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Annexin 2 Expression Is Reduced in Human Osteosarcoma Metastases

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Abstract Osteosarcoma is an aggressive primary bone cancer affecting primarily children and young adults. The development of valuable diagnostic indicators and therapeutic agents will be enhanced by the identification and characterization of genes that contribute to its aggressive behavior. We used representational difference analysis to isolate genes differentially expressed between primary human osteosarcoma tumors and subsequent metastatic lung lesions to identify genes potentially involved in metastatic potential. Several genes were differentially expressed between the two tumor populations, including annexin2. The levels of annexin2 mRNA and protein inversely correlated with metastatic potential in a subset of human osteosarcoma tumor specimens, as well as in a human osteosarcoma cell line selected for increased metastatic potential. Annexin2 has been described in several cellular localizations with various functional implications, many of which may be relevant to metastatic potential. Therefore, the subcellular localization of endogenous annexin2 protein was evaluated biochemically by subcellular fractionation and immunologically by flow cytometry and immunofluorescence in osteoblastic cells. Annexin2 was localized to the cytoplasm and intracellular aspect of the plasma membrane, excluded from the nucleus and undetectable on the cell surface or in the conditioned medium. Overexpression of annexin2 in osteosarcoma cells did not alter several in vitro phenotypes often used to assess metastatic potential including motility, adhesion, and proliferation. However, our previous data have implicated annexin2 in the mineralization process of osteoblastic cells in vitro. Consistent with an increase in differentiation-induced mineralization, there was diminished tumorigenicity and experimental metastatic potential of osteosarcoma cells overexpressing annexin2. These data suggest that annexin2 may downregulate osteosarcoma aggressiveness by inducing a more differentiated state in osteoblastic cells. J. Cell. Biochem. 92: 820–832, 2004.

Key words: differential expression; subcellular localization; differentiation; osteoblast

Osteosarcoma (OS) is the most common form of primary bone cancer and occurs predominantly in children and young adults. OS readily metastasizes and patients identified as having metastatic disease at diagnosis experience a 5-year survival rate as low as 20% [Meyers et al., 1993]. During the last few decades, a combination of surgical and chemotherapeutic treatment of OS primary tumors has brought the survival of patients *without* metastatic disease to nearly 80% [Meyers et al., 1992]. Clearly, a better understanding of the metastatic process in these patients is necessary to more effectively control the disease.

Several studies of OS primary tumors and cell lines have identified the overexpression of genes such as c-*fos*, *c-myc*, and *mdm2* [Wu et al., 1990; Miller et al., 1996; Gamberi et al., 1998]. Yet other studies have focused on genes down-

Abbreviations: AnxA2, annexin 2; DP1/2, RDA difference products 1 or 2; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; OS, osteosarcoma; PCR, polymerase chain reaction; RDA, representational difference analysis; RT-PCR, reverse transcription PCR.

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regulated in OS tumors. Loss of heterozygosity studies have identified chromosomal loss within 13q (*Retinoblastoma*), 17p (*p53*), 3q, 18q, and 10q [Toguchida et al., 1989; Scheffer et al., 1991; Yamaguchi et al., 1992; Kruzelock et al., 1997]. Altered gene expression has been associated predominantly with the tumorigenic phenotype yet the genetic program required for metastatic potential is likely to consist of an additional set of genes beyond those required for tumorigenesis. Therefore, we chose to use a molecular subtraction technique (representational difference analysis, RDA) to isolate genes differentially expressed in cells of a primary human OS tumor compared to those in a metastatic lesion.

To employ RDA for the isolation of metastasis-specific genes, we isolated RNA from primary human OS tumors and subsequent metastatic lung lesions to focus on genes involved in metastasis. Among the genes downregulated in metastatic lesions, we identified annexin 2 (*anxA2*). AnxA2 expression levels were characterized in OS tumors and cell lines and the protein was localized to the cytoplasmic and intracellular aspect of the plasma membrane. Annexins are characterized by conserved annexin repeat domains and an ability to bind negatively charged phospholipids in a calcium-dependent manner. Although the physiological roles of the annexins require further clarification, they have been described as participants in processes such as endo- and exocytosis, membrane fusion, membrane/cytoskeletal interactions, and voltage-dependent calcium channels [Drust and Creutz, 1988; Creutz, 1992; Harder et al., 1997; Faure et al., 2002; van de Graaf et al., 2003].

AnxA2 was first identified as a substrate for the Src kinase of Rous Sarcoma virus, which initially implicated anxA2 in a signaling cascade often involved in cancer [Gerke and Moss, 1997; Siever and Erickson, 1997]. Additional studies have failed to elucidate a common role of anxA2 in cancer and may suggest cell type specificity. In osteoblastic cells, the overexpression of anxA2 did not affect cell motility, adhesion, or proliferation when analyzed in normal culture conditions. However, previous studies from our laboratory have described a role for anxA2 in the process of osteoblastic mineralization in vitro. Studies described herein illustrate that cells overexpressing anxA2 have a decreased propensity to form tumors or metastasize when inoculated in vivo Therefore, anxA2 may modulate the aggressive phenotype of OS by affecting the osteoblastic differentiation program.

MATERIALS AND METHODS

Tissue and RNA Isolation

Primary OS tumor samples were obtained as part of the patient's diagnostic biopsy and stored in an -80°C archive. Metastatic nodules were obtained from those patients undergoing post-chemotherapeutic thoracic surgery for metastatic OS and were also stored in the frozen archive. The use of human tissues in these studies was approved by the Colorado Multiple Institutional Review Board and informed consent was obtained from the patients.

Frozen tumor tissue (12–24 mg) was removed from the tumor specimen with a scalpel and pulverized in the presence of liquid nitrogen. Lysis buffer (500 μl) containing 10 mM Tris, pH 8.4, 140 mM NaCl, 15 mM $MgCl_2$, 0.5% NP-40, and 500 U RNasin® (Promega, Madison, WI) was immediately added to the frozen, powdered tissue and allowed to thaw on ice for 5 min. The nuclei were pelleted at 2,000*g* for 5 min and the supernatant was extracted with 500 μl phenol:-chloroform:isoamyl alcohol (25:24:1) and 0.1% SDS. The aqueous phase was re-extracted twice. Total RNA was precipitated in ethanol, quantitated and stored in ethanol at -80°C.

Representational Difference Analysis

Approximately 0.5 μg of total RNA from each sample (primary and metastatic tumor) was used to generate first strand cDNA using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). The cDNA was size-selected on a CHROMA SPIN-1000 column (Clontech, Palo Alto, CA), and restricted with Csp61 (MBI Fermentas, Amherst, NY). RDA was performed essentially as described by Hubank and Schatz [1994] with modifications to the first primer set ("R" primers) for concordance with cDNA synthesis. The sequences of the modified "R" primers are as follows: RM12: 5′-TAGATCTGCGGT-3′, RM24: 5′-ACTCTCCAG-CCTCTCACCGCAGATC-3′ (Life Technologies, Rockville, MD). PCR amplification from these primers restored the *Dpn*II restriction site so the remainder of the Hubank and Schatz protocol was followed as described. cDNA was minimally amplified to generate 'representations' from the full complement of cellular mRNA. 'Tester' cDNA (the population from

which unique sequences were isolated) and 'driver' cDNA (the population used to eliminate common sequences) were derived from the respective representations. To enrich for potential metastasis suppressor genes or sequences unique to the primary tumor, the 'tester' cDNA was derived from the primary tumor biopsy. We also performed the reverse experiment to isolate sequences unique to, or upregulated in, the metastatic tumor. In these experiments, cDNA derived from the metastatic tumor biopsy was used as the 'tester' cDNA population. Following the first round of selective enrichment, the cDNA pool was referred to as difference products-1 (DP1). This partially 'subtracted' population of cDNA was then hybridized to another pool of 'driver' cDNA. The final, enriched, unhybridized pool of cDNA was referred to as difference products-2 (DP2).

Complementary DNA Cloning and Sequence Analysis

Complementary DNA from the DP1 and DP2 pools were separated by electrophoresis and purified using the GeneClean DNA isolation kit (Bio 101, Vista, CA). The purified cDNA was cloned into the pCR2.1 vector (Promega, Madison, WI) and propagated in Top10F′ (Invitrogen, Carlsbad, CA). In addition, the DP2 pool of clones was shotgun cloned into pCR 2.1 (Promega, Madison, WI) and cultured on LB plates containing kanamycin, IPTG, and x-gal. White colonies (1056) were arrayed into 96-well plates, frozen in 15% glycerol, and used to inoculate cultures for the amplification of plasmid DNA. DNA was purified from 5-ml cultures using the Mini Spin Extraction kit (Qiagen, Valencia, CA) and sequenced with Sequenase® 2.1 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The sequencing reactions were electrophoresed on an 8% polyacrylamide gel at 110 W for 4.5 h, dried and exposed to Kodak XAR film for 18 h at −80°C. All sequences were subject to BLAST analysis (www.ncbi.nlm.nih.gov) and aligned using Sequencher 3.0 (Molecular Diagnostics, San Diego, CA).

RT-PCR

Total cytoplasmic RNA was isolated as above and subjected to first strand cDNA synthesis using the Superscript cDNA synthesis kit (Life Technologies). First strand cDNA was amplified using sequence-specific primers (Life Technologies). Fifty-microliter PCR reactions containing 0.5–1.0 μg first strand cDNA were initiated and 10-μl aliquots were removed at the indicated number of cycles. PCR product was visualized on an agarose gel with ethidium bromide staining to provide a semi-quantitative analysis of specific mRNA expression.

Cells

The human OS cell lines SaOS2 and U2OS were obtained from the American Type Culture Collection. The metastatic variants of SaOS2, SaOSLM2 [Radinsky et al., 1994] and SaOSLM7 [Jia et al., 1999], were kindly provided by Robert Radinsky, PhD and E. Kleinerman, MD, respectively, University of Texas MD Anderson Cancer Center. Both of these metastatic variants were originally isolated from lungs following serial intravenous inoculations in athymic mice. SaOSLM2 cells stably transfected with either green fluorescent protein (GFP) or the AnxA2/GFP fusion construct were generated as previously described [Gillette and Nielsen-Preiss, 2004]. Cell lines were routinely cultured in RPMI 1640 supplemented with 10% FBS, nonessential amino acids, MEM vitamins, sodium pyruvate, and sodium bicarbonate.

Immunofluorescence and Flow Cytometry

Cells were cultured overnight on chambered slides (Nunc, Inc., Naperville, IL). Adherent cells were rinsed with PBS and fixed in 3.7% formaldehyde, 0.12-M sucrose in PBS for 10 min. The fixed cells were rinsed with PBS, permeabilized with methanol at −15°C for 15 min, and blocked in 15% donkey serum (Jackson Immuno Research, West Grove, PA) in PBS. AnxA2 primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted 1:1,000 and incubated overnight at ambient temperature in a humidified petri plate. In contrast, nonpermeabilized cells were washed with PBS then incubated with primary antibody for 1 h on ice. We were unable to reproducibly fix OS cells without resulting in an unacceptable level of permeabilization. The cells were then washed with cold PBS and fixed with 3.7% formaldehyde. Following fixation, cells from either experiment were blocked with 15% donkey serum in PBS and incubated with diluted FITC-conjugated donkey anti-goat antibody (1:100, Santa Cruz Biotechnology, Inc.) for at least 1 h at ambient temperature. Cells were washed with PBS, mounted with Vectashield

(Vector Laboratories, Burlingame, CA), and visualized on either a TE 200 Nikon inverted microscope with fluorescence or a Nikon PCM2000 confocal microscope.

For flow cytometry experiments, viable cells were incubated with primary antibody for 1 h at 4°C. Cells were washed with PBS and incubated with secondary antibody for 30 min at 4°C. Following a final wash with PBS, cells were analyzed on a Beckman Coulter XL (Beckman Coulter, Fullerton, CA) with the assistance of the personnel in the UC Cancer Center Flow Cytometry Core Facility. Cells incubated with only the appropriate secondary antibody served as the negative controls.

Immunoblot Analysis

Total cellular protein was isolated from cultured cells that had been rinsed with PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Igepal, 0.1% SDS, 0.5% deoxycholic acid) supplemented with a protease inhibitor cocktail (Boerhinger Mannheim, Germany). For subcellular fractionation studies, cells were swollen in intracellular buffer (0.25 M sucrose, 20 mM HEPES, 120 mM KCl, 12 mM NaCl, 1.62 mM $MgSO_4$, 1 mM EDTA, 0.35 mM $CaCl_2$) and gently lysed using a Dounce tissue grinder (Kontes, Vineland, NJ). During the course of disruption, cell integrity was monitored by microscopy. Intact nuclei were pelleted by centrifugation at 5,000 rpm for 5 min. The nuclear pellet was washed once with PBS. The membrane fraction was isolated following centrifugation at 100,000*g* for 1 h. The supernatant was concentrated tenfold in Centricon filter devices (Millipore Corporation, Before, MA) and treated as the cytoplasmic or soluble fraction. Protein aliquots were electrophoresed through a 10% SDS polyacrylamide gel (unless otherwise noted) and electrotransferred to a PVDF membrane (Schleicher & Schuell, Inc., Keene, NH) using the mini transblotter system (Bio-Rad Laboratories, Hercules, CA). The PVDF membranes were blocked in 5% nonfat dry milk and 0.2% Tween 20 in PBS. Primary anxA2 antibody (Santa Cruz Biotechnologies, Inc.) was diluted 1:1,000 in blocking reagent and the membrane was incubated at 4°C overnight. Membranes were washed in PBS/0.2% Tween 20 for 3 × 20 min. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies, Inc.) was diluted 1:1,000 in blocking reagent and incubated on the PVDF membrane for 2 h at ambient temperature. The membranes were washed as above and visualized using chemiluminescence according to the manufacturer's protocol (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

Analysis of Cell Motility and Adhesion

SaOSLM2 cells (+/− anxA2 overexpression) were plated at confluence in 6-well plates (500,000 cells per well) and allowed to adhere overnight. A 'wound' was introduced into the cell monolayer with a sterile 10-μl pipet tip. Dislodged cells were removed by two rinses of the monolayer with culture medium. The width of the 'wound' was documented by photomicroscopy. The cells were incubated for up to 24 h. At 4, 20, and 24 h, the width of the 'wound' was documented. Motility was measured as the rate at which cells entered and filled the 'wound' during two 4-h time periods. Adhesion, a phenotypic property of which tight regulation is required for successful migration, was measured using an inverted centrifugation assay. Briefly, cells were plated in 96-well plates and allowed to adhere for 1.5 h. The wells were filled with medium and covered with sterile adhesive sealing film (ThermalSeal, Research Products International Corp., Mt. Prospect, IL). The 96-well plates were inverted and centrifuged at the indicated g-forces for 5 min. The medium and dislodged cells were removed and 100-μl fresh medium was added to each well. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldridge, St. Louis, MO) (10 μl per well) was added and incubated for 3 h. The formazan crystals generated by enzymes from metabolically active mitochondria were solubilized in 10% SDS, 0.01M HCl and quantified at 570 nm to determine the relative number of adherent cells.

Proliferation Assay

Cell growth was analyzed using an MTT assay. Cells were plated in quadruplicate in 96-well plates (1,000 cells/well) after trypsinization. At each time point, the MTT reagent was added to the medium at a final concentration of 1.2 mM. The cells were allowed to incubate for an additional 3 h at 37°C at which time the formazan crystals were solubilized by the addition of 100-μl solubilization buffer (10% SDS, 0.01 M HCl). The optical density was recorded at 570 nm in a microplate reader (BioRad, Hercules, CA).

Cell Cycle Analysis

Cell cycle analysis was performed following fluorescence labeling of cellular DNA by propidium iodide. Cells were seeded in 6-well plates and incubated for 24 h (50% confluent) in complete medium. Cell monolayers were rinsed in PBS and incubated for 18 h in serum-free RPMI. The medium was then replaced with fresh serum-free medium for an additional 1 h. Cells were stimulated by the addition of medium containing 10% FBS, but in the presence of aphidicolin (14.8 μM) to further synchronize the cell population. Aphidicolin was removed and cells were stimulated in the presence of medium containing 10% FBS. At the indicated time points, monolayers were rinsed with PBS and trypsinized. The trypsin was inhibited by the addition of serum-containing medium and the cells were pelleted by low-speed centrifugation (400*g* for 5 min). Supernatant was aspirated and the cells were resuspended in 1 ml of propidium iodide stain (3.8 mM sodium citrate, 69 μM propidium iodide, 0.01% NP40, and 0.01 mg/ml RNase) and incubated overnight at 4°C. Cell cycle analysis was performed by the UCHSC Cancer Center Flow Cytometry Core facility.

In Vivo Analyses of Metastatic Potential

SaOSLM2 cells stably transfected with a GFP construct or an anxA2/GFP fusion construct were inoculated into 6-week-old male athymic mice. Cells (2×10^6) (in 100 μl) were suspended in serum-free medium and inoculated either subcutaneously in the flank with 100-μl matrigel or intravenously in the tail vein. The mice were sacrificed 8 weeks after inoculation at which time tumors were harvested and lungs were removed and analyzed for the presence of GFP-expressing metastatic cells.

RESULTS

Isolation of Sequences Differentially Expressed in Lung Metastases Relative to Primary OS Tumors

To identify alterations in gene expression specific to the metastatic phenotype, we performed a selective amplification technique, RDA, on mRNA isolated from a human primary OS and a metastatic lung lesion. The 'tester' cDNA populations were ligated to sequence-specific adaptors and allowed to hybridize with an excess of 'driver' cDNA. Following two rounds of hybridization and selective PCR amplification (Fig. 1A), we determined the efficiency of subtraction by amplifying a commonly expressed gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH, Fig. 1B). G3PDH product was easily detected in as few as 15 PCR cycles in the pre-subtraction cDNA 'representations' (Fig. 1B, lanes 2). In contrast, the ability to amplify G3PDH sequences was diminished by 50–60% by densitometry (Fig. 1B, lanes 5 and 9) following one round of selective amplification yielding the first difference product (DP1). Analysis of DP2, from the

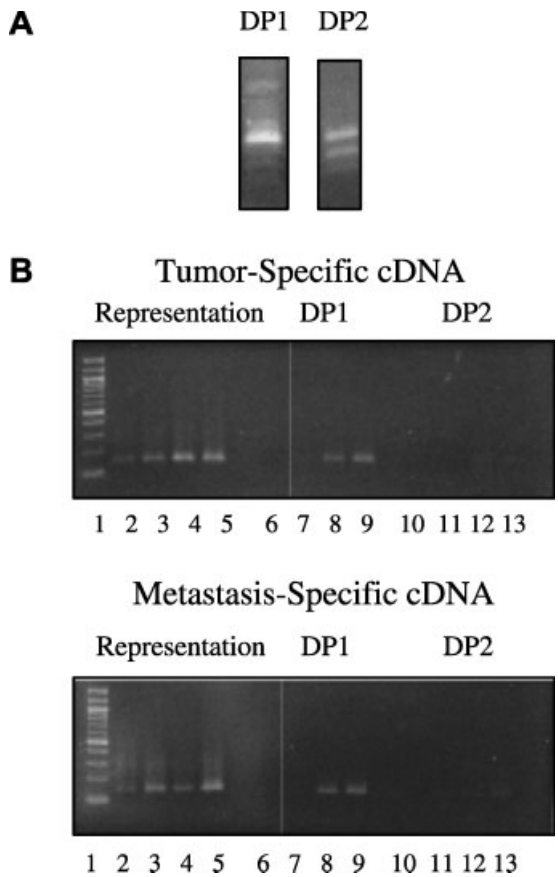

Fig. 1. Subtraction of G3PDH gene sequences following two rounds of representational difference analysis. **A**: RNA was isolated from a human primary OS and a corresponding lung metastasis. cDNA representations were generated and subjected to two rounds of RDA, yielding successively subtracted populations of cDNA, DP1, and DP2. **B**: cDNA (1 μg) from each the tumor representation (**lanes 2–5**), DP1 (**lanes 6–9**), and DP2 (**lanes 10–13**) was PCR amplified with primers specific for human G3PDH. Aliquots of each reaction were removed at 15 (lanes 2, 6, 10), 20 (lanes 3, 7, 11), 25 (lanes 4, 8, 12), and 30 (lanes 5, 9, 3) cycles, electrophoresed, and visualized with ethidium bromide. **Lane 1**, 100-bp DNA ladder.

second round of selective amplification, revealed that G3PDH sequences were nearly undetectable even after 30 cycles of PCR amplification (Fig. 1B, lanes 13).

The DP2 pools were considered depleted of sequences common to the two populations of cells from which the cDNAs were derived. Complementary DNA bands were isolated from agarose gels and, in addition to the DP2 cDNA pools, were cloned, arrayed, and cultured in 96-well plates. Analysis of the difference products revealed a few abundant sequences (i.e., L ferritin, downregulated in metastasis), but also several less abundant sequences that persisted through two rounds of enrichment. AnxA2 was isolated as a gene potentially downregulated in metastatic lesions and is the subject of the studies described herein. Mdm2 was isolated as a gene upregulated in metastasis. Regulation of p53, including by the upregulation of mdm2, has been well documented in a subset of OS [Ladanyi et al., 1993; Miller et al., 1996]. Therefore, the isolation of this gene further supported the integrity of the RDA technique.

RDA was initially performed on cDNA derived from tumor and metastatic tissue isolated from separate patients, but upon obtaining tumor and metastatic tissue samples from the same patient we repeated the subtraction experiment. AnxA2 was isolated as a downregulated transcript in metastases in each of these experiments.

AnxA2 mRNA Expression Is Reduced in a Subset of OS Metastases

AnxA2 expression was decreased in metastatic OS tumors in two separate RDA experiments, therefore we further investigated the frequency of differential expression of this gene product in a more broad panel of OS tumors. The expression of anxA2 in human OS primary tumors and metastatic lung lesions was evaluated using semi-quantitative reverse transcription (RT)-PCR. As seen in Figure 2A, anxA2 was expressed in each of the OS primary tumors analyzed and detected in as few as 25–30 PCR cycles. In contrast, amplification of anxA2 from the mRNA of metastatic tumors (Fig. 2B) required as many as 35–40 cycles for detection. We were unable to detect anxA2 PCR product in a subset of the metastatic tumors. The level of anxA2 in mRNA isolated from tumor and metastatic lesions of the same patient (matched patient samples) is shown in Figure 2C. Two of the three matched patients (99-1 and 98-5) showed diminished anxA2 expression in tissue from the metastatic lesions.

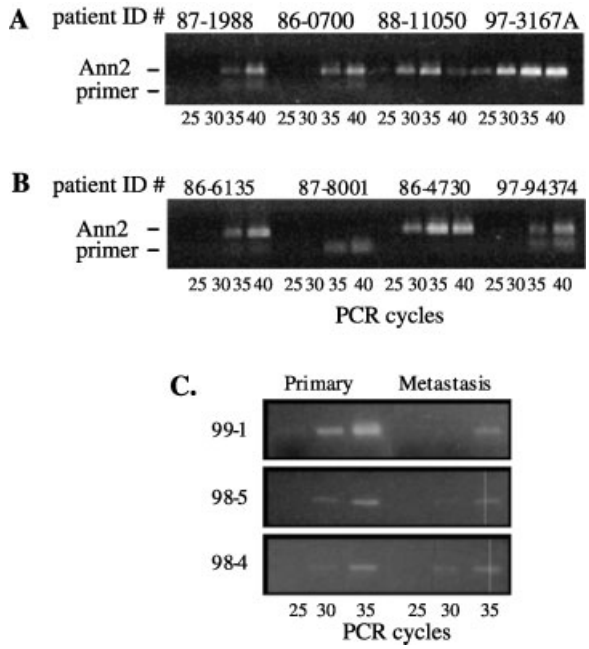

Fig. 2. AnxA2 mRNA is differentially expressed in primary tumors and metastases obtained from OS patients. Total cellular RNA was isolated from OS primary tumors (**A**) and metastatic lesions (**B**). The RNA was reverse transcribed and subjected to PCR amplification using anxA2-specific primers. Fifty microliter PCR reactions were initiated and 10-μl aliquots were removed after 25, 30, 35, and 40 cycles. **C**: AnxA2-specific RT-PCR was performed using RNA isolated from primary tumors and metastatic lung lesions isolated from matched patients.

AnxA2 Protein Is Differentially Expressed in Human OS Cell Lines Differing in Metastatic Potential

Next, we analyzed the level of anxA2 protein expression in OS cell lines that differ in metastatic potential. By immunoblot analysis, anxA2 protein was reduced in the SaOS metastatic variant cells (LM2 and LM7) two- to fourfold as compared to the nonmetastatic SaOS2 parental line (Fig. 3B,C). Consistent with these results, anxA2 expression was diminished in the metastatic SaOSLM7 cells by immunofluorescence (Fig. 3D). In contrast, the expression of PTEN, a protein we have found expressed in several human osteosarcoma cell lines [Nielsen-Preiss et al., 2003], was abundantly expressed in each cell line (Fig. 3C). Based on these studies, the quantity of anxA2 appears to be reduced at both the mRNA and protein levels in metastatic OS cells.

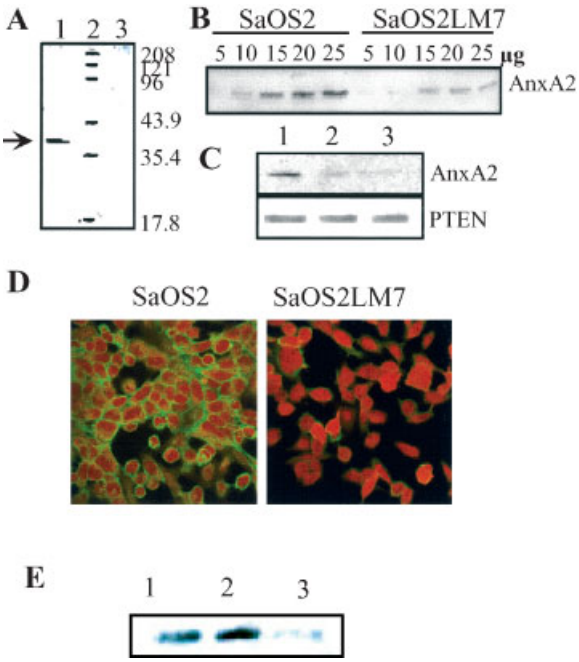

Fig. 3. AnxA2 protein is differentially expressed between metastatic and nonmetastatic OS cell lines. **A**: The specificity of the anxA2 antibody is illustrated by immunoblot analysis using an anxA2 blocking peptide (Santa Cruz Biotechnology). **Lane 1**, OS total cell lysate probed with the anxA2 antibody. **Lane 2**, mass standards indicated on the right side of the panel. **Lane 3**, OS total cell lysate probed with the anxA2-specific antibody following a pre-incubation with 1 µg of anxA2 blocking peptide. **B**: SaOS2 (nonmetastatic) and SaOSLM7 (metastatic) cells were grown to subconfluence, washed with PBS and lysed in RIPA buffer. Total cellular protein (5–25 µg/lane) was electrophoresed, transferred to a PVDF membrane and probed with an anxA2-specific antibody. **C**: The expression of anxA2 and PTEN were also evaluated in SaOSLM2 and SaOSLM7 cell lysates as compared to that of the parental SaOS2 cells (15 µg). **Lane 1**, SaOS2 cell lysate; **lane 2**, SaOSLM2 cell lysate; **lane 3**, SaOSLM7 cell lysate. **D**: SaOS2 and SaOSLM7 cells were cultured on slides. AnxA2 was detected using an anxA2-specific primary antibody with a FITC-conjugated secondary antibody. In the same cells, PTEN was detected using a PTEN-specific primary antibody with a Rhodamine-conjugated secondary antibody; 200× magnification. **E**: U2OS cells were lysed using Dounce homogenization in a hypotonic buffer and fractionated by differential centrifugation. The nuclei (**lane 3**) were pelleted by low-speed centrifugation and lysed in RIPA buffer. Membranous components (**lane 1**) were separated from soluble proteins (**lane 2**) by high-speed centrifugation. Each fraction was subjected to immunoblot analysis with an anxA2 antibody.

AnxA2 Is Localized to the Intracellular Compartment and Associated With the Plasma Membrane in Human OS Cells

We next wanted to evaluate the effects of exogenously expressed anxA2 in the osteoblastic cells. In preparation for those studies, we determined the subcellular localization of endogenous anxA2. The immunofluorescence studies shown in Figure 3D suggest that anxA2 is excluded from the nucleus and potentially associated with the plasma membrane. To further pursue the subcellular localization of anxA2, we used subcellular fractionation of both U2OS (Fig. 3E) and SaOSLM2 (data not shown) cells to evaluate whether anxA2 was associated with the membrane fraction. These biochemical studies determined that anxA2 could be isolated in association with cellular membranes, although a soluble proportion of anxA2 is present at variable levels. A small nuclear fraction of anxA2 was detected biochemically, however this is most likely due to contamination during the fractionation protocol since anxA2 is reported to have a nuclear export signal [Eberhard et al., 2001] and immunofluorescence studies suggested nuclear exclusion.

AnxA2 has been documented to be localized on either side of the plasma membrane [Raynal and Pollard, 1994]. AnxA2 associated with the outer aspect of the plasma membrane has been implicated in enzyme activation, whereas anxA2 associated with the inner aspect of the membrane may tether cytoskeletal proteins or transport vesicles. To refine the localization of membrane associated anxA2, we performed immunofluorescence with OS cells that were fixed and permeabilized to detect intracellular anxA2. We also analyzed nonpermeabilized OS cells to detect only extracellular anxA2. As seen in Figure 4A, anxA2 (panels 'a' and 'b') and F-actin (phalloidin staining-panels 'c' and 'd') could be detected only in permeabilized cells, whereas the cell surface protein CD44 could be detected in both the permeabilized and non-permeabilized cells (panels 'e' and 'f'). These data suggest anxA2 is not detectable on the cell surface of osteoblastic cells. To confirm these results, we used flow cytometry. AnxA2 could not be detected on the surface of intact U2OS (Fig. 4B) or SaOSLM2 cells (data not shown), whereas CD44 was readily observed on non-permeabilized cells.

AnxA2 Overexpression Does Not Affect Motility or Adhesion of Osteosarcoma Cells

Metastatic success can be determined, in part, by the ability of cells to migrate in vivo. Effective motility requires a balance between lamellipodia extension and adhesion followed by uropod release and retraction. In order to evaluate the ability of anxA2 to diminish

either cell motility or adhesion as a possible mechanism for metastatic suppression, we stably overexpressed anxA2 twofold in the SaOSLM2 cell line, which was chosen due to low levels of endogenous anxA2 expression [Gillette and Nielsen-Preiss, 2004]. As illustrated in Figure 5A, anxA2 overexpression did not alter the rate at which SaOSLM2 cells migrate into an artificial 'wound' created in a cell monolayer. Similarly, we were unable to detect an effect of anxA2 overexpression on the ability of cells to migrate in a transwell chamber assay (data not shown).

The ability of cells to migrate has been explained as a bell-shaped curve related to cell adhesion, where a median level of adhesion supports the most efficient motility [DiMilla et al., 1993]. Therefore, cell adherence may be affected even in the absence of changes in in vitro motility. Although we found SaOSLM2 cells to be more adherent than several other cell lines analyzed (data not shown), the level of anxA2 expression did not affect the ability of OS cells to attach (Fig. 5B).

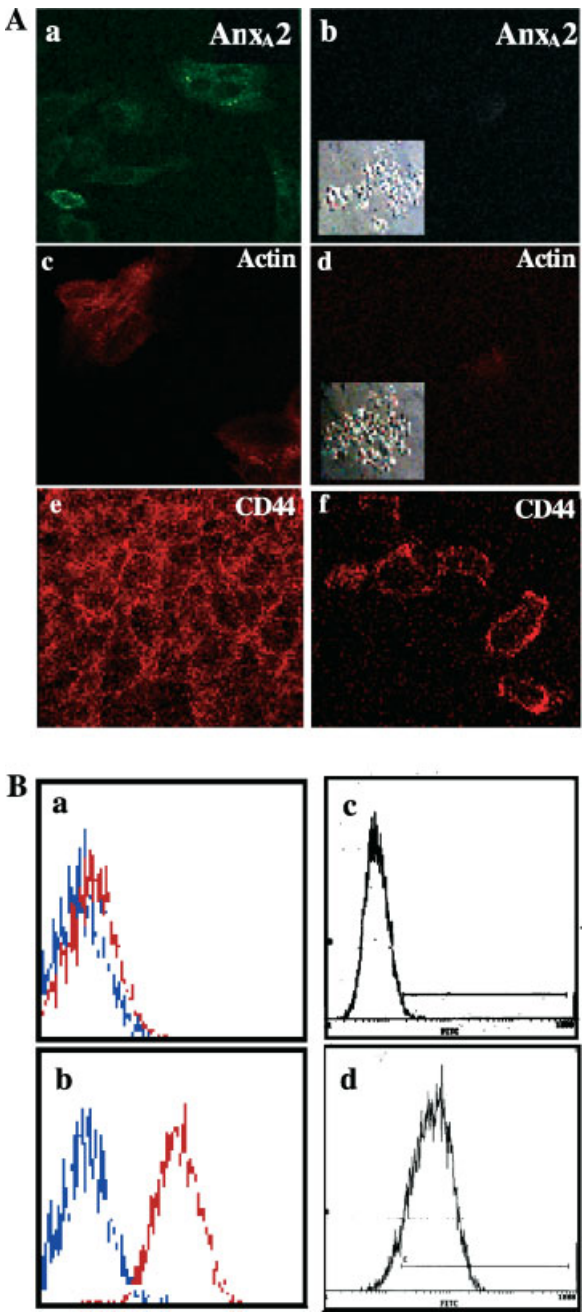

Fig. 4. AnxA2 is associated with the intracellular aspect of the plasma membrane in OS cells. **A**: U2OS cells were fixed and permeabilized (**panels a, c, e**) or incubated with primary antibody prior to fixation (**panels b, d, f**) and then subjected to immunofluorescence. AnxA2 was detected using a FITC-conjugated antibody, whereas CD44 was visualized with a Rhodamine-conjugated antibody. The inset in panels 'b' and 'd' display a lower magnification of cells present in the field using bright field microscopy. Panel 'f' represents a subconfluent monolayer, 200× magnification. **B**: Live and intact U2OS (**panels a, b**) or SaOSLM2 (**panels c, d**) cells were subjected to flow cytometry. The anxA2 peak (panel a: red) overlaps that of the secondary antibody alone (panel a: blue) in U2OS cells. SaOSLM2 cells exposed to an anxA2 antibody (with a FITC-conjugated secondary antibody) are shown in panel c. The CD44 peak in U2OS cells (panel b: red) is observed as a right-shifted peak distinguishable from the secondary antibody-only control peak (blue) in U2OS. SaOSLM2 cells exposed to CD44 antibody reveal a similar right-shifted peak (panel d).

AnxA2 Overexpression Does Not Affect Proliferation or Progression Through the Cell Cycle

Cancerous cells have commonly acquired survival and proliferative advantages, including alterations in control of the cell cycle. Therefore, we proposed that anxA2 suppresses metastatic potential by negatively affecting growth. The rate of proliferation was evaluated between cells expressing either a GFP or an anxA2GFP construct. As is illustrated in Figure 6A, anxA2 does not interfere with the ability of SaOSLM2 cells to proliferate under normal culture conditions. The MTT assay, although routinely used to evaluate growth, is fundamentally a metabolic assay. Therefore, we repeated the growth rate analysis studies using traditional cell counts and found no affect of anxA2 overexpression (data not shown). In addition, our data illustrate that anxA2 overexpression does not alter progression through the cell cycle as demonstrated in Figure 6B.

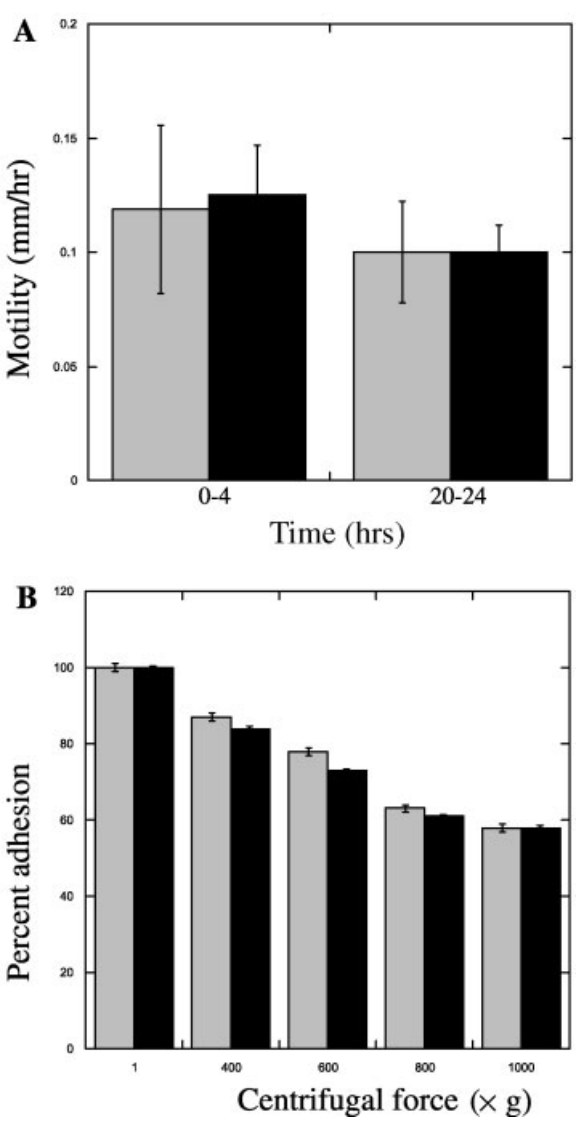

Fig. 5. Overexpression of anxA2 does not affect cell motility or adhesion. **A**: SaOSLM2 GFP (gray bars) and SaOSLM2anxA2GFP (black bars) cells were seeded at confluence in 6-well plates. The monolayer was 'wounded' and cell intrusion into the 'wound' was measured over two 4-h time periods. **B**: SaOSLM2GFP (gray bars) and SaOSLM2anxA2GFP (black bars) monolayers were subjected to the indicated forces in an inverted plate centrifugation assay. Following centrifugation, the relative number of cells remaining was determined by an MTT assay.

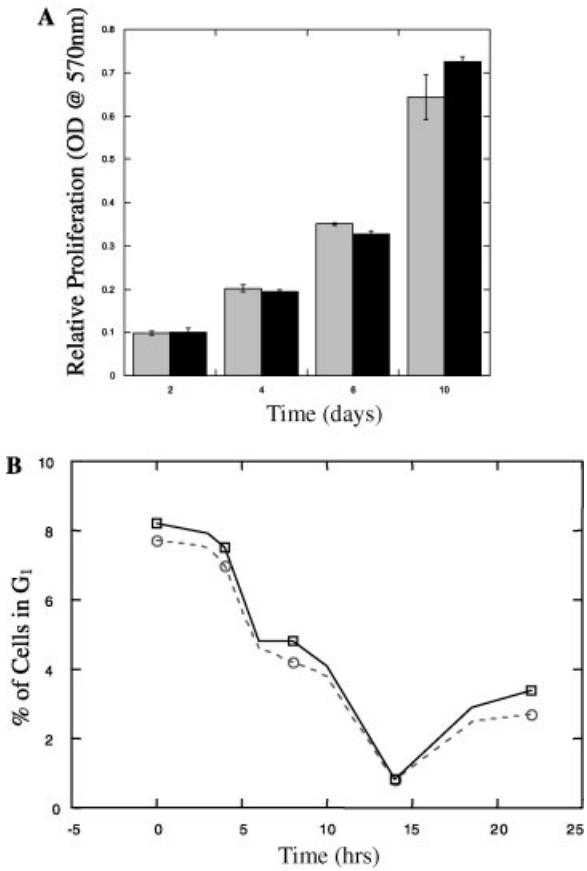

Fig. 6. AnxA2 overexpression does not affect OS proliferation. **A**: SaOSLM2 cells stably transfected with GFP (gray bars) or anxA2GFP (black bars) were seeded in 96-well plates and subjected to an MTT assay at various time points up to 10 days. **B**: SaOSML2 (dotted line) and SaOSLM2 anxA2GFP (solid line) cells were synchronized by serum starvation and an aphidicolin block. At time 0, cells were stimulated in medium containing 10% FBS and cell cycle progression was analyzed over 24 h.

AnxA2 Overexpression Diminishes Tumorigenic and Experimental Metastatic Potential In Vivo

The increase in in vitro mineralization that we observed previously suggests that anxA2 may shift osteosarcoma cells to a more differentiated state. To evaluate whether an anxA2-mediated shift toward differentiation negatively impacts tumor cell aggressiveness, cells expressing either GFP or overexpressing anxA2GFP were inoculated subcutaneously in athymic mice. Six weeks following inoculation, primary tumors were harvested and analyzed. Lungs were isolated from mice that had received tail vein inoculations. As is illustrated in Table I, overexpression of anxA2 decreased the ability of osteosarcoma cells to form primary tumors as well as to colonize the lungs following intravenous inoculation.

DISCUSSION

In this study, we used RDA to isolate sequences that were differentially expressed between cells of a primary human OS tumor and a metastatic lung tumor. We report the identi-

TABLE I. AnxA2 Overexpression Reduces Metastatic and Tumorigenic Potential of Osteosarcoma Cells

Cell type	Cell # per inoculation	Tumor rate[a]	Metastasis[b]
SaOSLM2GFP	2×10^6	3/8 (38%)	1/4 (25%)
SaOSLM2AnxA2GFP	2×10^6	0/10 (0%)	0/5 (0%)

[a]Cells were inoculated subcutaneously in the presence of matrigel in the flank of athymic mice.
[b]Metastasis to the lungs was evaluated, cells were inoculated into the tail vein of mice.

fication of *anxA2* as a gene with diminished expression in metastatic OS tumors that may therefore have a role in suppressing OS aggressiveness. The *anxA2* gene was found to be downregulated in a subset of human OS metastases and metastatic OS cell lines at the level of both mRNA and protein expression. Although anxA2 was diminished in only a subset of metastatic lesions or cells, other genes such as p53, have been shown to have a role in tumor aggressiveness, but only in a small proportion of the samples. The annexins belong to a large family of proteins that are characterized by conserved annexin repeat domains and an ability to bind negatively charged phospholipids in a calcium-dependent manner. While the annexin protein family is structurally related, it has a diverse cellular distribution and poorly defined functions [Raynal and Pollard, 1994; Gerke and Moss, 1997]. Knockout mice have been unable to define common roles for the annexin family members, since the anx7 knockout is lethal and the anx6 knockout lacks a clear phenotype [Hawkins et al., 1999; Srivastava et al., 1999]. In general, annexins have been described as participants in processes such as membrane fusion, endo- and exocytosis, membrane/cytoskeletal interactions, receptor signaling, protease activation at the cell surface, and membrane channel activation [Raynal and Pollard, 1994; Gerke and Moss, 1997, 2001; Hansen et al., 2002; van de Graaf et al., 2003].

Various roles for anx2 have been characterized in complex phenotypes including tumorigenesis and metastasis. Several studies suggest an inhibitory role for anxA2 in cancer and metastasis. For example, anxA2 antibodies were shown to enhance migration of Lewis lung carcinoma cells, indicating that anxA2 may inhibit migratory potential. Consistent with this interpretation, the addition of exogenous anxA2 inhibited wound closure and transwell migration [Balch and Dedman, 1997]. In yet another system, expression of anxA2 in PC12 rat adrenal pheochromocytoma cells was strongly increased after induction of differentiation [Fox et al., 1991]. Similarly, additional studies have suggested that anxA2 may be involved in neuronal cell differentiation [Jacovina et al., 2001]. Other studies suggest a positive role for anxA2 in cancer and metastasis. For example, studies with large cell lymphoma cells implicated anxA2 in adhesion between tumor cells and endothelial cells. AnxA2-specific antibodies were able to inhibit RAW117 cell adhesion to liver sinuosoidal endothelial cells [Tressler et al., 1993]. Yet other studies describe diminished anxA2 expression in prostate cancer samples suggesting that anxA2 may be involved in an early or common pathway of prostate carcinogenesis [Chetcuti et al., 2001]. Recent data suggest that the re-expression of anxA2 in prostate cancer cells inhibits transwell migration [Liu et al., 2003]. AnxA2 has also been described in pancreatic cancers in which anxA2 expression correlated with actively growing parts of tumors suggesting a role in cell proliferation. In combination, these studies implicate anxA2 in several forms of malignancy yet the variety of functional differences illustrate the lack of a common function and may reflect tissue specificity and differences in anxA2 localization.

Before initiating functional studies, we evaluated whether anxA2 was most likely involved in cell surface proteolytic events, nuclear proliferation events, or cytosolic events affecting protein transport and/or cytoskeleton rearrangements by determining the anxA2 subcellular distribution in OS cells. We were unable to detect anxA2 in the nucleus, on the cell surface, or secreted into the conditioned medium. Thus, the localization of anxA2 in OS cells most likely excludes a role in DNA binding or activation of proteolytic enzymes (such as plasminogen) on the cell surface. AnxA2 was localized to the intracellular compartment of osteoblastic cells with a plasma membrane-associated and a cytosolic component. The intracellular membrane localization of anxA2 is consistent with

previously characterized models of anxA2 function including (1) linkage of the cytoskeleton to the extracellular environment through plasma membrane microdomains [Gerke and Weber, 1984; Thiel et al., 1992; Harder et al., 1997; Oliferenko et al., 1999] and (2) regulation of secretory vesicles involved in protein transport and/or endo- and exocytosis [Creutz, 1992; Chasserot-Golaz et al., 1996; Konig et al., 1998]. These two potential mechanisms of anxA2 function could still impact several phenotypic parameters. Therefore, we overexpressed anxA2 and evaluated the effects of anxA2 on phenotypes associated with malignancy. Our data suggest that while anx2 overexpression did not alter cell motility, adhesion, or proliferation, an increase in differentiation potential was observed in osteoblastic cells. Generally, as cells undergo differentiation, there is a concomitant decrease in proliferation that was not observed in our studies. However, the cell cycle and proliferation data were collected from cells maintained in rich medium rather than in medium favoring differentiation. This increased differentiation was consistent with previous studies defining a role for anxA2 in osteoblastic mineralization mediated by lipid raft microdomains [Gillette and Nielsen-Preiss, 2004].

Other members of the annexin family have been identified in osteoblastic cells including A1, A4, A5, and A6. Based on functional and structural characteristics, anxA1, A5, and A6 would be the most likely candidates to provide compensatory functions. Of these, anxA5 has been implicated in the differentiation and mineralization program. AnxA5 was originally identified as a collagen binding protein and located in the extracellular matrix of calcifying cartilage [Mollenhauer and von der Mark, 1983]. Further studies discovered the calcium channel activity of anxA5, which is thought to be responsible for calcium influx in matrix vesicles [Genge et al., 1992; Liemann et al., 1996]. Recently studies employing an anxA5 knockout mouse have determined that anxA5 is not required for skeletal development [Brachvogel et al., 2003]. These studies suggest that anxA2 may be compensating for the loss of anxA5 although the authors were unable to detect an upregulation of anxA2 protein. Clearly, the functional relationships and compensatory potential of annexin family members need to be further explored.

A balance between proliferation and differentiation is required to maintain cellular homeostasis and a shift in this balance toward proliferation is often considered a hallmark of cancer. Therefore, disruption of the differentiation program, perhaps through the loss of anxA2, may induce a proliferative advantage that promotes OS aggressiveness. The production of a mineralized extracellular matrix can be used as an outcome variable to monitor osteoblastic mineralization and differentiation. We found previously that overexpression of anxA2 in osteoblastic cells in vitro increased the production of mineralized nodules [Gillette and Nielsen-Preiss, 2004] and therefore indicates a shift in differentiation. AnxA2-enhanced mineralization coincides with diminished OS aggressiveness as was observed in Table I. Interestingly, other studies have shown that overexpression of alkaline phosphatase, an enzyme important in bone differentiation, can also reduce malignancy of osteoblastic cells [Manara et al., 2000]. Anx2 expression has also been shown to induce chondrocyte mineralization in culture [Wang and Kirsch, 2002]. Consistently, upregulation of anxA2 has also been correlated with the pathologic mineralization and joint destruction associated with both rheumatoid and osteoarthritis [Justen et al., 2000; Kirsch et al., 2000]. In combination, these data support a mechanism whereby anxA2 decreases tumor aggressiveness by inducing differentiation. Studies to more clearly define the molecular role of anxA2 in osteoblastic mineralization are currently underway.

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REFERENCES

Balch C, Dedman JR. 1997. Annexins II and V inhibit cell migration. Exp Cell Res 237:259–263.

Brachvogel B, Dikshas J, Moch H, Welzel H, von der Mark K, Hofmann C, Poschl E. 2003. Annexin A5 is not essential for skeletal development. Mol Cell Biol 23: 2907–2913.

Chasserot-Golaz S, Vitale N, Sagot I, Delouche B, Dirrig S, Pradel LA, Henry JP, Aunis D, Bader MF. 1996. Annexin II in exocytosis: Catecholamine secretion requires the translocation of p36 to the subplasmalemmal region in chromaffin cells. J Cell Biol 133:1217–1236.

Chetcuti A, Margan SH, Russell P, Mann S, Millar DS, Clark SJ, Rogers J, Handelsman DJ, Dong Q. 2001. Loss of annexin II heavy and light chains in prostate cancer and its precursors. Cancer Res 61:6331–6334.

Creutz CE. 1992. The annexins and exocytosis. Science 258:924–931.

DiMilla PA, Stone JA, Quinn JA, Albelda SM, Lauffenburger DA. 1993. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. J Cell Biol 122:729–737.

Drust DS, Creutz CE. 1988. Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature 331:88–91.

Eberhard DA, Karns LR, VandenBerg SR, Creutz CE. 2001. Control of the nuclear-cytoplasmic partitioning of annexin II by a nuclear export signal and by p11 binding. J Cell Sci 114:3155–3166.

Faure A, Migne C, Devilliers G, Ayala-Sanmartin J. 2002. Annexin 2 "secretion" accompanying exocytosis of chromaffin cells: Possible mechanisms of annexin release. Exp Cell Res 276:79–89.

Fox MT, Prentice DA, Hughes JP. 1991. Increases in p11 and annexin II proteins correlate with differentiation in the PC12 pheochromocytoma. Biochem Biophys Res Comm 177:1188–1193.

Gamberi G, Benassi MS, Bohling T, Ragazzini P, Molendini L, Sollazzo MR, Pompetti F, Merli M, Magagnoli G, Balladelli A, Picci P. 1998. C-myc and c-fos in human osteosarcoma: Prognostic value of mRNA and protein expression. Oncol 55:556–563.

Genge B, Cao X, Wu LNY, Wuthier RE. 1992. Establishment of the primary structure of the two major matrix vesicle annexins by peptide and DNA sequencing. Bone Mineral 17:202–208.

Gerke V, Moss SE. 1997. Annexins and membrane dynamics. Biochim Biophys Acta 1357:129–154.

Gerke V, Moss SE. 2001. Annexins: From structure to function. Physiol Rev 82:331–371.

Gerke V, Weber K. 1984. Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin. EMBO J 3:227–233.

Gillette JM, Nielsen-Preiss SM. 2004. The role of annexin 2 in osteoblastic mineralization. J Cell Sci 117:441–449.

Hansen MD, Ehrlich JS, Nelson WJ. 2002. Molecular mechanism for orienting membrane and actin dynamics to nascent cell-cell contacts in epithelial cells. J Biol Chem 277:45371–45376.

Harder T, Kellner R, Parton RG, Gruenberg J. 1997. Specific release of membrane-bound annexin II and cortical cytoskeletal elements by sequestration of membrane cholesterol. Mol Biol Cell 8:533–545.

Hawkins TE, Roes J, Rees D, Monkhouse J, Moss SE. 1999. Immunological development and cardiovascular function are normal in annexin VI null mutant mice. Mol Cell Biol 19:8028–8032.

Hubank M, Schatz DG. 1994. Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res 22:5640–5648.

Jacovina AT, Zhong F, Khazanova E, Lev E, Deora AB, Hajjar KA. 2001. Neuritogenesis and the nerve growth factor-induced differentiation of PC-12 cells requires annexin II-mediated plasmin generation. J Biol Chem 276:49350–49358.

Jia SF, Worth LL, Kleinerman ES. 1999. A nude mouse model of human osteosarcoma lung metastasis for evaluating new therapeutic stratagies. Clin Exp Metastasis 17:501–506.

Justen H, Grunewald E, Totzke G, Gouni-Berthold I, Sachinidis A, Wessinghage D, Vetter H, Schulze-Ostoff K, Ko Y. 2000. Differential gene expression in synovium of rheumatoid arthritis and osteoarthritis. Mol Cell Biol Res Comm 3:165–172.

Kirsch T, Swoboda B, Nah HD. 2000. Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage. Osteoarthritis Cartilage 8:294–302.

Konig J, Prenen J, Nilius B, Gerke V. 1998. The Annexin II-p11 complex is involved in regulated exocytosis in bovine pulmonary artery endothelial cells. J Biol Chem 273: 19679–19684.

Kruzelock RP, Murphy EC, Strong LC, Naylor SL, Hansen MF. 1997. Localization of a novel tumor suppressor locus on human chromosome 3q important in osteosarcoma tumorigenesis. Cancer Res 57:106–109.

Ladanyi M, Cha C, Lewis R, Jhanwar SC, Huvos AG, Healey JH. 1993. MDM2 gene amplification in metastatic osteosarcoma. Cancer Res 53:16–18.

Liemann S, Benz J, Burger A, Voges D, Hofmann A, Huber R, Gottig P. 1996. Structural and functional characterization of the voltage sensor in the ion channel human annexin V. J Mol Biol 258:555–561.

Liu J-W, Shen J-J, Tanzillo-Swarts A, Bhatia B, Maldonado CM, Person MD, Lau SS, Tang DG. 2003. Annexin II expression is reduced or lost in prostate cancer cells and its re-expression inhibits prostate cancer cell migration. Oncogene 22:1475–1485.

Manara MC, Baldini N, Serra M, Lollini P-L, De Giovanni C;Vaccari M, Argnani A, Benini S, Maurici D, Picci P, Scotlandi K. 2000. Reversal of malignant phenotype in human osteosarcoma cells transduced with the alkaline phosphatase gene. Bone 26: 215–220.

Meyers PA, Heller G, Healey J. 1992. Chemotherapy for nonmetastatic osteogenic sarcoma: The Memorial Sloan-Kettering experience. J Clin Oncol 10:5–15.

Meyers PA, Heller G, Healey JH, Huvos A, Applewhite A, Sun M, LaQuaglia M. 1993. Osteogenic sarcoma with clinically detectable metastasis at initial presentation. J Clin Oncol 11:449–453.

Miller CW, Aslo A, Won A, Tan M, Lampkin B, Koeffler HP. 1996. Alterations of the p53, Rb and MDM2 genes in osteosarcoma. Cancer Res Clin Oncol 122:559–565.

Mollenhauer J, von der Mark K. 1983. Isolation and characterization of a collagen-binding glycoprotein from chondrocyte membranes. EMBO J 2:45–50.

Nielsen-Preiss SM, Silva SR, Gillette JM. 2003. Role of PTEN and Akt in the regulation of growth and apoptosis in human osteoblastic cells. J Cell Biochem 90:964–975.

Oliferenko S, Paiha K, Harder T, Gerke V, Schwarzler C, Schwarz H, Beug H, Gunthert U, Huber L. 1999.

Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. J Cell Biol 146:843–854.

Radinsky R, Fidler IJ, Price JE, Esumi N, Tsan R, Petty CM, Bucana CD, Bar-Eli M. 1994. Terminal differentiation and apoptosis in experimental lung metastases of human osteogenic sarcoma cells by wild type p53. Oncogene 9:1877–1883.

Raynal P, Pollard HB. 1994. Annexins: The problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta 1197:63–93.

Scheffer H, Kruize YCM, Osinga J, Kuiken G, Oosterhuis JW, Leeuw JA, Schraffordt Koops H, Buys CHCM. 1991. Complete association of loss of heterozygosity of chromosomes 13 and 17 in osteosarcoma. Cancer Genet Cytogenet 53:45–55.

Siever DA, Erickson HP. 1997. Extracellular annexin II. Int J Biochem Cell Biol 29:1219–1223.

Srivastava M, Atwater I, Glasman M, Leighton X, Goping G, Caohuy H, Miller G, Pichel J, Westphal H, Mears D, Rojas E, Pollard HB. 1999. Defects in inositol 1,4,5-triphosphate receptor expression, Ca2+ signaling, and insulin secretion in the anx7 (+/−) knockout mouse. Proc Natl Acad Sci 96:13783–13788.

Thiel C, Osborne M, Gerke V. 1992. The tight association of the tyrosine kinase substrate annexin II with the submembranous cytoskeleton depends on intact p11- and Ca^{2+}-binding sites. J Cell Sci 103:733–742.

Toguchida J, Ishizaki K, Nakamura Y, Sasaki MS, Ikenaga M, Kato M, Sugimoto M, Kotoura Y, Yamamuro T. 1989. Assignment of common allele loss in osteosarcoma to the subregion 17p13. Cancer Res 49:6247–6251.

Tressler RJ, Upkyke TV, Yeatman T, Nicolson GL. 1993. Extracellular annexin II is associated with divalent cation-dependent tumor cell-endothelial cell adhesion of metastatic RAW117 large-cell lymphoma cells. J Cell Biochem 53:265–276.

van de Graaf SF, Hoenderop JGJ, Gkika D, Lamers D, Prenen J, Rescher U, Gerke V, Staub O, Nilius B, Bindels RJM. 2003. Functional expression of the epithelial Ca^{2+} channels (TRPV5 and TRPV6) requires association of the S100A10-annexin 2 complex. EMBO J 22:1478–1487.

Wang W, Kirsch T. 2002. Retinoic acid stimulates annexin-mediated growth plate chondrocyte mineralization. J Cell Biol 157:1061–1069.

Wu JX, Carpenter PM, Gresens C. 1990. The proto-oncogene c-fos is overexpressed in the majority of human osteosarcomas. Oncogene 5:989.

Yamaguchi T, Toguchida J, Yamamuro T, Kotoura Y, Takada N, Kawaguchi N, Kaneko Y, Nakamura Y, Sasaki MS, Ishizaki K. 1992. Allelotype analysis in osteosarcomas: Frequent allele loss on 3q, 13q, 17p and 18q. Cancer Res 52:2419–2423.