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EXPRESSION AND FUNCTION OF SURVIVIN IN CANINE OSTEOSARCOMA

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Abstract

Osteosarcoma (OS) has a high mortality rate and remains in need of more effective therapeutic approaches. Survivin is an IAP family member protein that blocks apoptosis and drives proliferation in human cancer cells where it is commonly elevated. In this study, we illustrate the superiority of a canine OS model as a translational tool for evaluating **survivin-directed therapies,** owing to the striking similarities in gross and microscopic appearance, biologic behavior, gene expression and signaling pathway alterations. Elevated survivin expression in primary canine OS tissue correlated with increased histologic grade and mitotic index and a decreased disease free interval (DFI). Survivin attenuation in canine OS cells inhibited cell cycle progression, increased apoptosis, mitotic arrest and chemosensitivity, and cooperated with chemotherapy to significantly improve ***in vivo*** **tumor control.** Our findings illustrate the utility of a canine system to more accurately model human OS and strongly suggest that survivin-directed therapies might be highly effective in its treatment.

INTRODUCTION

Osteosarcoma (OS) is the most common primary bone tumor in dogs and humans (1-3), and is characterized by both aggressive local tissue infiltration and a very high metastatic rate. Despite the use of neoadjuvant and adjuvant chemotherapy, the 5-year survival rate is only 60% in humans and the 2-year survival rate is only 20% in dogs, illustrating that new therapeutics are needed (1).

The dog is a well-established model for spontaneous OS in humans, owing to striking similarity in biology and gene expression (3, 4). The dog's large size, relative outbreeding and immunocompetence increase their model potential. Furthermore, dogs with spontaneous tumors naturally develop therapy resistance and metastasis. Additionally, tumor burdens in spontaneously arising cancers of dogs are more similar to humans than the experimentally-induced tumors found in murine models, which may be important with regard to biologic factors such as hypoxia and clonal variation. The size of canine tumors also allows for serial imaging and tissue collection over time (3, 4).

Survivin is a 16.5 kD protein belonging to the Inhibitor of Apoptosis (IAP) family (5). The survivin gene has five known mRNA splice variants: Survivin, Survivin-2B, Survivin-ΔEx3, Survivin-3B, and Survivin-2α (6). Unlike other IAP family members, survivin has two known functions in cells: regulation of cell division and inhibition of apoptosis (7). Survivin is found as a monomer in the chromosomal passenger complex (CPC) and binds via its C terminal to the microtubules of the mitotic spindle during mitosis (8). Survivin is dimeric in its role as an IAP molecule (9) and is thought to have targets upstream of effector caspases or target effector caspases themselves (10, 11).

Evidence suggests that survivin plays a role in inhibiting both caspase-dependent and caspase-independent apoptosis (12, 13). Survivin may also indirectly inhibit apoptosis, via binding to the proapoptotic protein Smac/DIABLO, preventing it from binding to and inhibiting other IAP molecules (14). Some survivin is localized to the mitochondria and is thought to prevent apoptosis upon release as well as block the release of apoptosis-inducing factor (AIF) (13). Both cytoplasmic and mitochondrial survivin are thought to inhibit apoptosis via binding to other proteins, possibly the effector caspases or other associated proteins (10, 15). More recent evidence suggests that survivin also enhances telomerase activity, and may play a role in chemotherapy resistance and metastasis (16-19).

Normal cells do not require survivin for survival (20). In cancer cells, however, survivin is critical for its roles in cell division and inhibition of apoptosis (5). It also appears to have roles in tumorigenesis (15, 21) and drug resistance (17, 18). Hematopoietic progenitor cells, thymocytes, and T-cells express survivin at low levels for cell proliferation (22-24); however, differentiated cells do not express survivin. More specific to the purpose of our study, survivin is only expressed at very low levels in normal osteoblasts (25). Most types of cancer express survivin at very high levels and depend on it for continued proliferation (5, 20).

Survivin expression has prognostic significance in many types of human cancer (26). Small studies in human OS have suggested that survivin may be useful in determining prognosis and degree of malignancy (27-29); however, definitive studies regarding the role of survivin in human OS are lacking. Survivin expression is a negative prognostic factor in dogs with B-cell lymphoma (30), as has been

demonstrated in human B-cell lymphoma (31), and survivin expression has been identified in select other canine neoplasms (30).

In this study, we sought to determine the effects of survivin inhibition in canine OS cell lines and to evaluate a correlation between survivin expression and outcome in canine OS patients. We hypothesized, as observed in human OS, that survivin inhibition would decrease cell proliferation and increase apoptosis and chemosensitivity in canine OS cells, and that increased survivin expression would correlate with a poor prognosis in canine OS patients.

MATERIALS AND METHODS

Cell lines and conditions

The Abrams canine OS cell line was provided by Dr. William Dernell, and the D17 canine OS cell line was purchased from American Type Culture Collection (Rockville, MD). Both cell lines were serially passaged by trypsinization, and maintained in C/10 media [Minimum Essential Medium (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), and 10% heat inactivated fetal bovine serum (FBS) (Atlas, Fort Collins, CO)]. Cells were grown in a humidified incubator, with 5% CO_2 at 37°C. Both cell lines were confirmed to be of canine origin by multispecies multiplex PCR and identified by short tandem repeat analysis as described (32).

siRNA Transfection

A custom siRNA against canine survivin (430) and a scrambled siRNA control were designed using an online resource (BLOCK-iT™ RNA Designer, Invitrogen, Carlsbad, CA) and purchased from Invitrogen. Transfection was accomplished using HiPerFect transfection reagent (Qiagen, Valencia, CA) in 100 μL Opti-mem media (Invitrogen, Carlsbad, CA). Cells were harvested for analysis 24-72 hours following transfection. Detailed transfection methods are included in the **Supplemental Data.**

Survivin Expression

Detailed methods for the techniques described below are included in the **Supplemental Data.**

qRT-PCR – We evaluated survivin mRNA in the survivin siRNA transfected, sham transfected and control cells at 48 hours post transfection using real-time RT-PCR. Primers for canine survivin and the housekeeping gene, HPRT, were designed using Integrated DNA Technologies' (IDT) website and purchased from IDT (Coralville, IA) (**Table S1**).

Immunofluorescence – Transfected cells were washed, fixed methanol and air-dried, followed by incubation in rabbit polyclonal anti-survivin antibody (Novus Biologicals Bloomington, MN) followed by Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen). The slides were washed again, then mounted using VectaShield plus DAPI mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired using a Zeiss Axioplan 2 imaging microscope and Axio Vision Release 4.6 software (Carl Zeiss Micro-Imaging Inc, Thornwood, NY).

Western blot – At 48 hours post transfection, the cells were harvested, protein quantified and equal amounts loaded onto a 4-12% Bis-Tris gel (Invitrogen), followed by electrophoresis and transfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked in 5% non-fat dry milk in TBST, followed by incubation with rabbit polyclonal anti-survivin (Novus) followed by HRP conjugated goat anti-rabbit IgG (Pierce, Rockford, IL). Protein bands were detected using SuperSignal West Femto Maximum Sensitivity ECL Substrate (Thermo Scientific) using radiographic film (Kodak, Rochester, New York). The images were scanned and pixel density assessed using Image J (NIH).

Cell Number and Viability

To determine cell numbers, total and live/dead cell numbers were counted in triplicate at 24, 48, 72 hours post transfection using trypan blue.

Apoptosis

Caspase-3/7 Assay – To determine levels of apoptosis, we used a SensoLyte Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA). At 48 hours post transfection, the cells and their supernatants from each individual well were harvested from 6-well plates, and centrifuged at 1500 rpm for 7 minutes. The media was aspirated off and the cells were lysed with 1x lysis buffer (AnaSpec). Next the lysates were transferred to Eppendorf tubes and inverted for 30 minutes at 4˚C. Then the lysates were centrifuged at 2,500 g for 10 minutes at 4˚C. The supernatant was collected from each sample and transferred at 60 μL per well to a 384-well black-walled plate in duplicate. Twenty μL of caspase-3/7 assay reagent mix was added to each well and the plate was put on a plate shaker for ~60 minutes at 100-200 rpm in the dark at room temperature. The plate was then read on a microplate reader (Synergy HT, Bio-Tek, Winooski, VT) to measure fluorescence intensity at 360/460 nm. Fluorescence intensity measurements were termed 'Relative Fluorescent Units' or RFUs in graphical presentations.

TUNEL Assay – For further analysis of apoptosis, we used a commercial TUNEL kit (*In Situ* Cell Death Detection Kit, Roche Diagnostics, Mannheim, Germany). At 48 hours post transfection, 2-well chamber slides were rinsed in PBS and air-dried

overnight followed by fixation (4% paraformaldehyde in 1x PBS) for 1 hour at room temperature. The slides were rinsed in 1x PBS and incubated in permeabilization solution (0.1% Triton X 100 and 0.1% sodium citrate in 1x PBS) for 2 minutes at 4°C. The slides were rinsed 2 more times in 1x PBS, then 200 μL of TUNEL reaction mixture was added. Slides were incubated for 60 minutes in the dark at 37°C, rinsed 3 times in 1x PBS, and mounted using VectaShield plus DAPI mounting medium. Microscopic images of random 20x fields were obtained.

Cell Cycle Analysis

Propidium iodide staining and flow cytometry was used to evaluate changes in cell cycle distribution following siRNA transfection. The cells were trypsinized, resuspended in 1.5 mL 1x PBS and 3.5 mL ice-cold 100% EtOH was added dropwise while vortexing slowly. The cells were then placed on ice for 30 minutes or overnight at -20°C. After this incubation, the cells were centrifuged at 1500 rpm for 7 minutes, washed once in PBS, then resuspended in 250 μL 1x PBS. Two hundred μL of extraction buffer (192 μL of 0.2 M Na_2HPO_4, 8 μL of 0.1 M citric acid), followed by 500 μL PI-RNAse reagent (50 μg/mL propidium iodide, 125 Worthington U/mL RNAse) was added. Cells were filtered through a 40 μm nylon cell strainer and incubated at 37°C for 30 minutes. Samples were then run on a FACscan flow cytometer (BD Biosciences, Durham, NC). Cell cycle analysis on samples was performed using FlowJo Software (Tree Star, Ashland, OR).

Chemosensitivity

To determine sensitivity to carboplatin (CPT, Amatheon, Miami, FL) or doxorubicin (DOX, Bedford Laboratories, Bedford, OH), cells were incubated with no drug, 21.55 μM CPT, or 125 nM DOX. Drug was added for 36-48 hours directly after a 24 hour transfection. Cells were then harvested from their individual wells with their supernatants and total cell count for each well was determined. Cells were lysed and processed according to the SensoLyte Homogenous AMC Caspase-3/7 Assay Kit as above. RFUs for each sample were normalized to cell count (RFUs/cell) and results expressed as fold-change versus control (untreated cells). Degree of synergy between survivin knockdown and chemotherapy was evaluated using the Bliss independence model (See **Supplemental Data**).

Murine Xenograft Experiment

Female 6-8 week old nu/nu mice were purchased from the National Institutes of Health. EZN-3042, a survivin inhibiting locked nucleic acid antisense oligonucleotide (33, 34), and EZN-3046 (scrambled control oligonucleotide) were supplied by Enzon Pharmaceuticals (Piscataway, NJ). After demonstration of gene knockdown efficacy *in* vitro (not shown), mice were injected subcutaneously with $2x10^6$ Abrams OS cells. Tumors were grown to 7 mm diameter, size-matched and then allocated into 4 groups (n=8 per group). Group 1 received saline and EZN-3046, group 2 received DOX (Bedford) and EZN-3046, group 3 received saline and EZN-3042, and group 4 received DOX and EZN-3042. EZN-3042 and EZN-3046 were administered intraperitoneally at 100 mg/kg every three days starting day 0 until the end of the study (day 55). Doxorubicin or an equivalent volume of saline was administered intravenously at 3

mg/kg on day 4 and repeated every two weeks until the end of the study. Mice were weighed and tumors measured every three days. Mice were sacrificed at 15 mm tumor diameter or at the end of the study if they were still alive. A separate group of tumor-bearing mice were randomized similarly at 10 mm tumor diameter. They received EZN-3042 or EZN-3046 on days 0 and 3 (AM) and DOX or saline on day 3 (PM) and sacrificed on day 4. Tumors were harvested and snap-frozen or paraffin embedded for analysis of survivin expression by qRT-PCR as described above and immunohistochemistry as described below.

Canine Osteosarcoma Patient Population

The population of canine appendicular OS patients studied was a subset of patients from a previously reported randomized, prospective clinical trial (35). The study was approved by the Institutional Animal Care and Use Committees of the participating institutions. All dogs underwent amputation followed by 5 cycles of adjuvant DOX, with or without an investigational matrix metalloprotease inhibitor and had decalcified primary tumor tissue blocks available for analysis. Inclusion/exclusion criteria, staging, and follow-up procedures were standardized as previously reported (35). Histologic grading (from 1 to 3) was performed in a subset of cases by one author (BEP) utilizing a published schema incorporating amount of matrix, percent necrosis, nuclear pleomorphism, nucleolar size/number and mitosis score (35). Mitotic index was also calculated by counting the number of mitotic figures per 10 random 400X fields.

Survivin Immunohistochemistry

Slides of canine OS tissues were prepared from paraffin blocks. Slides were put through a hydration process of xylene baths to graded alcohol, then immersed in Target Retrieval Solution (DakoCytomation) and put through a pressure cooker cycle and cooled to room temperature. The slides were then washed in TBST, blocked with Biocare Sniper (Biocare Medical) for 10 minutes, then washed again. Incubation in primary rabbit polyclonal anti-survivin antibody, at 1:600 dilution occurred overnight at 4°C. The slides were washed 3 times before a 15-minute incubation in 3% hydrogen peroxide at room temperature and washed 3 additional times. Incubation in secondary antibody, Envision+ Dual Link System Peroxidase (Dako) for 30 minutes occurred at 4°C. The slides were washed 3 more times, chromogen stained for 10 minutes using DAB Peroxidase Substrate Kit, (Vector) washed once more and lightly counterstained with hematoxylin. The slides were graded based on survivin stain intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong, 4 = intense) and proportion of cells with positive survivin staining (0 = 0%, 1 = 1-10%, 2 = 10-25%, 3 = 25-50%, 4 > 50%). A final immunoreactivity score for each tissue sample was calculated by multiplying the percentage score by the intensity grade (possible score of 0-16). Scoring was performed by 2 individuals blinded as to patient outcome and the final survivin score averaged across the 2 raters. This scoring system has previously been used in immunohistochemical scoring of canine OS samples (36), and for survivin scoring in canine lymphoma (30).

Statistical analysis

Statistical analysis of *in vitro* data was performed using GraphPad Prism for

Macintosh Version 5.0b (GraphPad Software, La Jolla, CA). Survivin expression levels were summarized by standard descriptive statistics in terms of means and standard deviations. The comparisons of survivin expression, cell numbers, caspase activity and apoptosis levels between experimental conditions was performed using a two-sample t-test with a two-sided significance level. Tumor growth between groups was evaluated using a 1-way ANOVA. The Shapiro Wilk test was used to verify the normality assumption. P-values of less than 0.05 were considered statistically significant.

The Intra-Class Correlation Coefficient (ICC) was used to evaluate the reproducibility of the survivin immunoreactivity scores between the two independent raters. The ICC can be interpreted as follows: 0 – 0.2 (slight), 0.2 – 0.4 (fair), 0.4 – 0.6 (moderate), 0.6 – 0.8 (substantial) and 0.8 – 1.0 (almost perfect) (37).

Statistical analysis of survival data was performed using a combination of Prism and SAS software version 9.2 (SAS Institute, Cary, NC, USA). Correlations between survivin expression levels and other markers on a continuous scale were evaluated using linear regression analysis. Analysis of variance was used to evaluate the association between survivin expression levels and categorical markers. The median disease free interval (DFI) was estimated using the Kaplan-Meier method. Markers were categorized into a low risk and high risk group (with respect to predicting DFI) using the recursive portioning method (38). This method selects the best predictor variables using recursive splitting. It starts with the best possible predictor from the data set and successively splits the data into categories predicted to observe the event or not. As a splitting method, the exponential scaling method was used. The splitting process stopped when a minimum of five patients per groups was reached or when

there was no further decrease in prediction error. The associations between the categorized markers and DFI were evaluated using the log-rank test. Multivariate Cox proportional hazard regression analysis was used to determine the prognostic significance of the markers for predictive value of DFI. Predictive markers were selected by backward selection procedures with a p-value cut off of <0.10. A previously deleted variable was allowed to re-enter the final model if its p-value was <0.05. The likelihood ratio test was used to compare various models. The proportional hazard assumption was verified using plots of the log(-log) survival curves and Schoenfeld residuals.

RESULTS

siRNA-mediated knockdown decreases survivin expression. After preliminary validation of survivin as a viable target by confirming elevated survivin expression in 4 or 4 canine OS cell lines by western analysis (data not shown), we proceeded with survivin knockdown experiments. To verify the efficiency of the survivin knockdown in our survivin siRNA transfection, we harvested mRNA from the survivin knockdown, sham knockdown, and control cells at 48 hours post siRNA transfection. Analysis of the qRT-PCR data revealed ~20 fold and ~9 fold decreases in survivin mRNA expression in Abrams and D17 respectively in the survivin knockdown cells when compared to control and sham knockdown cells (**Fig. 1a**).

Since inhibition of survivin mRNA expression does not confirm decreased survivin protein, we performed further experiments to verify decreased protein expression. Western blot analysis of Abrams and D17 cells confirmed approximately 85% reduction in protein expression in survivin siRNA transfected groups compared to the sham transfected and control groups (**Fig. 1b, 1c**). Immunofluorescence cytochemistry further confirmed reduced survivin protein expression. Based on immunofluorescence assessment, knockdown efficacy was 80.2 +/- 7.1% for Abrams and 76.2 +/- 9.4% for D17 (**Fig. S1**).

Survivin inhibition decreases total cell number and cell viability. Total and live/dead cell counts were performed at 24, 48, and 72 hours post survivin knockdown in both cell lines. At all three time points, there were significantly decreased total cell numbers and significantly higher percent dead cells in the survivin siRNA transfected cells compared to both the sham transfected and control groups (**Fig. S2**).

Survivin inhibition increases apoptosis. To determine if the cell death observed was via apoptosis, we evaluated caspase-3,7 activity in the survivin siRNA transfected, sham transfected, and control cells 48 hours post transfection. There was a significant increase in caspase activity in survivin siRNA transfected cells compared to their respective sham transfected and control cells (**Fig. 2a**). This was confirmed by observation of increased DNA fragmentation 48 hours post siRNA transfection, as assessed via TUNEL (**Fig. 2b**).

Survivin inhibition results in failure of normal mitosis. We used flow cytometry to analyze cell cycle distribution 48 hours post siRNA transfection. In addition to an increase in the sub-G1 population consistent with previous observations regarding apoptosis, we observed an increase in a 4N population of cells, consistent with failure of mitosis, in survivin siRNA transfected cells. Compared with control transfected cells, the sub-G1 population increased an average of 3.0 and 1.9-fold in Abrams and D17 respectively, and the super-G2 (4N) population increased an average of 3.5 and 2.5-fold in Abrams (**Fig. 3a,b**) and D17 (**Fig. 3c)** respectively.

Survivin inhibition increases chemosensitivity. Doxorubicin and platinum drugs form the mainstays of medical therapy for the treatment of both canine and human OS. To determine if survivin was important in mediating resistance to chemotherapy in canine OS, we incubated canine OS cells with CPT or DOX, with or without concurrent survivin or sham siRNA transfection. Survivin knockdown increased caspase activity in both cell lines in the presence of DOX and CPT (**Fig. 4**).

Survivin inhibition in canine OS xenografts results in increased doxorubicin sensitivity. Survivin knockdown *in vivo* was accomplished using the locked nucleic acid

antisense inhibitor EZN-3042. Mice bearing established xenografts of Abrams canine OS cells were randomized to receive DOX or saline, +/- EZN-3042 or the scrambled control oligo EZN-3046. Knockdown was confirmed *in vivo* using both qRT-PCR (not shown) and immunohistochemistry following two intravenous oligo treatments (**Fig. 5a**). Notably there was a significant increase in survivin expression in the DOX/EZN-3046 group compared to the control (saline/EZN-3046) (**Fig. 5a**), suggesting possible survivin induction as a DOX response mechanism. Tumor growth rate was significantly reduced in the combined EZN-3042/DOX treated group compared to the other treatment groups (**Fig. 5b**).

Survivin protein expression in canine OS tissues correlates with histologic features and clinical outcome. Survivin expression was studied via immunohistochemistry in 67 primary canine OS tissues from dogs that underwent standardized staging, treatment and follow-up as part of a previously reported prospective clinical trial (35). The Intra-Class Correlation Coefficient of the final survivin immunoreactivity score between the two independent raters was 0.90 (95% CI: 0.84 – 0.93) indicating a very high level of reproducibility. Demographic information regarding the patient population is reported in **Table S2**. Survivin was expressed in 65 of 67 cases evaluated, with expression intensity ranging from modest to heavy (**Fig. S3**). Median survivin immunoreactivity score was 5 (range, 0 to 12). Staining was predominantly nuclear, although a combination of nuclear and cytoplasmic staining was observed in most samples. Survivin immunoreactivity score correlated positively with both histologic grade and mitotic index (**Figs. 6a and b**).

The overall median DFI in the studied patient population was 211 days (range 43

– 1,393+ days). Upon univariate analysis, histologic grade (1/2 vs. 3), bone-specific alkaline phosphatase (BALP) activity (≤ 48 vs. >48) and survivin immunoreactivity score (≤ 2.75 vs. >2.75) were identified as significant predictors of DFI (**Table 1, Fig. 6c)**. The median DFI in patients with a lower survivin immunoreactivity score (≤ 2.75) was 331 days versus 173 days in patients with a higher survivin immunoreactivity score (>2.75) ($p = 0.046$).

As observed in the original clinical trial (35), drug assignment did not impact DFI. Upon multivariate analysis, BALP and histologic grade retained prognostic significance (**Table 1**). Survivin immunoreactivity was not identified as an independent significant predictor of DFI in the multivariate analysis, owing in large part to the strong positive correlation between survivin immunoreactivity and histologic grade.

DISCUSSION

To assess the utility of canine OS as a potential model for survivin-directed therapeutics, we sought to determine the impact of survivin inhibition on canine OS cell lines *in vitro*, as well as the prognostic significance of survivin expression in primary canine OS treatment with surgery and chemotherapy. Survivin inhibition in Abrams and D17 canine OS cell lines induced apoptosis, cell cycle arrest, and increased caspase activity in the presence of CPT and DOX. Furthermore, inhibition of survivin was associated with an increase in the efficacy of DOX in a murine OS xenograft. Other research groups have reported similar results when indirectly and directly inhibiting survivin in OS. In one recent paper, inhibition of STAT3 activity (which caused down regulation of survivin expression) in canine and human OS decreased cell proliferation and viability, and induced caspase-3/7 mediated apoptosis in treated cells (39). Another group inhibited survivin in HeLa cells and observed caspase-dependent cell death as well as mitotic failure, resulting in multinucleated cells, up to 8 and 16N (40). This observed increase in 4N(+) population could be attributed to the importance of survivin in the chromosomal passenger complex (8) and its association to the mitotic spindle during mitosis (7, 20). Additionally, siRNA-mediated survivin inhibition in human MG-63 OS cells and shRNA-mediated survivin inhibition of human SAOS2 OS cells enhanced sensitivity to cisplatin and DOX (41, 42).

We observed significantly increased apoptosis in survivin knockdown compared to the sham knockdown and control cells in both canine OS cell lines in the absence of any pro-apoptotic stimulus (e.g. serum withdrawal or chemotherapy). There was also modestly increased apoptosis in the sham knockdown compared to the control for both

cell lines. We speculate that the enhanced basal apoptosis observed in the survivin knockdown was possibly due to the cellular stress imparted by the siRNA transfection process combined with the survivin inhibition. The modest increase in apoptosis observed in the sham knockdown cells supports this observation.

It is interesting to note that the growth curves in the murine xenografts did not begin to diverge until after the second DOX dose. It is possible that multiple EZN-3042 treatments enhanced gene knockdown beyond what was observed at the time we evaluated expression (following 2 injections), leading to a more pronounced effect on survivin expression. It is also worth noting that cotreatment with EZN-3042 and DOX resulted in tumor stabilization rather than regression in the Abrams xenograft model. This could be a function of the DOX dose intensity (supported by the lack of single-agent effect of DOX in this experiment), or dosage and scheduling of EZN-3042 treatments relative to DOX.

Elevated survivin protein immunoreactivity in canine OS tissue samples correlated with increased histologic grade and mitotic index as well as decreased DFI upon univariate analysis. Survivin immunoreactivity lost prognostic significance upon multivariate analysis owing to a strong correlation between survivin score and histologic grade. The correlation of increased survivin protein immunoreactivity to increased mitotic index is not surprising considering our *in vitro* findings that survivin inhibition caused mitotic failure and survivin's known roles in mitosis and the cell cycle (43).

Our results in canine OS are comparable with the limited information regarding survivin expression and outcome in human OS. One group has reported that nuclear localization of survivin correlated with a positive outcome, but did not report whether

overall survivin expression had an impact on DFI or survival (27). Another group associated survivin expression with histologic grade, differentiation and proliferation index (28). Most recently, high survivin mRNA expression has been correlated with both presence of metastasis and overall survival (29).

Survivin is a viable target for therapy. YM155, a small-molecule suppressor of survivin, is currently in phase II clinical trials in human cancer. Single-agent objective responses have been observed in patients with melanoma and non-small cell lung cancer (44, 45) as well as regression of established human hormone-refractory prostate cancer in xenograft models (46). Studies in combination with chemotherapy are ongoing. EZN-3042, the antisense oligonucleotide utilized in these *in vivo* experiments, is capable of inhibiting survivin expression and tumor growth *in vivo* (47) and improves chemotherapeutic response *in vitro* (48). EZN-3042 is currently in phase I clinical trials in human cancer. Survivin is also being considered as an immunotherapy target (49, 50). Phase I and phase II clinical trials of survivin-targeted vaccines are currently under way.

In conclusion, we have demonstrated that transient survivin knockdown in canine OS cells results in decreased total and viable cell numbers, increased apoptosis and mitotic arrest, and enhanced sensitivity to carboplatin and doxorubicin. Furthermore, elevated survivin expression in canine OS tissue correlates with increased histologic grade, increased mitotic index and decreased DFI. These findings are consistent with those in human OS, and indicate that survivin may be a viable therapeutic target for evaluation in canine OS as a preclinical model for human OS. There remains substantial room for improvement in the medical therapy for OS, and canine OS may

provide a novel translational model for the investigation of survivin-directed therapeutics.

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Table 1: *Univariate/Multivariate Analysis of Factors Associated with Outcome in Dogs with Appendicular Osteosarcoma Treated with Amputation and Doxorubicin.*

Univariate Analysis					
	Hazard Ratio	p-value	95% CI	Median (Days)	Range (Days)
Survivin score	0.512	0.0460	0.26 - 0.98		
≤2.75				331	43 - 1116
>2.75				173	45 - 1393+
BALP	0.393	0.0032	0.21 - 0.75		
≤48				239	43 – 1393+
>48				148	45 – 878+
Histologic Grade	0.487	0.0316	0.25 - 0.96		
1/2				231	43 – 1393+
3				153	70 - 486
Multivariate Analysis					
	Hazard Ratio	p-value	95% CI		
BALP	0.272	0.0052	0.11 – 0.69		
≤48					
>48					
Histologic Grade	0.275	0.0088	0.11 – 0.72		
1/2					
3					

FIGURE LEGENDS

Figure 1: *Efficiency of survivin gene knockdown in canine osteosarcoma cells.* **A.** qRT-PCR for survivin mRNA in Abrams and D17 cells showed a ~20 fold and ~9 fold decrease, respectively, in survivin siRNA when compared to the control and sham knockdown groups. **B:** Western blot analysis confirms survivin knockdown in both cell lines. **C:** Using image J analysis we measured the integrated density of the survivin protein bands following three separate survivin siRNA transfections. There was ~85% reduction in survivin protein expression in both cell lines. 430 = survivin siRNA, scrm = sham knockdown (scrambled) siRNA**.** Error bars in C represent standard deviation. *P < 0.05 vs. control and sham transfection.

Figure 2: *Survivin knockdown induces apoptosis in canine osteosarcoma cells.* **A.** 48 hours following survivin siRNA knockdown, activated caspase-3,7 activity (**A**) and TUNEL staining (**B**) were evaluated using ELISA and immunofluorescence respectively. A significant increase in caspase activity and TUNEL reactivity was observed following survivin knockdown. Error bars indicate standard deviation. *P < 0.0001 vs. control and sham transfection.

Figure 3: *Cell cycle changes following survivin knockdown in canine osteosarcoma cells.* Cell cycle analysis was performed 48 hours following survivin knockdown. A representative histogram for the Abrams cell line is shown in panel **A**, demonstrating increases in both the sub-G1 (apoptotic) and super-G2 (4N) populations, as indicated

by arrows. **B:** Means (+/- SD) of 4 independent transfections demonstrating significantly increased sub-G1 and super-G2 populations following survivin knockdown in the Abrams cell line. Error bars indicate standard deviation. *P < 0.05 compared with control and sham-transfected cells. **C:** Means (+/- SD) of 4 independent transfections demonstrating significantly increased super-G2 population following survivin knockdown in D17 cell line. There is also a trend toward increased sub-G1 populations following survivin knockdown. Error bars indicate standard deviation. *P < 0.05 compared with control and sham-transfected cells.

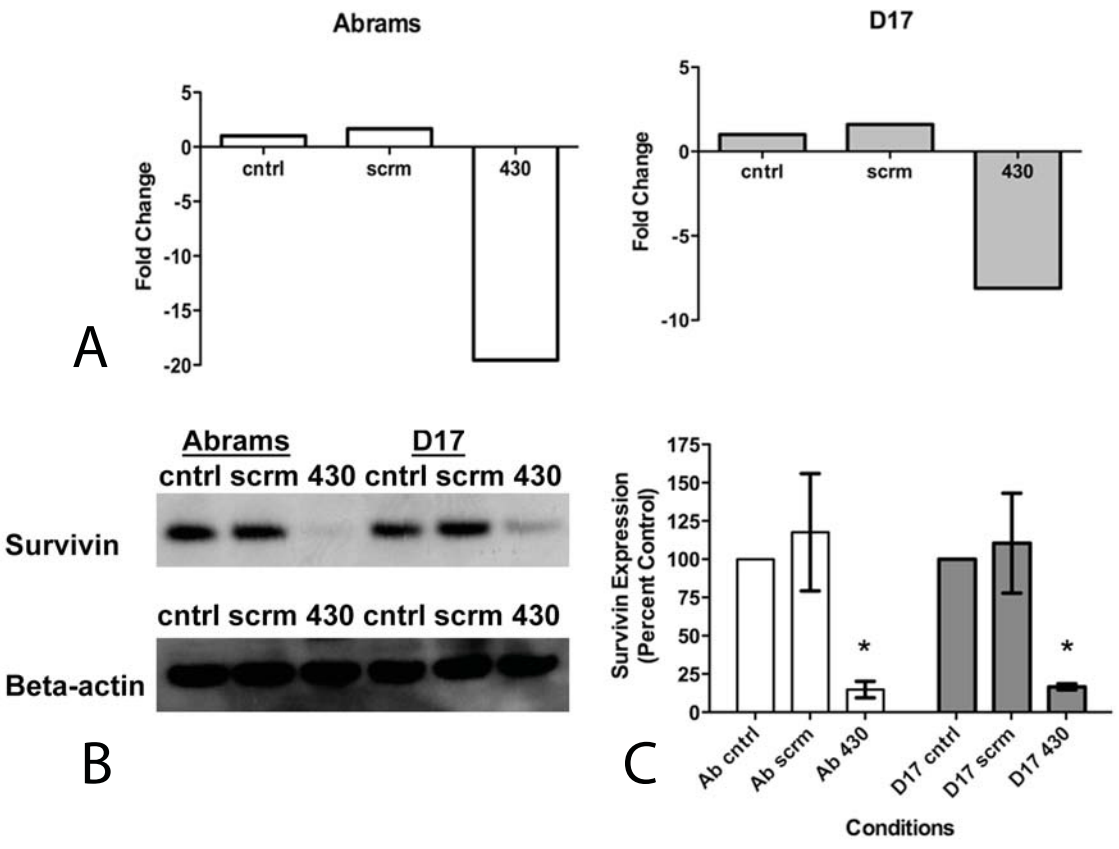
Figure 4: *Survivin knockdown increases canine osteosarcoma chemosensitivity.* Abrams and D17 cells were treated for 48 hours with 21.55 μM carboplatin or 125 nM doxorubicin, +/- survivin knockdown. Caspase-3,7 activity was then determined by ELISA. Survivin knockdown significantly enhanced caspase activity in cells exposed to carboplatin and doxorubicin. Degree of synergy was assessed with Bliss analysis. * = additive enhancement of caspase activity, ** = synergistic enhancement of caspase activity.

Figure 5: *Survivin inhibition in vivo results in decreased tumor growth when combined with doxorubicin.* **A.** EZN-3042, a survivin inhibiting locked nucleic acid antisense oligonucleotide, caused a significant decrease in survivin protein expression in Abrams canine OS xenografts compared to tumors treated with a scrambled control oligonucleotide (EZN-3046). *P < 0.01 compared with saline/EZN-3046. **P < 0.05 compared with saline/EZN-3046 and DOX/EZN-3046. **B.** Mice treated with DOX/EZN-3042 had significantly decreased tumor growth when compared to other treatment

groups. Arrows represent DOX treatments.

Figure 6: *Survivin immunoreactivity correlates with grade, mitotic index and outcome in canine osteosarcoma.* **A.** Primary canine appendicular osteosarcomas of histologic grade 2 or 3 had higher survivin immunoreactivity scores than did grade 1 tumors. **B.** There was a significant correlation between survivin immunoreactivity and mitotic index in primary canine osteosarcomas. **C.** Canine osteosarcoma patients treated with amputation and doxorubicin with high survivin immunoreactivity scores had a significantly inferior disease-free interval on univariate analysis.

FIGURE 1



Author Manuscript Published OnlineFirst on November 8, 2011; DOI: 10.1158/0008-5472.CAN-11-2315
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

FIGURE 2

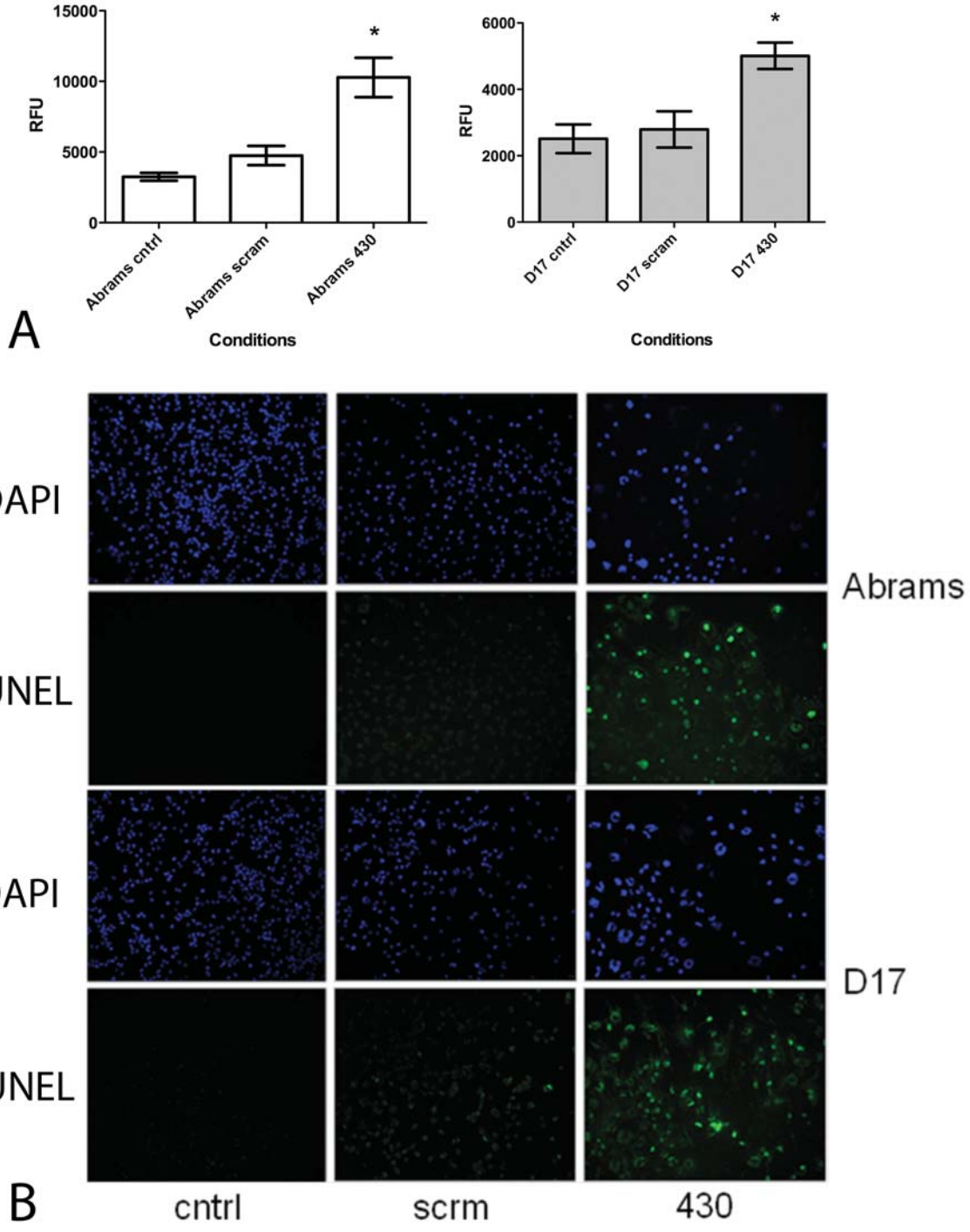


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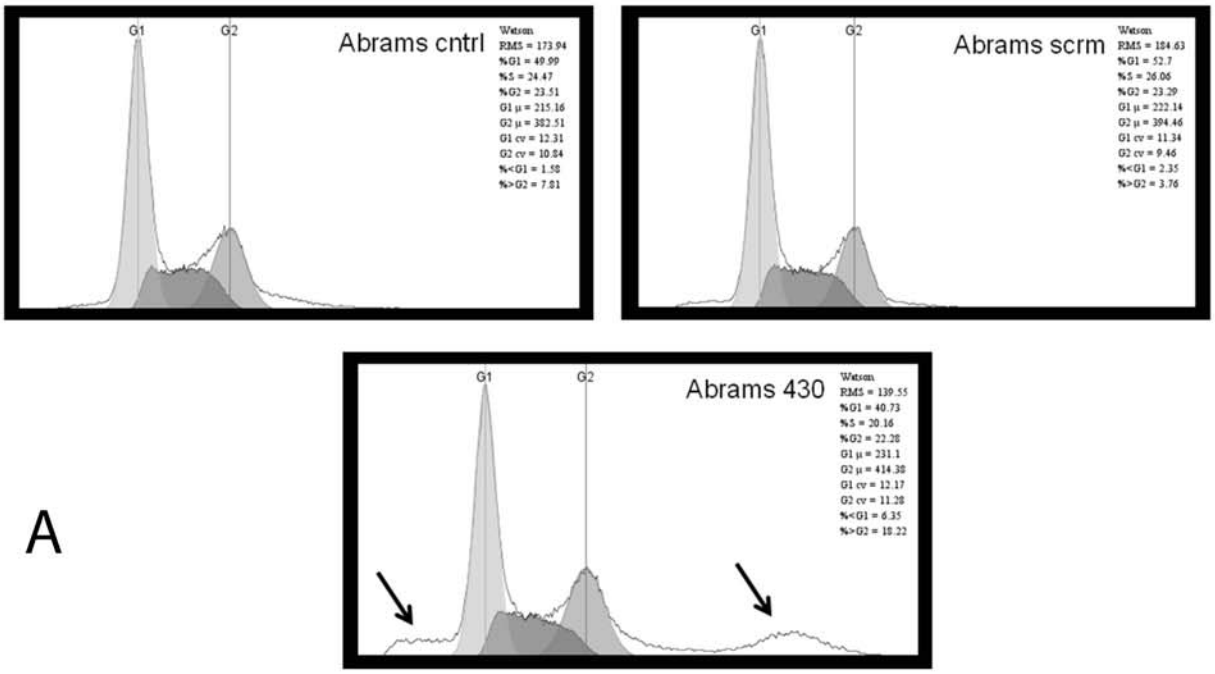
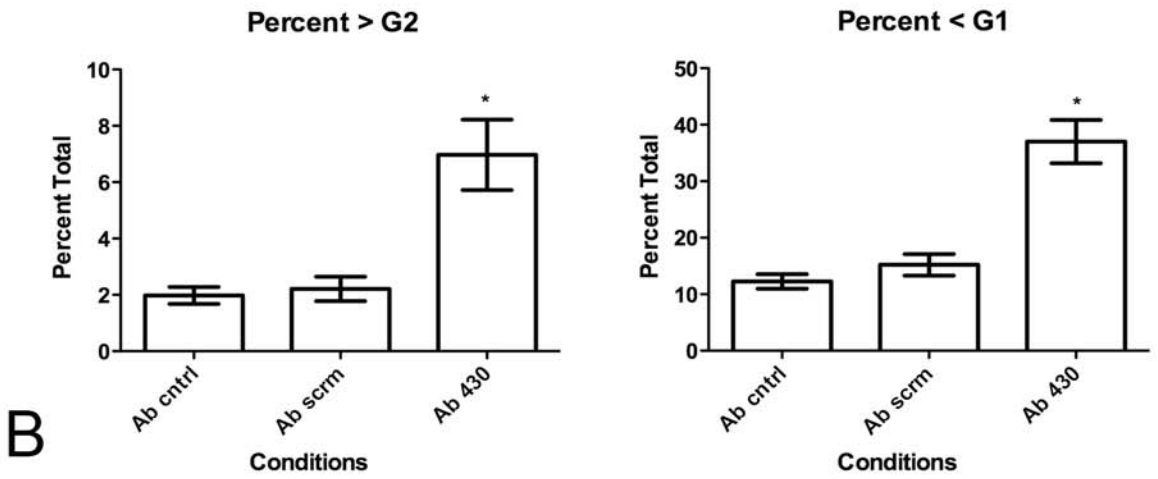
FIGURE 3





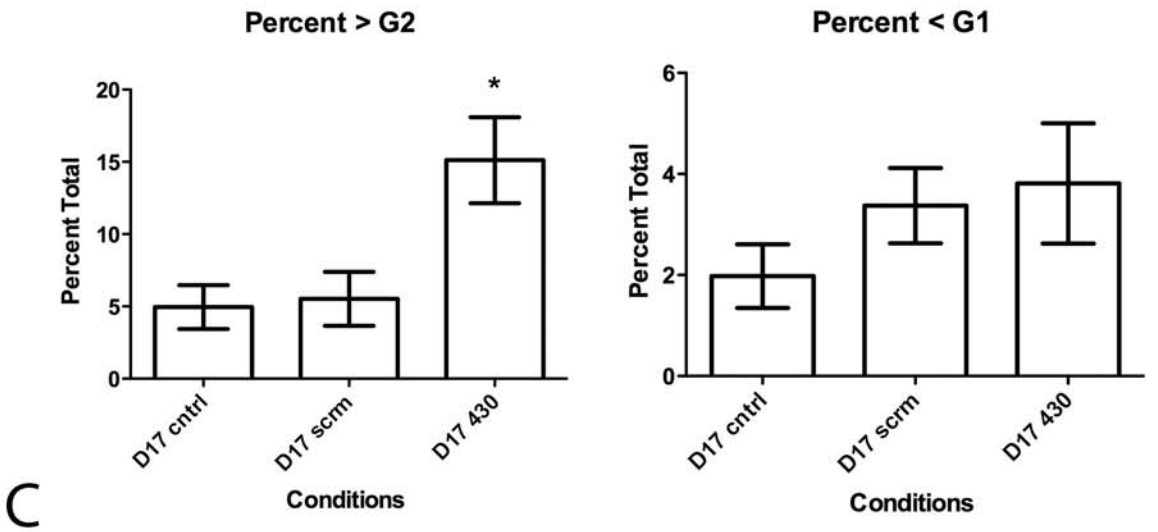


FIGURE 4

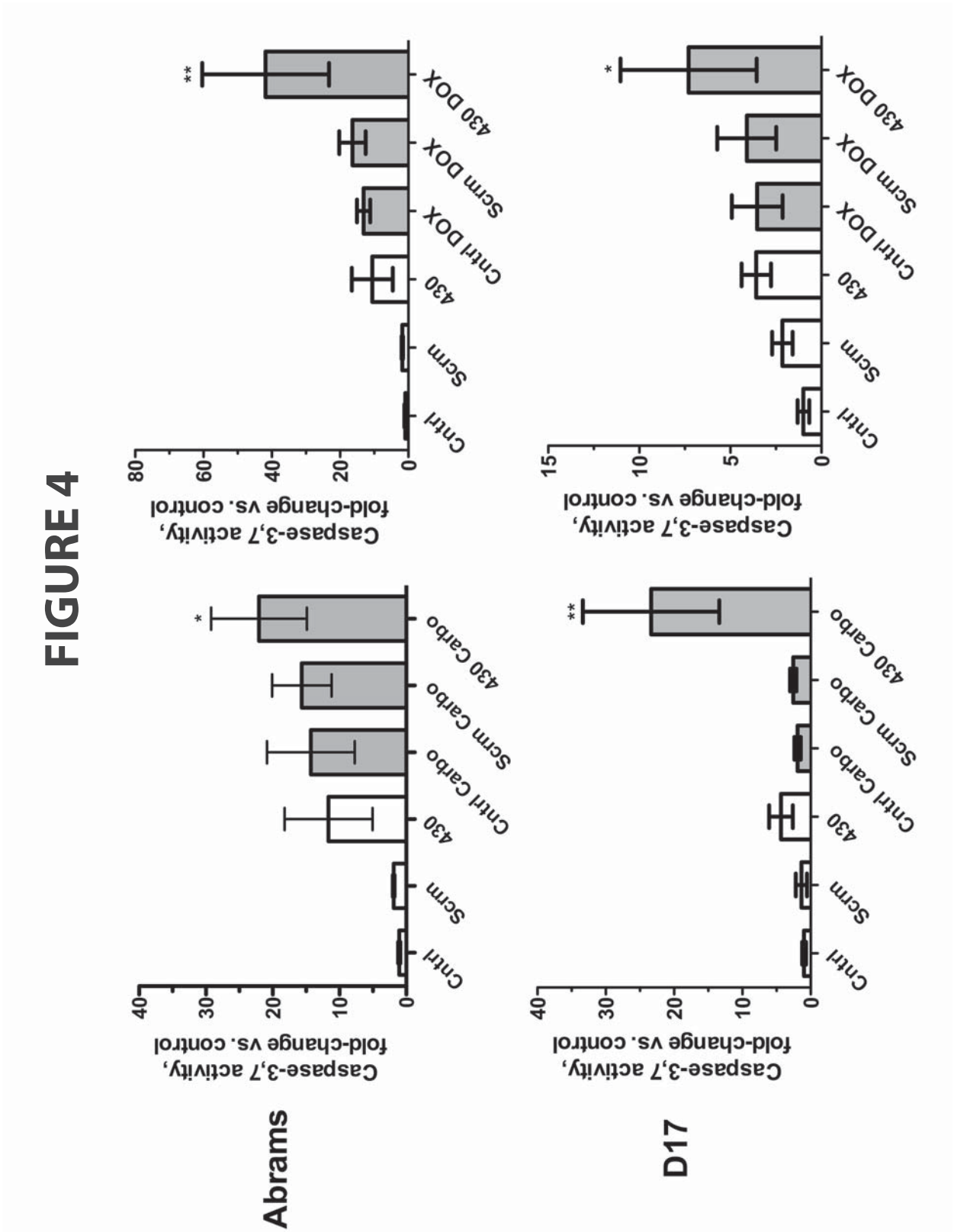


FIGURE 5

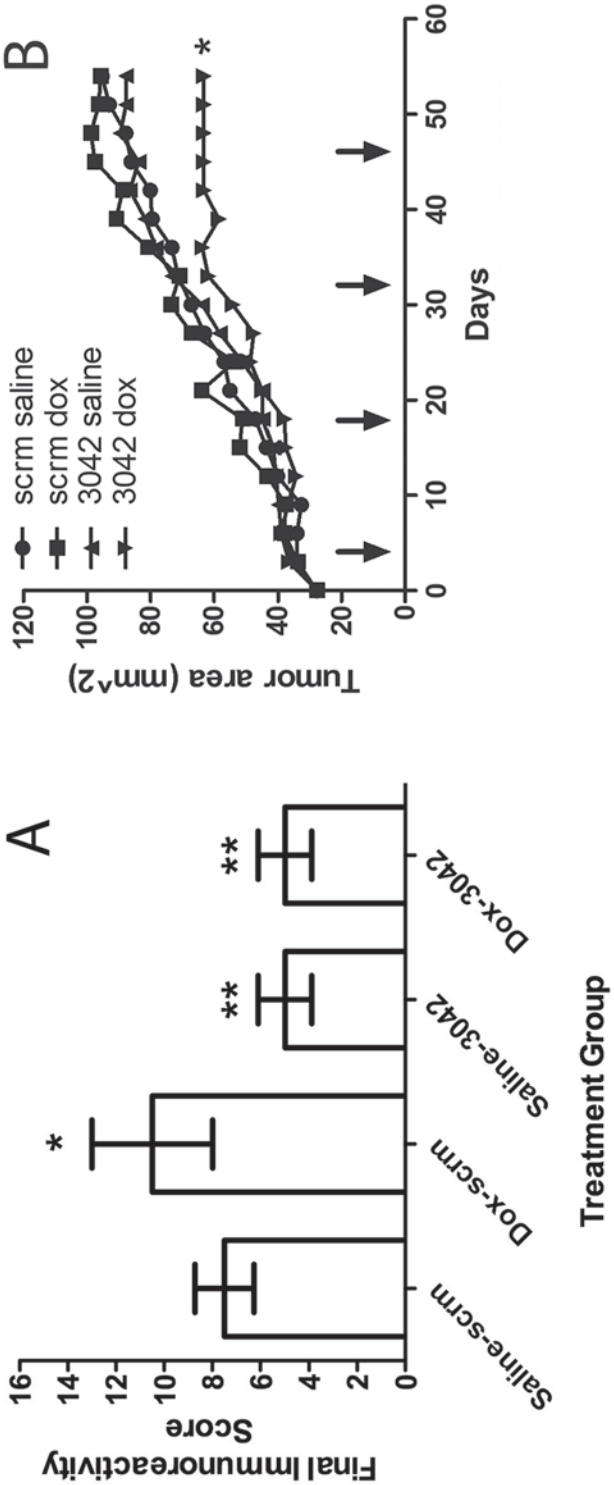
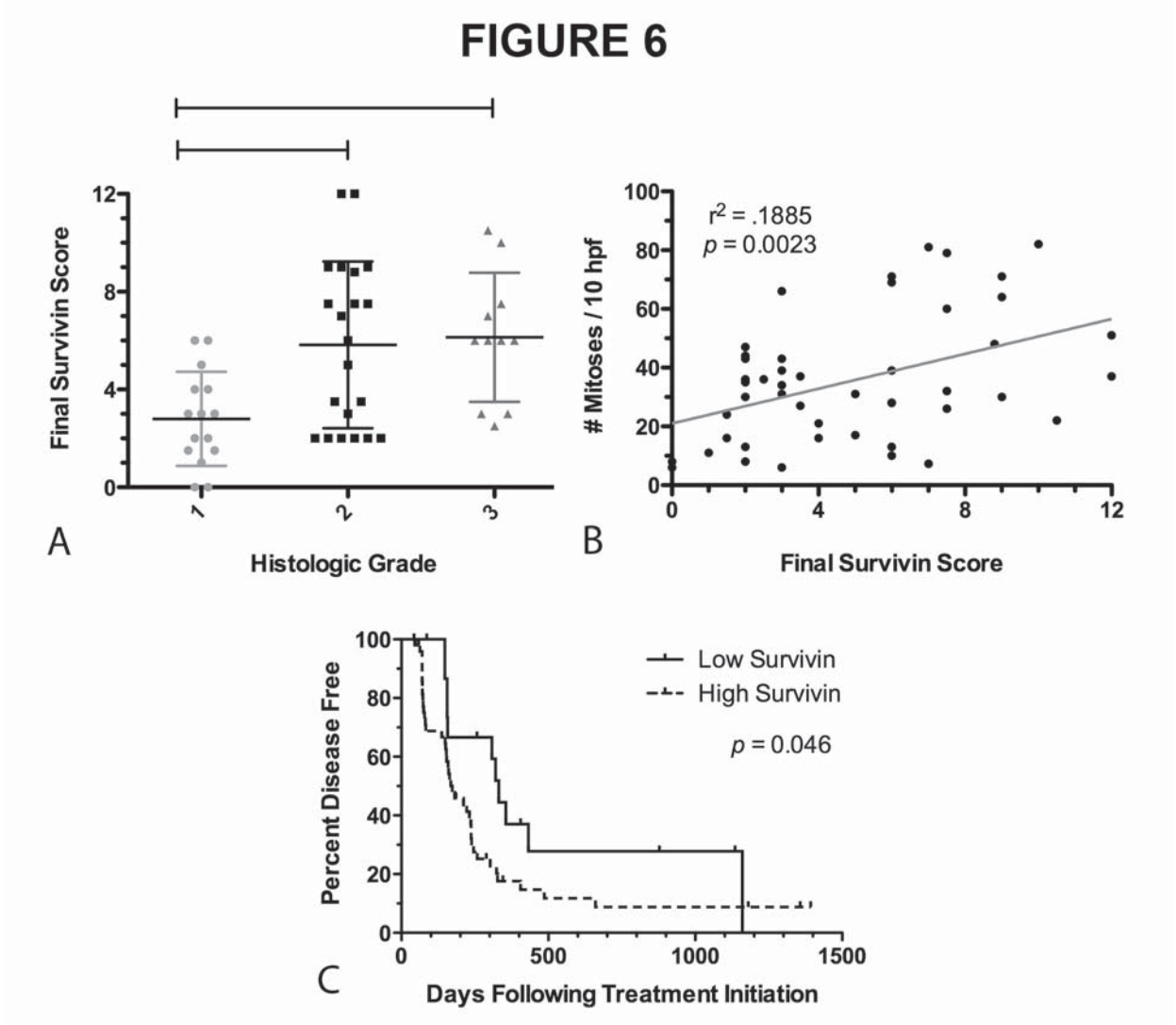


FIGURE 6



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