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IGF-1 Receptor Contributes to The Malignant Phenotype in Human and Canine Osteosarcoma

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Abstract To further define the role of insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) in osteosarcoma (OS), human OS cell lines with low (SAOS-2) and high (SAOS-LM2) metastatic potential and three canine OS-derived cell lines were studied. Cell lines were evaluated for: IGF-1R expression; expression of IGF binding proteins (IGFBPs); effect of IGF-1 on tumor cell growth, invasion, expression of urokinase plasminogen activator (uPA), and soluble uPA receptor (suPAR), and; ectopic and orthotopic tumorigenicity of the canine OS cells in athymic mice. All cell lines exhibited steady-state mRNA expression of IGF-1R. The SAOS-2 and SAOS-LM2 cells expressed 9,138 and 10,234 cell-associated binding sites, respectively. Canine OS cells expressed from 1,728 to 3,883 binding sites. Two IGF-1-treated cell lines displayed enhanced proliferation. Two cell lines formed colonies in semisolid media, and IGF-1 increased colony number. Matrigel invasion was enhanced in one cell line following IGF-1 treatment. uPA and suPAR were unchanged in SAOS-2 and SAOS-LM2 cells following IGF-1 treatment, but the highly metastatic OS line SAOS-LM2 expressed five times more suPAR and displayed enhanced invasion compared to the parental, low metastatic SAOS-2. IGFBP-5 was detected in four of five cell lines, and IGFBP-3 was detected in two canine OS cell lines. Two canine OS lines were tumorigenic, and one metastasized spontaneously. In conclusion, OS cells express IGF-1R, which can contribute to their growth and invasion. There is suggestive evidence that increasing receptor number may contribute to in vivo tumorigenesis. Additional studies are needed to determine how IGF-1/IGF-1R interactions contribute to the malignant phenotype of OS. J. Cell. Biochem. 92: 77–91, 2004.

Key words: growth factor; IGF-1R; sUPAR; invasion; metastasis; IGF binding protein; dog; bone

Osteosarcoma (OS) is a highly malignant and metastatic cancer, usually diagnosed in children and young adults [Cordon-Cardo, 1997]. Despite improvements in treatment, about 40% of patients eventually succumb to metastatic disease. A large number of growth factors, such as platelet-derived growth factor, hepatocyte growth factor (HGF), and insulin like growth factor-1 (IGF-1) have been shown to influence tumor growth and invasion, and to contribute to the malignant phenotype for various tumors, including OS [Radinsky, 1991; Galimi et al., 1993; Weiner et al., 1994; Alman et al., 1995; Rong et al., 1995; Rubin and Baserga, 1995; Maier et al., 1996].

IGF-1 is a polypeptide that exhibits structural homology to proinsulin and exerts growth promoting and metabolic effects. The IGFs are the most abundant growth factors in bone and contribute about 50% to basal bone cell proliferation [Canalis et al., 1988]. Relative to other growth factors, the IGFs are abundant in the

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circulation. Osteoblasts and OS cells in culture have Type I IGF receptors, and can proliferate in response to IGF-1 [Kappel et al., 1994]. Activation of IGF-1R regulates tumor growth, both in vitro and in vivo, by at least four mechanisms: (a) it is mitogenic; (b) it plays a role in the establishment of the transformed phenotype; (c) it protects cells from apoptosis, and; (d) it promotes angiogenesis [Kaleko et al., 1990; Harrington et al., 1994; Baserga, 1995; Rubin and Baserga, 1995; Goad et al., 1996; Reinmuth et al., 2002].

The IGF-1 receptor belongs to the small family of homologous receptors that include the insulin receptor and an orphan receptor-related receptor [Shier and Watt, 1989]. The receptor exists as an $\alpha_2-\beta_2$ heterodimer, with several $\alpha-\alpha$ and $\alpha-\beta$ disulfide bridges. The tyrosine kinase domains of the IGF-1 and insulin receptor are over 80% homologous [Baserga, 1995].

Other investigators [Sell et al., 1994; Baserga, 1995] have shown that IGF-1R is not required for mouse NIH 3T3 fibroblast growth, but is required for 3T3 oncogene transformation, an event necessary for immortalization. In another study, a neuroblastoma cell line with minimal expression of IGF-1R displayed morphologic alterations and partial loss of contact inhibition when transfected with IGF-1R [Singleton et al., 1996]. Furthermore, IGF-1 has also been shown to protect OS and other cells from apoptosis [Sell et al., 1995; Herzlieb et al., 2000; Schmid et al., 2001]. Overexpression of IGF-1R in pancreatic tumor cells has been shown to increase invasiveness and metastatic potential [Lopez and Hanahan, 2002], and IGF-1R is upregulated in breast cancer cells capable of brain metastasis [Nishizuka et al., 2002]. Despite these findings, two studies have failed to demonstrate an effect of IGF-1 or IGF-1R expression on the clinical behavior of human OS [Burrow et al., 1998; Rodriguez-Galindo et al., 2001].

Inhbition of IGF-1R expression or function in melanoma cells results in inability to grow in soft agar and decreased viability [Resnicoff et al., 1994; All-Ericsson et al., 2002]. Antisense strategies blocking IGF-1R expression induce growth inhibition, decrease tumorigenesis, and decrease metastasis in human and murine carcinomas [Long et al., 1995; Chernicky et al., 2000; Sun et al., 2001], and induce tumor regression or apoptosis in rhabdomyosarcoma [Shapiro et al., 1994], Ewing's sarcoma [Scotlandi et al., 2002], and central nervous system tumors [Singleton et al., 1996; Liu et al., 1998; Andrews et al., 2001]. Antibodies directed at the IGF-1R, antisense oligonucleotides targeted to IGF-1R mRNA, and strategies reducing systemic IGF-1 levels have been reported to inhibit proliferation and tumor xenograft growth in OS and colon cancer [Hirschfeld and Helman, 1994; Pinski et al., 1995, 1996; Reinmuth et al., 2002]. Dominant negative mutants of the IGF-1R can also reverse the transformed phenotype or inhibit tumorigenesis or metastasis [Prager et al., 1994; Dunn et al., 1998]. Thus, IGF-1 and IGF-1R are compelling targets for anticancer therapeutic strategies.

The activity of IGF-1 is influenced by IGF binding proteins (IGFBPs). The IGFBPs can have inhibitory, stimulatory, and IGF-independent effects on cell growth [Clemmons et al., 1993]. Seven IGFBPs have been identified [Shimasaki and Ling, 1991; Oh et al., 1996]. IGFBP-3 is the most abundant in serum and plays a key role in IGF-1 bioavailability. IGFBP-3 has been shown to inhibit cell growth, to induce apoptosis, and to mediate contact inhibition, in an IGF-dependent or independent fashion, depending on cell line [Cohen et al., 1993; Oh et al., 1993; Rajah et al., 1997; Schmid et al., 2001]. However, IGFBP-3 has also been shown to be mitogenic for UMR-106.01 rat OS cells in culture [Slootweg et al., 1995]. OS cells have been shown to express IGFBP-2, IGFBP-3, IGFBP-4, and IGFPB-5 [Cohen et al., 1993; Lalou et al., 1994; Zumkeller et al., 1996]. Gene expression of IGFBP-3 is influenced by TGF-B_1 [Oh et al., 1995], retinoic acid [Gucev et al., 1996; Zumkeller et al., 1996], TNF-α [Yateman et al., 1993], and p53 [Buckbinder et al., 1995], as well as by IGF-1 itself [Lalou et al., 1994; Rosato et al., 2001].

The regulation of pathologic local bone destruction by OS and the proteolytic enzymes involved are largely unknown. OS cells can produce various metalloproteinases as well as urokinase type plasminogen activator (uPA) [Fawthrop et al., 1992; Kariko et al., 1993; Hackel et al., 1994; de Bart et al., 1995], which is upregulated by interleukin-1α [de Bart et al., 1995], transforming growth factor-alpha, epidermal growth factor (EGF) [Mars et al., 1996], HGF [Rosen et al., 1994; Jeffers et al., 1996; Paciucci et al., 1998], and IGF-1 [Dunn et al., 2000]. uPA is a serine protease, and when bound

to its high-affinity receptor (uPAR), is thought to be involved in tissue remodeling and cell migration processes [Blasi, 1993]. In addition, uPA plays a central role in catalyzing extracellular matrix-basement membrane (ECM/BM) degradation and there is a strong association between uPA expression and the invasive-metastatic phenotype. Normal rat and human osteoblasts produce low levels of uPA and tissue-type plasminogen activator [Hoekman et al., 1991; Fawthrop et al., 1992]. The involvement of uPA in tumor development and metastasis has been demonstrated both in vivo and in vitro [Mignatti et al., 1986; de Vries et al., 1994], and uPA has been shown to participate in the local invasion and metastasis of murine and human OS [Hackel et al., 1994, 1998; Fisher et al., 2001; Kushlinskii et al., 2001].

Canine OS is a spontaneous malignant and highly metastatic tumor occurring most commonly in large breed dogs, and is a superb model for human OS [Vail and MacEwen, 2000]. Despite amputation and adjuvant chemotherapy, 75% of dogs die of metastasis within 2 years [Dernell et al., 2001]. Using human and canine OS cells, the objectives of this study were to: (1) characterize OS cell lines for expression of IGF-1R, and IGFBPs; (2) determine their response to IGF-1 using growth and invasion assays, and; (3) determine if IGF-1R expression correlates with/contributes to the malignant phenotype following in vivo transplantation in nude mice.

MATERIALS AND METHODS

Cells and Culture Conditions

D17 canine OS cells were obtained from ATCC (CCL 183), Abrams canine OS cells were kindly provided by Dr. W. Dernell, Colorado State University, and Grey canine OS cells were established in our laboratory from a spontaneous lung metastasis. The human SAOS-2 cells were obtained from ATCC (HTB 85) and the SAOS-LM2 cells were obtained from Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX). The highly metastatic SAOS-LM2 cells were developed following serial passage in nude mice as described [Radinsky et al., 1994]. The cell lines were passaged on plastic in minimal essential medium supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, twofold vitamin solution (C/10, GIBCO, Grand Island, NY), and incubated in 5% CO_2 at 37°C. All cell lines were tested for mouse viruses (MAP test, NCI-Frederick Cancer Research and Development Center, Fredrick, MD) prior to in vivo transplantation into mice.

mRNA Analysis

OS cells were cultured in C/10 or starved for 24 h in 0% FBS complete MEM to determine if serum starvation would alter IGF-1R expression. Total cellular RNA was extracted from 1×10^7 tumor cells growing in culture using a modified FastTrack™ mRNA isolation system (Invitrogen, Inc., San Diego, CA). For Northern blot analyses, poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography, fractionated on a 1% denaturing formaldehyde–agarose gel (5 μg mRNA/lane), and electrotransferred at 0.6–0.8 A to a GeneScreen nylon membrane with 120,000 μJ/cm^2 using the Stratalinker 1800 (Stratagene, La Jolla, CA). Filters were washed two to three times at 55°C with 30 mM NaCl–3 mM sodium citrate (pH7.2)–0.1% $NaDodSO_4$ (w/v) [Radinsky et al., 1995].

Hybridization Probes

The cDNA probes used were a 4.2-kilobase *Bam*HI–*Xba*I restriction endonuclease fragment from the plasmid pCVN human IGF-IR cDNA (courtesy of Dr. R. Baserga, Thomas Jefferson University, Philadelphia, PA), and a 1.3-kilobase *Pst*I gene fragment corresponding to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Fort et al., 1985]. Each cDNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, Inc., LaJolla, CA), and radiolabeled by the random primer technique using [α-^{32}P] deoxyribonucleotide triphosphates [Feinberg and Vogelstein, 1983].

Tumor Cell Proliferation Assays

Two methods were used to assess proliferation; [^{3}H]thymidine incorporation [Radinsky et al., 1990] and MTT reduction [Mosmann, 1983]. All cells were plated in quadruplicate in 10% FBS-containing complete medium at 5 or 10×10^3 cells/well into 96-well plates. The cells were allowed to adhere for 18 h, then the medium was removed, cells were washed 2× with Hank's balanced salt solution (HBSS), and medium was replaced with 0, 1, or 10% FBS-containing medium. Recombinant human (rh) IGF-1 (R & D Systems, Inc., Minneapolis, MN) was added at 0, 10, or 100 ng/ml, and cells were

incubated (37°C, 5% CO_2) for 72 h. To assess DNA synthesis, 0.5 μCi/ml [^{3}H]thymidine (specific activity 10–20 Ci/mMol, Amersham Corp., Arlington Heights, IL) was added for an additional 24 h before harvesting. The medium was aspirated, adherent cells rinsed 2× with HBSS, and the cells were solubilized with 50 μl of 1% SDS in 0.1 M NaOH for 1 h. Lysates were collected using a cell harvester system and counted on a beta counter. Percent stimulation was calculated by the formula [B−A]/A×100, where A is the counts per minute (cpm) for control cells and B is the cpm of the treated cells.

To assess relative viable cell number we used a tetrazolium-based colorimetric assay that measures the reduction of MTT (Sigma Chemical Co., St. Louis, MO) by live cells to violet-colored formazan crystals as described [Mosmann, 1983]. Briefly, plates were set up as above and incubated for 72 h. Following incubation, 50 μl of MTT (2 mg/ml) was added to each well and the plates were incubated for 4 h. Wells were then washed with HBSS and 100 μl of DMSO was added to each well to lyse the cells and dissolve the formazan crystals. Optical density (OD) for each well was determined on a micro-ELISA plate reader at 560 nm. OD correlates to the number of live cells per well.

Anchorage Independent Growth

Colony formation in agarose was determined as described [Li et al., 1989]. Briefly, tumor cells were plated in 6-well tissue culture plates at 5×10^3 cells/well and incubated in semi-solid agarose (0.3%) with 10% FBS in the presence or absence of rhIGF-1 (0, 10, or 100 ng/ml) overlying a base layer of 0.6% agarose. Cells were allowed to incubate for 3–6 weeks and the number and size of colonies were quantitated. All experiments were performed in duplicate.

Matrigel Invasion Assay

Each lot of Matrigel invasion chambers (Becton Dickinson Labware, Bedford, MA) was evaluated and standardized for invasiveness using a human fibrosarcoma cell line HT 1080 obtained from ATCC (CCL 121) as a positive control and NIH 3T3 fibroblasts (ATCC) as a negative control. The Matrigel invasion chamber consists of an 8.0 μm pore size filter (1×10^5 pores/cm^2, 0.31 cm^2) coated with a consistently uniform layer of Matrigel, containing laminin, collagen type IV, heparan sulfate proteoglycan, entactin, and other components in a 24-well format. Serum-free medium (0.1% BSA) with 0 (control) or 50 ng/ml of rhIGF-1 was then added to the lower chamber and 1×10^5 cells in serum-free medium added to the upper chamber. The chambers were incubated for 24 h at 37°C in 5% CO_2. The non-invading cells in the upper chamber were then wiped completely clean with a cotton swab, the filters were stained with Diff-Quick™, and cells were counted.

Urokinase Plasminogen Activator (uPA) and Soluble uPA Receptor (suPAR) Expression

Tumor cells (2.5×10^5 per well) were plated in a 6-well plate in complete MEM in 10% FBS. Cells were allowed to adhere overnight, then the medium was removed and the cells washed 2× with HBSS. The medium was replaced with complete MEM in 0% FBS with 0 (control) or 50 ng/ml IGF-1. Supernatant was collected after 48 h and analyzed for uPA and suPAR concentration using ELISA kits #894 and #893, respectively (American Diagnostics, Greenwich, CT) according to manufacturer directions. Both kits are based on a double-antibody sandwich technique in which two different monoclonal antibodies are used to capture and detect human uPA and suPAR.

Ligand-Binding Assay for IGF-1 Cell Surface Receptors

OS cells were plated in 24-well plates at 2×10^5 cells/well in 10% FBS in MEM. Forty-eight hours later, the cells were washed with warm (37°C) HBSS and ^{125}I-IGF-1 (Amersham, Inc., Arlington Heights, IL) at 125,000 cpm in binding buffer (20 μM HEPES in serum-free MEM, 0.1% purified BSA) was added to each well, followed by addition of the indicated concentrations of rhIGF-1 (0–200 molar excess IGF-1) to a final volume of 2 ml. After a 4-h incubation at 4°C, the cells were washed twice with cold HBSS, solubilized with 200 μl Triton-X 100, and collected in 12 × 75 mm tubes. Assays were performed at least twice and cell associated radioactivity was measured in a gamma counter.

Scatchard analysis was performed as described using increasing concentrations of IGF-1 (unlabeled) up to 400 molar excess with a known amount of ^{125}I-IGF-1 to cause maximal binding [Radinsky et al., 1995]. Data were analyzed using Prism software (GraphPad, SanDiego, CA).

Western Ligand Blot for IGFBPs

For collection of conditioned medium (CM), 5×10^5 cells/well were plated in 6-well plates overnight. The cells were then washed 2× with HBSS, suspended in serum-free medium, and incubated in 37°C for 48 h. The CM was collected, centrifuged after the addition of protease inhibitors (2 mM EDTA, 1 mM PMSF, 20 μM leupeptin, 0.15 U/ml aprotinin), and concentrated with Centricon-10 concentrators (Amicon, Beverly, MA). Concentrated proteins were separated by 12% SDS–PAGE under non-reducing conditions, and transferred to nitrocellulose. For ligand blotting, membranes were incubated overnight at 4°C with 1.5×10^5 cpm of ^{125}I-IGF-1 or blocked with unlabelled rhIGF-1 in TBS buffer containing 0.1% Tween-20, and autoradiography was performed according to the method of Hossenlopp [Hossenlopp et al., 1986].

Xenograft Experiments

Male athymic nude mice (NCI-nu/nu) were obtained from the animal production area of the National Cancer Institute-Frederick Cancer Research and Development Center. The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and National Institutes of Health. The mice were used according to institutional guidelines when they were 8–10 weeks old.

Canine OS cells in log phase were harvested by trypsinization, washed in HBSS, and resuspended in serum-free culture medium at a concentration of 2×10^6 cells/100 μl. Mice were injected with 2×10^6 cells subcutaneously or 1×10^6 cells (100 μl) intravenously (three mice per group). In a separate experiment, three mice were injected with 2×10^6 canine Abrams cells into the medullary cavity of the distal femur using a 26 g needle. All animals were observed on a daily basis and once tumors were palpable, tumors were measured weekly. The mice receiving subcutaneous injections were euthanized when the subcutaneous tumor diameter reached 1 cm. The mice injected into the orthotopic site (distal femur) had the affected leg amputated by coxofemoral disarticulation while the mice were under general anesthesia using methoxyflurane. Mice were then examined frequently and euthanized when moribund. A complete necropsy was performed and the lungs were visually and microscopically evaluated.

Data Analysis

Student's unpaired two-tailed t-test was used to determine differences between means. Differences were considered significant when $P < 0.05$.

RESULTS

Northern Analysis for IGF-1R mRNA

Northern blot analyses using RNA from three canine and two human OS cell lines in log growth phase demonstrated steady-state expression of IGF-1R in all cell lines (Fig. 1). For SAOS-2 and SAOS-LM2, a 0.7-kilobase cDNA fragment of human IGF-1R hybridized with 11.0 and 7.0-kilobase mRNA transcripts. For the canine OS cells, the same probe for human IGF-1R hybridized with the 11.0-kilobase fragment and a <7.0-kilobase fragment. Serum starvation for 24 h did not alter mRNA expression (Fig. 1).

IGF-1 is Mitogenic to Osteosarcoma Cells

We next determined if IGF-1 was mitogenic to human and canine OS cells grown under anchorage-dependent conditions in low versus high serum concentrations. All five cell lines were grown in serum concentrations of 0, 1, and 10% FBS in the absence or presence of rhIGF-1 (0, 10, and 100 ng/ml). Mitogenic activity in response to IGF-1 was detected only in low serum concentrations (0 and 1%). This was confirmed using both [^{3}H]thymidine to detect DNA incorporation, and the MTT assay to determine relative viable cell number (Fig. 2). As shown in Figure 2A, [^{3}H]thymidine incorporation increased 100–125% in SAOS-2 and 175–300% in SAOS-LM2. Canine OS cells exhibited no significant increase in proliferation in response to rhIGF-1.

Relative viable cell number, as measured by the MTT assay, correlated with the [^{3}H]thymidine assay results. Both SAOS-2 and SAOS-LM2 proliferated in response to rhIGF-1 in low serum concentrations (Fig. 2B).

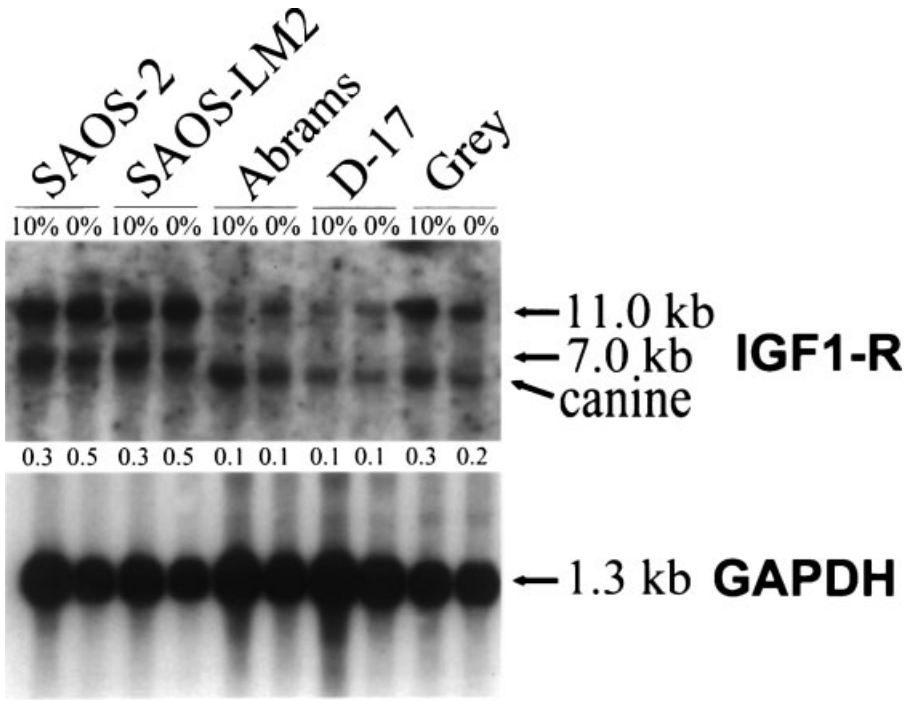

Fig. 1. Steady-state mRNA expression of IGF-1R in OS cell lines. Polyadenylated mRNA from OS cells grown in 10 or 0% FBS, was used in all cases. The hybridization probe used was a 0.7-kilobase cDNA fragment of human IGF-1R, which hybridizes with 11.0 and 7.0-kilobase mRNA transcripts in human cells. As a control, a 1.3-kilobase fragment of human GAPDH was also employed, in which a 1.3-kilobase transcript is expected (38).

When the OS cells were grown in 0.3% agarose (anchorage-independent growth conditions), only D17 and Abrams cells formed colonies, and IGF-1 treatment increased colony number compared to untreated controls (Fig. 2C). IGF-1 resulted in a 20–40% increase in colony number for D17 and a 140% increase for Abrams. No colony formation was observed in SAOS-2, SAOS-LM2, or gray OS cells.

Matrigel Invasion Assay

To examine whether IGF-1 could influence the invasive phenotype in the OS cells, we performed in vitro assays assessing ability to invade and migrate through a basement membrane (Matrigel)-coated filter in response to rhIGF-1. Cells were incubated for 24 h in the presence or absence of IGF-1 in the bottom chamber. Of the five OS cell lines tested, only the canine grey cells showed an increase in Matrigel invasion following treatment with IGF-1. The SAOS-LM2 cells showed greater invasion compared to the parental SAOS-2 under basal and IGF-1 supplemented conditions ($P = 0.01$), correlating with their metastatic phenotype (Fig. 3).

uPA and suPAR Expression

No detectable uPA or suPAR was identifiable in conditioned medium (CM) from the canine cell lines using the human ELISA kits. The level of uPA (<0.1 ng/ml) was similar in human SAOS-2 and SAOS-LM2 lines. uPA activity was marginally lower in CM from SAOS cells cultured with IGF-1 ($P = 0.07$). No difference in uPA production was noted in the SAOS-LM2 cells as a result of IGF-1 treatment (Fig. 4A). IGF-1 did not alter suPAR concentration in the CM, however, the concentration of suPAR was fivefold greater in the SAOS-LM2 compared to the parental SAOS-2 (Fig. 4B).

Ligand-Binding Assay for IGF-1 Cell Surface Receptors

The relative binding for ^{125}I-IGF-1 for all cell lines is shown in Figure 5. Table I summarizes the Scatchard analysis data. For the three canine OS cell lines, IGF-1 binding sites ranged

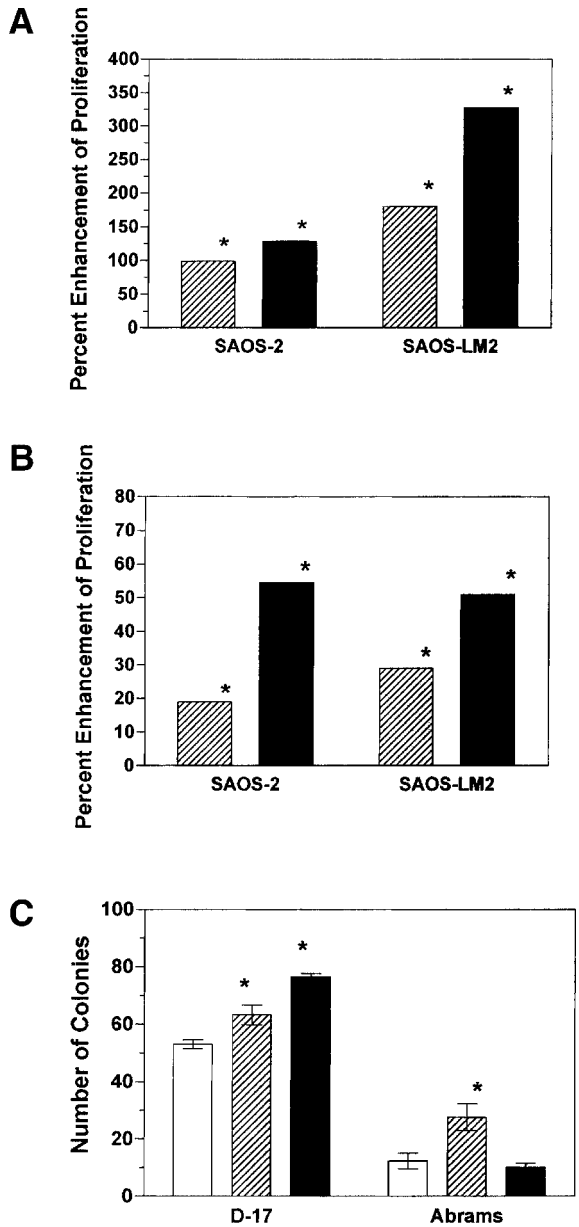

Fig. 2. Growth response to IGF-1. OS cells were plated in 10% FBS MEM for 24 h, washed, and cells were then cultured in 0% FBS MEM with IGF-1 added at 0 (control, open bar), 10 (striped bar), or 100 (solid bar) ng/ml. Results shown in **A** and **B** are the percent change observed when cells were exposed to IGF-1 compared to control. Asterisk denotes $P < 0.05$ compared to control. Data are presented from those cell lines that showed a difference compared to control. A: 72 h [^{3}H]thymidine incorporation assay. B: 72 h MTT assay. **C**: Agarose assay: cells were plated in 0.3% solid agarose in 10% FBS MEM with 0 (control), 10, or 100 ng/ml IGF-1 and colonies counted at week 5.

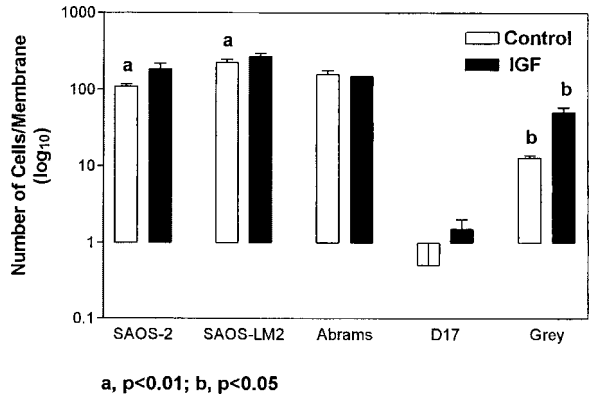

Fig. 3. Matrigel invasion assay. OS cells were plated in Matrigel invasion chambers, with IGF-1 used as a chemoattractant. The SAOS-LM2 cells displayed significantly greater invasion compared to the parental SAOS-2 cells (**a**, $P < 0.01$). Of the five OS cell lines treated, only grey showed significantly increased invasion in response to IGF-1 (**b**, $P < 0.05$).

from 1,728 to 3,883 per cell. The IGF-1R numbers for SAOS-2 and SAOS-LM2 OS cells were 9,138 and 10,234 per cell, respectively. A representative saturation binding curve and Scatchard plot for SAOS-2 is presented in Figure 6.

Western Ligand Blot for IGFBPs

IGFBPs were detected in all cell lines. Faint bands for IGFBP-5 (28 kDa) were detected in the human SAOS-2 and SAOS-LM2 and canine D17. The Abrams canine OS line showed bands for IGFBP-5 (28 kDa) and IGFBP-3 (46 kDa). A strong band for IGFBP-3 (46 kDa) was detected in gray OS cells (Fig. 7).

Xenograft Experiments

The tumorigenicity and metastatic potential of SAOS-2 and SAOS-LM2 in nude mice have been reported previously [Radinsky et al., 1994]. SAOS-2 is tumorigenic but poorly metastatic in nude mice, and SAOS-LM2 forms lung metastases when injected intravenously. As summarized in Table II, D17 and Abrams were tumorigenic following subcutaneous injection. Tumors were detected by 4 weeks and measured 5–10 mm by 6 weeks. All mice were necropsied when the tumors reached >15 mm in diameter and no metastases were noted. When tumor cells were injected intravenously, metastasis was detected in mice injected with the human SAOS-LM2 and the canine Abrams OS cells (Table II).

To further study the metastatic potential of the Abrams cells, these cells were injected orthotopically (distal femur), and the leg was amputated at the coxofemoral joint when the tumor mass measured 10 mm in diameter

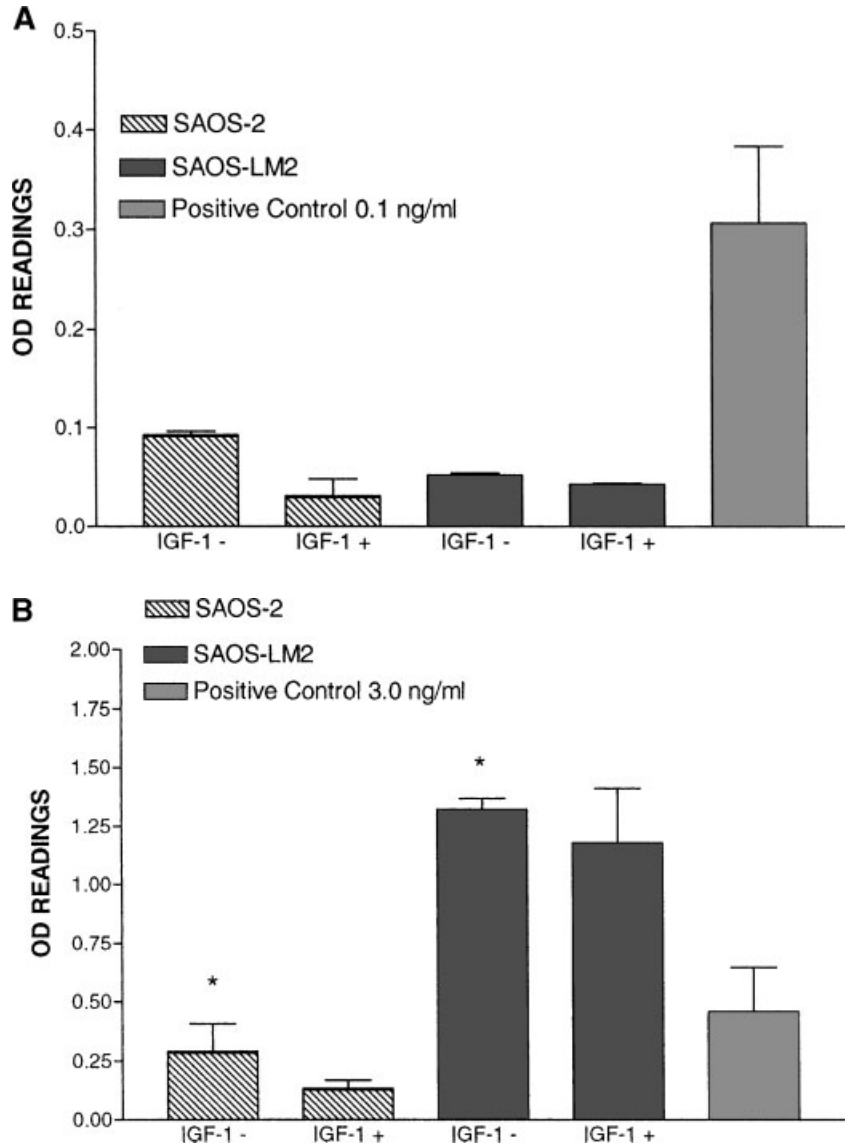

Fig. 4. uPA and suPAR expression in SAOS-2 and SAOS-LM2 cells. OS cells were cultured for 48 h in serum-free MEM ± 100 ng/ml rhIGF-1 and supernatants were collected and assayed for uPA (**A**) and suPAR (**B**). A: IGF-1 marginally ($P = 0.07$) reduced uPA levels in SAOS-2 cells. B: No effect of IGF-1 was detected, however, SAOS-LM2 produced five times more suPAR than SAOS-2 (*$P < 0.05$).

(approximately 4–5 weeks). Mice were euthanized and necropsied 6 weeks later, and gross and microscopic lung metastasis were detected in two of three mice evaluated (Fig. 8). Karyotypic analysis confirmed that the lung metastases were of canine origin (data not shown).

DISCUSSION

We sought to characterize the expression and functionality of IGF-1R in selected human and canine OS cell lines to determine whether IGF and/or IGF-1R expression correlated with phenotypic changes associated with malignancy and metastasis.

Based on the Northern analyses and confirmed by the radioligand studies, IGF-1R is expressed by human SAOS-2, SAOS-LM2, and the three canine OS cell lines studied. Scatchard analysis was linear in two canine OS cell lines (Abrams and D17) and both human OS cell lines (SAOS-2 and SAOS-LM2). However, it showed a complex pattern in Grey OS. Generally, non-linearity in Scatchard analysis implies ligand binding to more than one type of binding site. The IGF-1R expression levels we describe

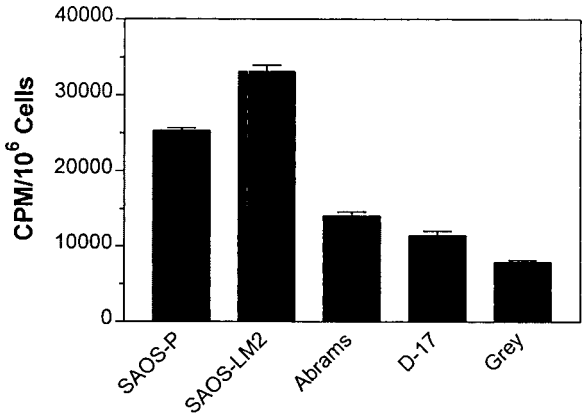

Fig. 5. Relative ^{125}I-IGF-1 binding. 2×10^5 cells were plated in triplicate wells and grown in 10% FBS MEM, washed, and ^{125}I-IGF-1 was added to each well and allowed to incubate for 4 h. Cells were then washed, lysed, and counted in a beta counter. ^{125}I-IGF-1 binding was blocked using excess IGF-1 for all cells or anti-IGF-1R (aIR3) for human cells only. Data is presented as cpm/10^6 cells.

TABLE I. Scatchard Analysis

Cell line	B-Max pM/L	KD pM/L	IGF-1 receptor #
SAOS-2	2.581	0.9187	9,138
SAOS-LM2	3.145	0.7596	10,234
Abrams	1.936	11.560	3,883
D17	1.617	6.1310	2,949
Grey	1.119	1.998	1,728

in the human and canine cells compare favorably with the numbers of IGF-1R (based on Scatchard analysis) reported for various human cell lines, which range from 1,200 to 15,200 per cell [Remacle-Bonnet et al., 1992; Nagamani and Stuart, 1996; Velez-Yanguas et al., 1996; van den Berg et al., 1996]. The Scatchard results obtained in this study must be interpreted taking into account the potential contribution of the IGFBPs, which may significantly alter the binding of IGF-1 to its receptor. Recently, a study reported that SAOS-2 cells expressed 55,000 IGF-1R per cell. However, these investigators used ^{125}I-des(1-3)IGF-1 to determine the receptor content [Ohlsson et al., 1998]. This ligand exclusively binds to the IGF-1R and does not bind to IGFBPs. This may account for the discrepancy between their results and the IGF-1R number reported in our study.

Although IGF-1 has been shown to be mitogenic for human OS cells [Pollak et al., 1990; Kappel et al., 1994] and our data support this work, we are the first to demonstrate that IGF-1 is mitogenic for canine OS-derived cells. Both Abrams and D17 showed an increase in colony formation in response to rhIGF-1 when grown in agarose. Growth of cells in agarose is usually associated with a more aggressive and

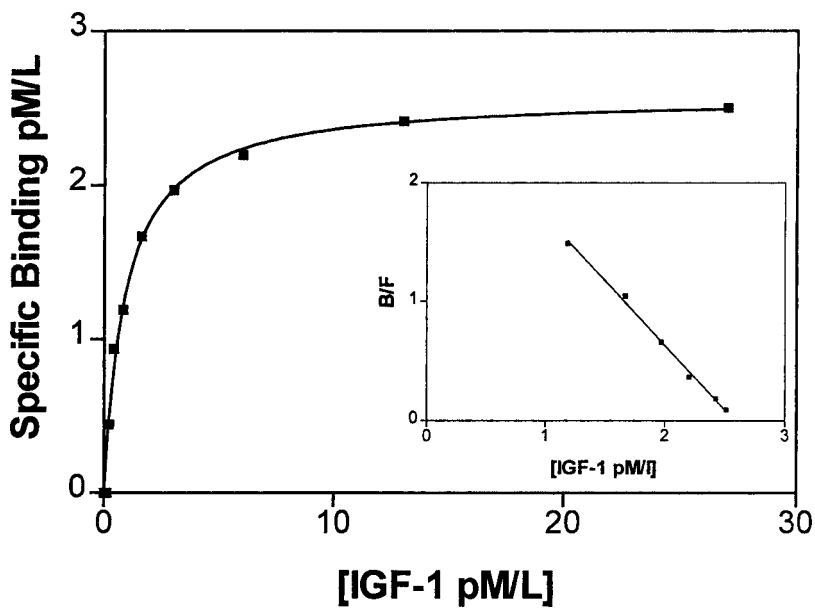

Fig. 6. Saturation binding curve and Scatchard analysis. Representative saturation curve showing specific binding of ^{125}I-IGF-1 to SAOS-2 cells measured in the presence of various concentrations of unlabeled IGF-1. Inset: Scatchard representation of the data for the ^{125}I-IGF-1 binding displacement by unlabeled IGF-1 analyzed by the GraphPad Prism software program. The solid line represents a computer-generated best fit for a one-site binding model. The abscissa shows pM of bound ^{125}I-IGF-1; the ordinate indicates the ratio of bound IGF-1 to free IGF-1.

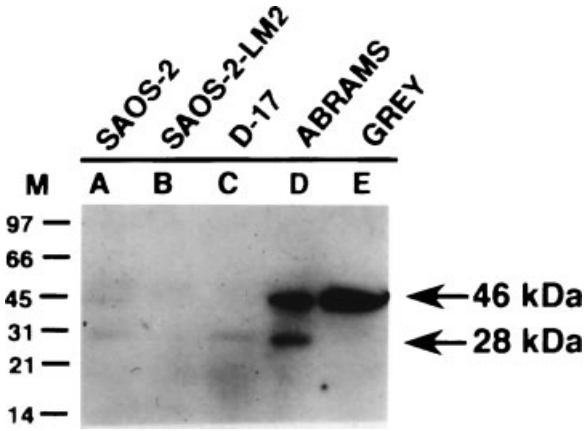

Fig. 7. Western ligand blot for IGFBPs. IGFBPs were detected by affinity cross-linking with ^{125}I-IGF-1. OS cells were cultured in serum-free medium for 48 h, and the conditioned media was concentrated by ultrafiltration and analyzed by Western ligand blotting. IGFBP-5 (28 kDa) was detected in human SAOS-2, SAOS-LM2, and canine D17 (faint bands) and Abrams (strong band). Canine OS cells (Abrams and Grey) showed strong bands for IGFBP-3 (46 kDa).

TABLE II. Tumorigenicity of Osteosarcoma Cell Lines

		In nude mice	
Cell line	Source	Tumorigenic	Metastatic
SAOS-2	Human	Yes	No
SAOS-LM2	Human	Yes	Yes
D17	Dog (ATCC)	Yes	No
Abrams	Dog (primary)	Yes	Yes
Grey	Dog (lung met)	No	No

metastatic phenotype. Previous studies have shown a correlation between anchorage-independent growth capacity and metastatic behavior for murine fibrosarcoma and human melanoma, breast cancer, and colon carcinoma cells [Price, 1986; Li et al., 1989]. It is noteworthy that only the colony-forming lines Abrams and D17 were tumorigenic following transplantation in nude mice, and that these were the canine lines with the highest IGF-1R number.

uPA is a serine protease that regulates multiple pathways involved in matrix degradation, cell motility, and angiogenesis [Blasi, 1993; Hudson and McReynolds, 1997]. uPAR is anchored to the cell membrane by a glycosylphosphatylinositol moiety and uPAR is hypothesized to focus and prolong the activity of uPA [Blasi, 1993; Nykjaer et al., 1994]. Many tumors have been shown to express uPAR and suPAR, i.e., uPAR protein without the gylcolipid anchor, and the expression has been associated with cell migration and tumor invasion [Kariko et al., 1993; Bianchi et al., 1994; Pyke et al., 1994; Stahl and Mueller, 1994; Hudson and McReynolds, 1997]. Cell lines lacking uPAR do not invade Matrigel [Hudson and McReynolds, 1997]. Studies of clinical material have shown that elevated plasma levels of suPAR are associated with reduced survival in non-small cell lung cancer [Pappot et al., 1997], metastatic breast cancer [Stephens et al., 1997], and colorectal cancer. Growth factors such as EGF and HGF have been shown to enhance matrix metalloproteinase (92 kid gelatinase/type IV

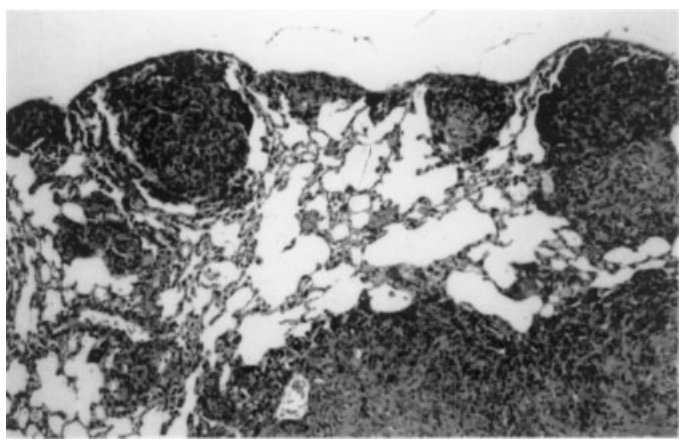

Fig. 8. Photomicrograph of lung metastasis (hematoxylin and eosin, 40×). Three nude mice were injected with Abrams canine OS cells into the distal femur and tumors developed within 5 weeks. The rear leg was amputated and the mice sacrificed and necropsied 6 weeks later. Lung metastases were detected grossly and histologically in two of three mice.

collagenase) [Price et al., 1996; Zeigler et al., 1996; Kondapaka et al., 1997] and uPA activity, and thus increase invasiveness [Jeffers et al., 1996; Long and Rose, 1996; Mars et al., 1996]. Antisense strategies reducing uPA expression in OS cells result in reduced Matrigel invasion [Haeckel et al., 1998]. In this study, IGF-1 marginally lowered the level of uPA in the SAOS-2 cells, indicating that IGF-1 can influence the production of uPA in OS cells. These findings are in agreement with a published study using MG-63 OS cells, indicating that uPA production was reduced following treatment with rhIGF-1 [Lalou et al., 1994].

We found in this study that only one OS cell line tested showed enhanced Matrigel invasion following treatment with IGF-1. Several studies have addressed the effect of IGF-1 on tumor cell invasion [Dunn et al., 1998, 2000; Long et al., 1998a; Mira et al., 1999]. One recently published study using PA-III prostate carcinoma cells reported that reduction of IGF-1R expression, using an antisense approach, reduced bone invasion in an in vivo model [Burfeind et al., 1996]. In another study, overexpression of IGF-1R in lung carcinoma cells enhanced invasion into Matrigel [Long et al., 1998b]. Enhanced expression of uPA and/or the matrix metalloproteases by IGF-1 may be responsible for its pro-invasive effects [Long et al., 1998a; Mira et al., 1999; Dunn et al., 2000].

The IGFBPs have been postulated to play important roles in controlling the activity of IGF [Ritvos et al., 1988; Ernst and Rodan, 1990; LaTour et al., 1990; Culouscou and Shoyab, 1991; Andress and Birnbaum, 1992; Lee et al., 1993; Slootweg et al., 1995; Zumkeller et al., 1996]. The IGFBPs may either inhibit or potentiate IGF action, and some IGFBPs may have direct independent inhibitory effects on cells [Culouscou and Shoyab, 1991; Rajah et al., 1997]. IGF-1 and IGFBPs are major regulators of osteoblast physiology. IGF-1 and IGF-II are involved in osteoblast mitogenesis and stimulating bone matrix protein synthesis, and IGFBPs can regulate the bioavailability of IGFs. In plasma, IGFBP-3 is the major IGFBP and has a dual inhibitory mechanism of action, either through IGF-1 dependent effects or directly [Moerman et al., 1993; Velez-Yanguas et al., 1996]. IGFBP-3 gene expression has been shown to be regulated by p53 signaling [Buckbinder et al., 1995]. IGFBP-3 may sequester IGF-1 and prevent receptor binding and more recently, was shown to induce apoptosis and may contribute to senescent growth [Moerman et al., 1993; Rajah et al., 1997]. In this study, Western radioligand binding demonstrated the expression of IGFBP-3 and -5 in OS cells. IGFBP-3 and IGFBP-5 have been shown to stimulate mitogenesis for osteoblasts or rat OS cells without exogenous IGF-1 [Andress and Birnbaum, 1992; Slootweg et al., 1995]. SAOS-2 cells have been shown by others to express IGFBP-5 [Schmid et al., 1995].

One important and significant finding from this study was the demonstration of spontaneous lung metastasis of canine Abrams OS cells following orthotopic transplantation. Another interesting observation was the correlation of IGF-1R protein expression to growth and metastasis in the nude mice. A similar observation can be made with the human SAOS cells. The metastatic SAOS-LM2 shows higher IGF-1R expression (based on Scatchard analysis) than the non-metastatic SAOS-2. The canine OS cells displaying an aggressive in vivo phenotype, as evidenced by local tumor growth and metastasis, also showed the highest IGF-1R density based on Scatchard analysis (Table I). These results are supportive of our hypothesis relating IGF-1R expression to the malignant phenotype. The association between IGF-1R expression and malignant phenotype needs further study.

Collectively, our results show that human and canine OS cells express IGF-1R based on Northern analyses and radioligand binding. Although not consistent in all OS cells tested, IGF-1 enhanced cell proliferation and colony formation in soft agar. Invasion through Matrigel was minimally increased following exposure to IGF-1. Furthermore, we have identified a canine OS cell line which, when injected into the orthotopic site (distal femur), metastasizes spontaneously following amputation. This canine OS cell line had the highest expression of IGF-1R of the canine cells tested based on Scatchard analysis.

In conclusion, this study provides additional evidence for the significant role of IGF-1 and IGF-1R on OS cell growth and invasion. Furthermore, the identification of IGF-1R and response to IGF in canine OS cells derived from spontaneous tumors supports the use of spontaneous canine OS as a model to further investigate the importance of the IGF-1 system in OS development and metastasis.

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