al.* [28] have suggested that α2 integrin might be associated with progression of osteosarcoma. In the present study, fibronectin might be potential for stimulating growth

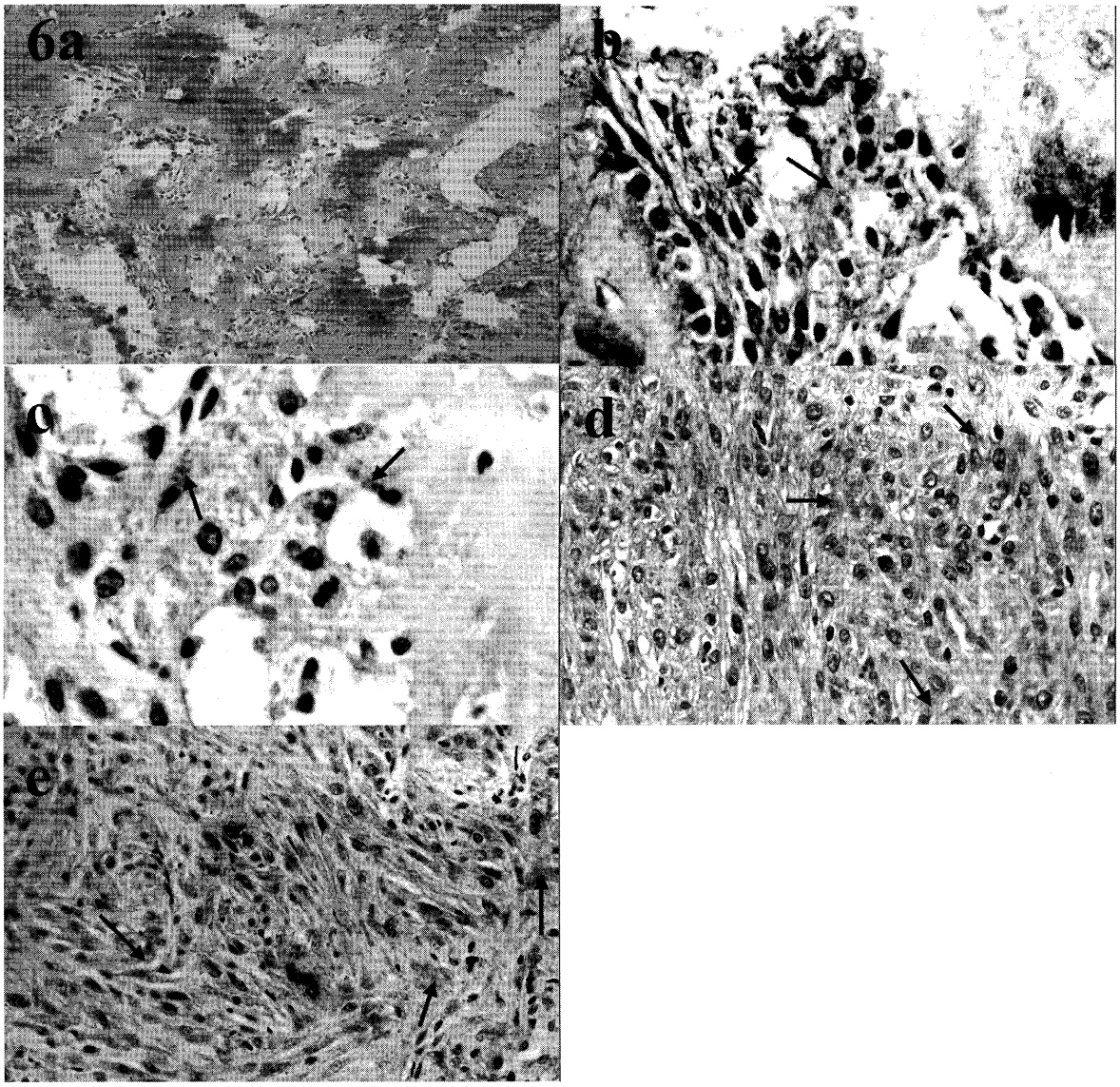

Fig. 6. Transplanted tumor. a. The formation of woven-bone trabeculae is observed. HE stains. Original magnification, × 100. b. Immunostaining for BMP-2/4. The osteoblastic cells are intensely positive for BMP-2/4 (arrows). Original magnification, × 400. c. Immunostaining for BMPRII. The osteoblastic cells are intensely positive for BMPR II (arrows). Original magnification, × 400. d. Immunostaining for BMP-2/4. The fibroblastic cells show mild immnunoreactivity for BMP-2/4 (arrows). Original magnification, × 200. e. Immunostaining for BMPRII. The fibroblastic cells show weak immunoreactivity for BMPRII (arrows). Original magnification, × 200.

Table 2. Results of immunohistochemistry of xenotransplanted tumor

	Xenotransplantation No. 1		Xenotransplantation No. 2	
Antibodies	Osteoblastic cells	Fibroblastic cells	Osteoblastic cells	Fibroblastic cells
BMP-2/4	3+	+	3+	+
BMPR II	3+	±	3+	±

– =0%, ± = 0–5%, + = 5–10%, 2+ = 10–50%, and 3+ = >50% positive cells.

of MCO-Y4 cells, suggesting that MCO-Y4 cells could be used for study on interaction between proliferative activity of osteosarcoma and fibronectin.

No metastasis of the inoculated cells was observed in the SCID mice in the present study. Many factors are reported to be involved in the metastasis or progression of tumors. In human osteosarcoma, some proteases are suggested to be important for invasion or metastasis of osteosarcoma cells [7, 20]. Yoshikawa *et al.* [42] have reported that BMPs are the potential factors involved in the metastasis or progression of osteosarcoma. In the present study, the xenotransplanted tumor showed mild immunoreactivity for BMP-2/4 and intense immunoreactivity for BMPR II. Moreover in human melanoma, Rothhammer *et al.* [32] suggested that BMP-2, -4, and -7 might be important factors for tumor invasion or migration. Although we were unable to obtain any evidence for a link between the expression of BMP-2/4 or BMPR II and the lack of metastatic lesions observed in the present study, MCO-Y4 might be useful for further investigations of the association between BMPs and invasion or migration of osteosarcoma.

BMP-6 has been shown to induce apoptosis in the epidermis of murine skin [37]. Hamdy *et al.* [11] suggested that high levels of BMP-6 expression might be associated with bone formation in bone metastases from prostate cancer. Therefore, BMPs, BMPRs and Smads have been considered to be important factors for suppression, rather than progression or metastasis, of prostate and cutaneous tumors [14, 18, 22]. However, expressions of BMPs and their receptors have been confirmed in a human osteosarcoma cell line, suggesting a mechanism involving the simultaneous activation of BMPs and BMPRs in osteosarcoma [8]. In addition, expression of BMP-2 and BMPR II in osteosarcoma has been shown to be associated with poor prognosis [10, 41]. The present study demonstrated that the fibroblastic cells in the xenotransplanted tumor and the cultured cells were positive for BMP-2/4 and BMPR II and that no tumor matrix was present in the culture dishes. These observations suggested that the intense immunoreactivity for BMPs and BMPR II in the neoplastic cells might be associated with not only bone formation and calcification but also some other role in the neoplastic cells. The growth assay revealed that noggin might be potential for inhibiting the cell proliferative activity in MCO-Y4 cells. This result supported the hypothesis that BMP-4 might be an important factor for the malignant behavior of osteosarcomas [3]. Moreover, our results suggested that fibronectin might cause growth stimulation in MCO-Y4 cells. Further experiments will be needed to clarify the role of BMPs and BMPR II in canine MGO.

In conclusion, our MCO-Y4 that has been newly established from a canine MGO is characterized by proliferation of cells and ossification ability in the xenotransplanted tumor. Moreover our results suggested that BMP signal and fibronectin might play roles in proliferative activity in MCO-Y4 cells. Hence, MCO-Y4 cells might be useful for the further study of progression and malignant behavior of canine osteosarcomas.

REFERENCES

1. Adams, J., C. and Watt, F. M. 1993. Regulation of development and differentiation by extracellular matrix. *Development* **117**: 1183–1198.
2. Anderson, H. C., Hsu, H.H., Raval, P., Hunt, T. R., Schwappach, J. R., Morris, D. C. and Schneider, D. J. 1995. The mechanism of bone induction and bone healing by human osteosarcoma cell extracts. *Clin. Orthop.* **313**: 129–134.
3. Asai, T., Ueda, T., Itoh, K., Yoshioka, K., Aoki, Y., Mori, S. and Yoshikawa, H. 1998. Establishment and characterization of a murine osteosarcoma cell line (LM8) with high metastatic potential to the lung. *Int. J. Cancer* **76**: 418–422.
4. Auclair, P. L., Langloss, J. M., Weiss, S. W. and Corio, R. L. 1986. Sarcomas and sarcomatoid neoplasms of the major salivary gland regions. A clinicopathologic and immunohistochemical study of 67 cases and review of literature. *Cancer* **58**: 1305–1315.
5. Benediktsdottir, K., Lagerberg, F., Lundell, L. and Thulin, A. 1980. Osteogenic sarcoma of the breast. Report of a case. *Acta. Pathol. Microbiol. Scand.* **88A**: 161–165.
6. Damsky, C. H. and Werb, Z. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* **4**: 772–781.
7. Fisher, J. L., Mackie, P. S., Howard, M. L., Zhou, H. and Choong, P. F. M. 2001. The expression of the urokinase plasminogen activator system in metastatic murine osteosarcoma: An *in vivo* mouse model. *Clin. Cancer Res.* **7**: 1654–1660.
8. Gobi, G., Sangiorgi, L., Lenzi, L., Casadei, R., Canaider, S., Strippoli, P., Lucarelli, E., Ghedini, I., Donati, D., Fabbri, N., Warzecha, J., Yeoung, C., Helman, L. J., Picci, P. and Carinci, P. 2002. Seven BMPs and all their receptors are simultaneously expressed in osteosarcoma cells. *Int. J. Oncol.* **20**: 143–147.
9. Going, J. J., Lumsden, A. B. and Anderson, T. J. 1986. A classical sarcoma of the breast: histology, immunohistochemistry and ultrastructure. *Histopathology* **10**: 631–641.
10. Guo, W., Gorlick, R., Ladanyi, M., Meyers, P. A., Huvos, A. G., Bertino, J. R. and Healey, J. H. 1999. Expression of bone morphogenetic proteins and receptors in sarcoma. *Clin Orthop.* **365**: 175–183.
11. Hamdy, F. C., Autzen, P., Robinson, M. C., Horne, C. H. W., Neal, D. E. and Robson, C. N. 1997. Immunolocalization and messenger RNA expression of bone morphgenetic protein-6 in human benign and malignant prostatic tissue. *Cancer Res.* **57**: 4427–4431.
12. Hellmen, E. and Lindgren, A. 1989. The expression of intermediate filaments in canine mammary glands and their tumors. *Vet. Pathol.* **26**: 420–428.
13. Hong, S-H., Kadosawa, T., Mochizuki, M., Matsunaga, S., Nishimura, R. and Sasaki, N. 1998. Establishment and characterization of two cell lines derived from canine spontaneous osteosarcoma. *Jpn. J. Vet. Med. Sci.* **60**: 757–760.
14. Horvath, L. G., Henshall, S. M., Kench, J. G., Turner, J. J., Golovsky, D., Brenner, P. C., O'Neil, G. F., Kooner, R., Stricker, P. D., Grygiel, J. J. and Sutherland, R. L. 2004. Loss of BMP-2, Smad 8 and Smad 4 expression in prostate cancer progression. *Prostate* **59**: 234–242.
15. Huhtala, P., Humhries, M. J., McCarthy, J. B., Tremble, P.M., Werb, Z. and Damsky, C. H. 1995. Cooperative signaling by alpha 5 beta1 and alpha 4 beta1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J. Cell. Biol.* **129**: 867–879.
16. Kawai, A. 1990. A newly established human osteosarcoma cell

line with osteoblastic properties. *Clin. Orthop.* **259**: 256–267.
17. Killick, S. B. and McCann, B. G. 1995. Osteogenic sarcoma of the breast associated with fibrosarcoma. *Clin. Oncol.* **7**: 132–133.
18. Kim, Y. I., Lee, D-H., Ahn, H-J., Tokunaga, H., Song, W., Devereaux, M. L., Jin, D., Sampath, K. T. and Morton, A. R. 2000. Expression of bone morphogenetic protein receptors Type-IA, -IB, and II correlates with tumor grade in human prostate cancer tissues. *Cancer Res.* **60**: 2840–2844.
19. Kingsley, D. M. 1994. TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organism. *Genes Dev.* **8**: 133–146.
20. Krueger, S., Haeckel, C., Buehling, F. and Roessner, A. 1999. Inhibitory effects of antisense cathepsin B cDNA transfection on invasion and motility in human osteosarcoma cell line. *Cancer Res.* **59**: 6010–6014.
21. Kusumi, T., Nishi, T., Tanaka, M., Tsuchida, S. and Kudou, H. 2001. A murine osteosarcoma cell line with a potential to ossification upon transplantation. *Jpn. J. Cancer Res.* **92**: 649–658.
22. Kwan, K. M., Li, A. G., Wang, X-J., Wurst, W. and Behringer, R., R. 2004. Essential roles BMPR-1A signaling in differentiation and growth of hair follicles and in skin tumorigenesis. *Genesis* **39**: 10–25.
23. Langenbach, A., Anderson, M. A., Dambach, D. M., Sorenmo, K. U. and Shofer, F. D. 1998. Extraskeltal osteosarcoma in dogs: a retrospective study of 169 cases (1986–1996). *J. Am. Ani. Hosp. Assoc.* **34**: 113–120.
24. Misdorp, W., Else, W., Hellmen, E. and Lipscomb, T. P. 1999. Histological classification of the mammary tumors of the dog and the cat. pp. 1–59, *In*: World Health Organization International Histological Classification of Tumors of Domestic Animals second series, vol.7, AFIP, Washington, D.C.
25. Moursi, A. M., Damsky, C. H., Lull, J., Zimmerman, D., Doty, S. B., Aota, S. and Globus, R. K. 1996. Fibronectin regulates calvarial osteoblast differentiation. *J. Cell Sci.* **109**: 1369–1380.
26. Moursi, A. M., Globus, R. K. and Damsky, C. H. 1996. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation *in vitro*. *J. Cell Sci.* **110**: 2187–2199.
27. Nishida, Y., Kohno, F., Furuya, Y., Nakatani, T.,. Kaneko, S., Sashikata, T., Fujiwara, O. and Saitoh, Y. 1989. Mammary fibroadenoma showing osseous metaplasia: a case report. *Jpn. J. Cancer Clin.* **35**: 1461–1465.
28. Nissimen, L., Westermarck, J., Koivisto, L., Kahari, V-M. and Heino, J. 1998. Transcription of α2 integrin gene in osteosarcoma cells is enhanced by tumor promoters. *Exp. Cell Res.* **243**: 1–10.
29. Patride, N. C., Alcorn, D., Michelageli, V. P., Ryan, G. and Martin, T. J. 1983. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. *Cancer Res.* **43**: 4308–4314.
30. Patnaik, A. K. 1990. Canine extraskeltal osteosarcoma and chondrosarcoma: a clinicopathologic study of 14 cases. *Vet.Pathol.* **27**: 46–55.
31. Raval, P., Hsu, H. T. T., Schneider, D. J., Sarras, M. P. Jr., Masuhara, K., Bonewald, L. F. and Anderson, H. C. 1996. Expression of bone morphogenetic proteins by osteoinductive and non-osteoinductive human osteosarcoma cells. *J. Dent. Res.* **75**: 1518–1528.
32. Rothhammer, T., Poser, I., Soncin, F., Batalle, F., Moser, M. and Bosserhoff, A.K. 2005. Bone morphogenetic proteins are overexpressed in malignant melanoma and promote cell invasion and migration. *Cancer Res.* **65**: 448–456.
33. Sampath, T. K. and Reddi, A. H. 1981. Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **78**: 7599–7603.
34. Sasano, Y., Mizoguguchi, I., Takahashi, I., Kagayama, M., Saito, T. and Kuboki, Y. 1997. BMPs induce endochondral ossification in rats when implanted ectopiocally within a carrier made of fibrous glass membrane. *Anat. Rec.* **247**: 472–478.
35. Sasano, Y., Ohtani, E., Narita, K., Kagayama, M., Muruta, M., Saito, T., Shigenobu, K., Takita, H., Mizuno, M. and Kuboki, Y. 1993. BMPs induce direct bone formation in ectopic sites independent of the endochondral ossification *in vivo*. *Anat. Rec.* **236**: 373–380.
36. Smith, W. C. and Harland, R. M. 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**: 829–840.
37. Wach, S., Schimacher, P., Protschka, M. and Blessing, M. 2001. Overexpression of bone morphogenetic protein-6 (BMP-6) in murine epidermis suppresses skin tumor formation by induction of apoptosis and downregulation of fos/jun family members. *Oncogene* **53**: 7761–7769.
38. Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., Luxenberg, D. P., Sibley, B. S. and Wozney, J. M. 1988. Purification and characterization of other distinct bone-inducing factors. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 9484–9488.
39. Wang, E. A., Rosen, V., D'Alessandro, J. S., Bauduy, M., Cordes, P., Luxenberg, D. M., McQuaid, D., Moutsatsos, I. K., Nove, J. and Wozney, J. M. 1990. Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. U.S.A.* **87**: 2220–2224.
40. Yamashita, H., Ten Dijke, P., Heldin, C. H. and Miyazono, K. 1996. Bone morphogenetic protein receptors. *Bone* **19**: 569–574.
41. Yoshikawa, H., Rettig, W. J., Takaoka, K., Alderman, E., Rup, B., Rosen, V., Wozney, J. M., Lane, J. M., Huveos, A. G. and Garin-Chesa, P. 1994. Expression of bone morphogenetic proteins in human osteosarcoma: immunohistochemical detection with monoclonal antibody. *Cancer* **74**: 842–847.
42. Yoshikawa, H., Nakase, T., Myoui, A. and Ueda, T. 2004. Bone morphogenetic proteins in bone tumors. *J. Orthop. Sci.* **9**: 334–340.