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MMP13, Birc2 (cIAP1) and Birc3 (cIAP2), Amplified on Chromosome 9, Collaborate with p53 Deficiency in Mouse Osteosarcoma Progression

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Abstract

Osteosarcoma is the primary malignant cancer of bone and particularly affects adolescents and young adults, causing debilitation, and sometimes death. As a model for human osteosarcoma we have been studying p53^{+/−} mice, which develop osteosarcoma at high frequency. To discover genes that cooperate with p53 deficiency in osteosarcoma formation we have integrated array comparative genomic hybridization, microarray expression analyses in mouse and human osteosarcomas, and functional assays. In this study we found seven frequent regions of copy number gain and loss in the mouse p53^{+/−} osteosarcomas, but have focused on a recurrent amplification event on mouse chromosome 9A1. This amplicon is syntenic with a similar chromosome 11q22 amplicon identified in a number of human tumor types. Three genes on this amplicon, the matrix metalloproteinase gene MMP13, and the anti-apoptotic genes Birc2 (cIAP1), and Birc3 (cIAP2) show elevated expression in mouse and human osteosarcomas. We developed a functional assay using clonal osteosarcoma cell lines transduced with lentiviral shRNA vectors to show that downregulation of MMP13, Birc2, or Birc3 resulted in reduced tumor growth when transplanted into immunodeficient recipient mice. These experiments revealed that high MMP13 expression enhances osteosarcoma cell survival and that Birc2 and Birc3 also enhance cell survival, but only in osteosarcoma cells with the chromosome 9A1 amplicon. We conclude that the anti-apoptotic genes Birc2 and Birc3 are potential oncogenic drivers in the chromosome 9A1 amplicon.

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Keywords

p53; osteosarcoma; MMP13; Birc2; Birc3

Introduction

Osteosarcoma is the most common malignant bone tumor in children and young adults and accounts for about 60% of malignant bone tumors in the first two decades of life (1). About 900 cases are diagnosed per year in the U.S. With modern adjuvant chemotherapy and surgery, the five year survival rate has been improved to 50~65% (2,3). However, 25–50% of patients with initial non-metastatic disease later develop metastatic disease, which is generally fatal. There remains a need for more effective and less toxic therapies.

In contrast to other sarcomas with signature chromosomal aberrations, osteosarcomas do not have specific molecular genetic abnormalities that serve as a tumor-specific marker (3), although they exhibit high frequencies of genome instability (4). A small fraction of osteosarcomas are associated with inherited cancer syndromes, providing insights into genes which might be relevant to osteosarcomagenesis (1). For example, Li-Fraumeni syndrome patients with a defective germline p53 allele are susceptible to an array of tumor types, with soft tissue sarcomas and osteosarcomas considered as signature tumors (5). The p53 protein is a prototypical tumor suppressor, mediating the cellular stress response and guarding genome integrity (6). p53 mutations occur in 15–30% of human osteosarcomas (7), and the presence of p53 mutations correlates with increased genome instability (8).

As with humans, sporadic osteosarcomas in inbred laboratory strains of mice are generally rare (9). However, in the p53 knockout mouse model generated by our laboratory, 38% of p53+/- mice developed osteosarcomas (10). p53-/- mice developed thymic lymphomas at an early age rather than osteosarcomas, but recent studies of conditional p53 knockout models, in which both p53 alleles are deleted in the osteoblast compartment, show early osteosarcoma development (11). The osteosarcomas in p53-deficient mice arise primarily in the long bones and spine and exhibit histopathology similar to that of human osteosarcomas. The propensity of humans and mice with germline p53 mutations to develop osteosarcomas indicates that p53 deficiency may affect aspects of osteoblast signaling that make these cells specifically susceptible to oncogenic transformation.

In this study, we developed integrated strategies to identify genes with functional relevance for osteosarcoma development. We accumulated an archive of p53+/- mouse osteosarcomas and characterized these tumors by DNA array-based comparative genomic hybridization (array CGH). Array CGH analyses performed on 41 p53+/- osteosarcomas revealed 7 frequent regions of copy number gain or loss. One region of copy number gain on mouse chromosome 9 (band A1) contained a cluster of ten matrix metalloproteinase (MMP) genes and two anti-apoptotic genes, Birc2 (cIAP1) and Birc3 (cIAP2). Interestingly, DNA amplification centered on Birc2 and Birc3 has also been observed in mouse liver cancers (12) and human lung cancer (13), liver carcinomas (12), oral squamous cell carcinomas (14,15), medulloblastomas (16), glioblastomas (17), and pancreatic cancers (18). To identify those amplicon genes likely to be important in osteosarcomagenesis, we examined RNA expression in mouse as well as human osteosarcomas. We further performed functional assays to determine whether alteration of candidate gene expression levels affected *in vivo* tumor growth rates. We found that the matrix metalloproteinase MMP13 gene and the anti-apoptotic genes Birc2 (cIAP1) and Birc3 (cIAP2) are important in promoting osteosarcomagenesis.

Materials and Methods

Array-based comparative genomic hybridization

The mouse BAC (bacterial artificial chromosome) arrays contained 19,000 BACs (printed in triplicate) covering the mouse genome with a resolution of less than 0.5 Mb. BAC array-CGH was performed on 41 p53^{+/-} osteosarcomas and 10 p53^{+/-} rhabdomyosarcomas as previously described (19). Normal genomic DNA from C57BL/6 mouse was used as diploid reference DNA, and mouse Cot-1 DNA was used to block repetitive sequences in sample probes and on BAC chips. Each sample/control pair was done twice by reciprocal labeling of sample or control with Cy5 or Cy3 dye to remove ratio artifacts. CGH data was analyzed as described in (19). Briefly, the fluorescent ratio representing the relative amounts of target sequences in the probe mix was analyzed by comparing the fluorescent intensity of corresponding individual spots after background subtraction. A control experiment comparing wild-type C57BL/6 and wild-type 129/SvEv genomic DNA copy number differences was performed in order to filter out strain-specific polymorphisms.

Isolation and culture of primary tumor cells

Tumor fragments were collected from p53^{+/-} mice, washed twice with sterile PBS, minced, and incubated with 0.25% trypsin solution for two consecutive 15 minute incubations at 37°C with shaking. The cell solution was filtered through a 70 µm cell strainer (BD Falcon) to remove clumps, and the filtrate was centrifuged at 1500 rpm for 5 minutes at 4°C. Cells were collected and cultured in DMEM with 10% FBS, 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂. Clonal populations of osteosarcoma cells were isolated by limiting dilution for both A+ (chromosome 9 amplicon positive) and A- (chromosome 9 amplicon negative) cell lines. The osteosarcoma origin of all lines was confirmed by alkaline phosphatase (ALP) staining.

Quantitative real-time PCR

Quantitative real-time PCR was performed with RT-3000 equipment (Corbett Research). Quantitation of genomic copy number was based on standard curves derived from sequential dilutions of genomic DNA from wild-type C57BL/6 mouse tail. For mRNA quantitation, total mRNA was extracted with RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. Standard curves were performed by sequential dilutions of mRNA followed by reverse transcription with Superscript III First-strand Synthesis System (Invitrogen), and real-time PCR with iQ SYBR Green Super Mix (Bio-Rad) using gene-specific primers. After determination of the optimal reaction condition for the primer set, real-time PCR was done in triplicate for each sample, and expression of target genes was normalized to β-actin levels. Primer sequences used in the real-time PCR experiments are listed in the Supplementary Methods.

shRNA lentivirus construction, packaging and transduction

For MMP13, chemically synthesized siRNAs were purchased from Qiagen (#SI00177765 and #SI00177772), tested for efficient knockdown of siMMP13 compared to control siRNA (Ambion #4611). DNA oligonucleotides were synthesized based on the siRNA sequences with the best knockdown efficiency and were incorporated into the FG12 lentiviral vector, which contains GFP-expression sequences driven by a UbiC promoter (20). Viruses were packaged by co-transfecting 293T cells with FG12-shMMP13 vector plasmid, packaging constructs pRSVREV, pMDLg/pRRE, and the VSV-G expression plasmid pHCMVG. Viruses were harvested and transduced into osteosarcoma cells as described previously (20). Positively transduced cells were cell sorted for GFP expression. For Birc2, Birc3, and Yap1, virus packaging and transduction was done as previously described (12). Positively transduced

clones were achieved by selection with puromycin at pre-determined concentrations (2ug/ml for A+ cell line and 4ug/ml for A- cell line). Real-time PCR confirmed the target gene (MMP13, Birc2, Birc3 or Yap1) knockdown in positively selected cells, and only those with knockdown efficiency of more than 75% were used in allograft studies.

Allograft study

Transduced cells were harvested, washed, counted and resuspended in HBSS (Invitrogen). 2×10^6 of A+ or 0.5×10^6 of A- cells were used for subcutaneous injection, by injecting dorsally above the scapula of 8–10 week Balb/c-nu/nu (Charles River Laboratories) mice. Tumor growth was monitored and measured every other day, and tumor volume (V) was determined by the formula $V = (4/3) * \pi * [(L+W)/4]^3$, L being the longer cross-section and W being the shorter.

Western blotting

Tumor tissue or cell pellets were lysed in NP40 lysis buffer using PT100 homogenizer (Polytron). Equal amounts (25ug) of protein were separated on NuPAGE® Novex 4–12% Bis-Tris Gel (Invitrogen) and transferred to PVDF membranes. The blots were probed with antibodies against MMP13 (Santa Cruz, #sc-30073, 1:500), Birc2 (12), Birc3 (Santa Cruz, #sc-7944, 1:100), Yap1 (12), or β -actin (Santa Cruz, #sc-1616, 1:2,000).

Immunohistochemistry

MMP13 staining was performed on the tumor sections with antibody from Santa Cruz (sc-30073). For cell proliferation experiments, BrdU solution (7mg/ml) was injected intraperitoneally into mice at 0.01 ml per gm of body weight. Mice were sacrificed 2 hours after injection and tumors were harvested, fixed with 4% paraformaldehyde, paraffin embedded and sectioned for immunohistochemistry. BrdU staining was performed with BrdU *In-Situ* Detection Kit (BD Biosciences #550803), and TUNEL staining was performed with In Situ Cell Death Detection Kit, Fluorescein (Roche #11 684 795 910), following manufacturer instructions. For the quantitation of BrdU staining and TUNEL staining, tumor sections from five mice were stained and counted for each group. Five fields from each tumor were randomly selected, both positively-stained cells and total cell number were counted, and the percentage was calculated by dividing positively-stained cell number by total cell number.

CT imaging

MicroCT imaging was performed on a Gamma Medica Xspect scanner at 50 μ m resolution at the BCM Mouse Phenotyping Core facility. Data were acquired with the system with X-ray tube voltage of 78kV(p), tube currents of 205 uA, and exposure matrix 512 $\times$ 512 views. A water phantom was included with each scan. Three-dimensional CT images of the tumors were reconstituted with Amira®.

Gene Expression Microarray Analysis

RNA was extracted from rhabdomyosarcoma or osteosarcomas (about 3mm cube) or mc3T3 cell (60mm dish) using the RNeasy Mini Kit (Qiagen). Following quality check of the RNA, microarray analysis was performed at the Baylor Microarray Core Facility (www.bcm.edu/mcfweb/) with Affymetrix Mouse Genome 430 2.0 Chips. Microarray data was analyzed with Genesifter software (www.genesifter.net).

Results

Array-CGH identifies genome instability in p53+/- murine tumors

To identify copy number changes in the genomes of p53+/- sarcomas, we carried out microarray-based comparative genomic hybridization (array-CGH) analysis on genomic DNA isolated from 41 p53 +/- osteosarcoma and 10 rhabdomyosarcoma samples. For the 41 osteosarcomas, seven frequent regions of copy number gain or loss were observed (Table S1). Among these, three were regions of copy number gain and four were regions of copy number loss. Analysis of the mouse genome sequence within the regions of copy number losses revealed no obvious tumor suppressor candidates. However, two of the three regions of copy number gain had known candidate oncogenes and were on mouse chromosomes 9 and 15. Seven of 41 osteosarcomas had strong amplifications on chromosome 15 with the *c-myc* oncogene at the amplicon epicenter (Figure 1A). This was consistent with observations that human osteosarcomas also exhibit amplification and overexpression of *c-myc* (9, 21, 22). The chromosome 9 (band A1) amplicon was observed in 5 of 41 osteosarcomas and contains a cluster of 10 matrix metalloproteinase genes (Figure 1B,C,D). Moreover, the epicenter of this amplicon is near the *Birc2* (*cIAP1*) and *Birc3* (*cIAP2*) genes, which encode inhibitors of apoptosis (Figure 1D). As indicated earlier, DNA amplification centered on *Birc2* and *Birc3* has been observed in multiple human tumor types (12–17). Finally, the *Yap1* gene, shown to be oncogenic in some contexts (12, 23), is also located in this amplicon.

Because of its previous association with various human tumors, we focused on the chromosome 9A1 amplicon, at chromosomal position 5–9 Mb (Figure 1D). The cluster of matrix metalloproteinase (MMP) genes within the amplicon is a family of extracellular endopeptidases that collectively cleave all the components of the extracellular matrix (ECM). MMP proteins may be important for osteosarcoma cell invasion if overexpressed (24, 25). The MMPs play important roles under both physiological and pathological conditions, including tumorigenesis, and are potential targets for cancer treatment (25–27).

Birc2, *Birc3* and *Yap1* are located in the chromosome 9 amplicon about one Mb downstream of the MMP cluster. Birc proteins, or the Baculoviral IAP Repeat-Containing proteins, are also called IAPs (Inhibitor of Apoptosis Proteins). This family is grouped by their characteristic BIR domains and is thought to be inhibitors of apoptosis (12,28). *Birc2* (*cIAP1*) and *Birc3* (*cIAP2*) may regulate apoptotic pathways by multiple mechanisms. They bind caspases and *Birc2* can affect the TNF α pathway by binding and ubiquitinating the TNFR-associated factor-2 (TRAF2) (29), whereas *Birc3* preferentially binds and ubiquitinates TRAF1 (30). In addition, degradation of *Birc2* has been shown to lead to NF- κ B activation and TNF α secretion. The autocrine TNF α signaling in turn induces caspase 8 activation (31–33). *Birc2* also affects the intrinsic apoptotic pathway by mediating the ubiquitination of Smac/DIABLO (34). Importantly, *Birc2* acts as an oncogene in a mouse hepatocellular cancer model containing a similar chromosome 9A1 amplicon as described here (12).

Yap1, or Yes-Associated Protein 1, also resides on the chromosome 9A1 amplicon. *Yap1* is a co-activator of p73-mediated apoptosis (35,36), while others have shown that it is a potential oncoprotein (12,23). Importantly, *Yap1* has been shown to exhibit oncogenic activity in mouse hepatocellular cancers that contained similar chromosome 9A1 amplicons to those described here (12). Based on these findings, we decided to examine the roles of MMP13, *Birc2*, *Birc3* and *Yap1* in osteosarcomagenesis.

Southern blot hybridization of the osteosarcoma DNAs with MMP13 and MMP7 probes confirmed copy number gain in those tumors with chromosome 9A1 amplification measured by array CGH (Figure S1). Since these tumors were derived from p53+/- mice, we assessed p53 allele status, and found that the wildtype p53 allele was retained in some tumors, but was

lost in cell lines derived from the same tumors (Figure S1). Historically, we have found that about half of all p53^{+/−} tumors retain the wildtype p53 allele and about half undergo loss of the wildtype allele (37).

MMP13 is overexpressed in osteosarcomas

To determine whether a particular gene or set of genes in the chromosome 9A1 amplicon could be driving osteosarcomagenesis, we analyzed the RNA expression status of all genes within the chromosome 9A1 amplicon in both amplicon-positive and amplicon-negative osteosarcomas by microarray expression analysis and real-time PCR. MMP genes localized within and outside of this amplicon were first analyzed. A murine osteoblast cell line mc3T3, as well as normal bone, were used as controls for comparison. We found that only MMP13 and MMP12 (within the 9A1 amplicon), and MMP9 and MMP23 (outside the 9A1 amplicon) had high expression in osteosarcomas relative to the normal osteoblast control (Figure 2A, Figure S2, Figure S3). However, within the chromosome 9A1 amplicon, MMP1, MMP7, MMP8, MMP10 or MMP20 did not exhibit elevated RNA expression. This was confirmed by RNA microarray expression analysis on all of the MMP genes (Figure S2, Figure S3). Furthermore, MMP expression in the mouse osteosarcomas was consistent with corresponding gene expression in human osteosarcomas (except MMP19) (Figure 2A, Figure S3).

These results suggested that overexpression of the chromosome 9A1 amplicon-associated MMP13 gene might play a role in osteosarcoma progression. MMP13 (collagenase-3) expression is most abundant in adult bone compared to other tissues (38). MMP13-null mice show interstitial collagen accumulation and cartilaginous growth plate abnormalities. MMP13 knockdown increases apoptosis in squamous cell carcinomas (39). Real time RT-PCR analysis of MMP13 gene expression in 15 p53^{+/−} osteosarcomas showed that virtually all of the osteosarcomas exhibited high RNA expression relative to osteoblast cells (Figure 2B). High MMP13 RNA expression was not dependent on gene amplification, as most tumors were diploid at the MMP13 locus (Figure 2B). High MMP13 RNA expression is also observed in many human osteosarcomas relative to normal human osteoblast cells, as measured by microarray expression analyses on 40 human osteosarcoma samples (Figure 2C). In contrast to high MMP13 expression in osteosarcomas, all six examined rhabdomyosarcomas exhibited low MMP13 expression. These expression patterns were confirmed at the protein level by immunohistochemistry staining of osteosarcoma and rhabdomyosarcoma sections (Figure 2D). No MMP13 staining was evident in rhabdomyosarcoma sections, while osteosarcoma sections showed both cytoplasmic and cell surface MMP13 staining. Other MMP genes did not show a pattern of elevated expression in human osteosarcomas (Figure S3).

Reduction of MMP13 expression decreases osteosarcoma growth rate

As a prelude to assessing the functional importance of high MMP13 expression in the murine osteosarcomas, we developed clonal cell lines from a primary osteosarcoma with chromosome 9A1 amplification (A+) and from primary osteosarcomas without this amplification event (A−). All clonal lines, when injected subcutaneously into immunodeficient Balb/c-*nu/nu* mice, formed osteosarcomas histopathologically indistinguishable from primary p53^{+/−} osteosarcomas within several weeks following injection (Figure S4). Lentiviral vectors expressing two MMP13 shRNAs targeting different domains on MMP13 mRNA were constructed and transduced into 9A amplicon-positive and amplicon-negative osteosarcoma cells. Stably transduced cells were selected by flow cytometric cell sorting for GFP fluorescence (expressed only in vector-transduced cells). MMP13 mRNA levels in transduced osteosarcoma cells were reduced over 80% compared to empty vector-transduced cells (data not shown). Equal numbers of MMP13 shRNA-transduced or empty vector-transduced cells were subcutaneously injected into the dorsal flanks of nude mice, and tumor volume was monitored over time. Osteosarcomas formed by the shMMP13-transduced A+ and A−

osteosarcoma cells grew at significantly reduced rates compared to their empty vector-transduced counterparts, suggesting that MMP13 plays an important role in osteosarcoma progression (Figure 3A,C, Figure S5). Western blots confirmed reduced MMP13 protein expression in the shRNA-expressing tumor allografts compared to empty vector-containing tumors (Figure 3B and 3D, upper panels).

The reductions in tumor growth rates caused by MMP13 knockdown could be a result of either reduced tumor cell proliferation or increased tumor cell apoptosis. To examine the effect of MMP13 shRNA on proliferation *in vivo*, we injected tumor bearing nude mice with bromodeoxyuridine (BrdU) for 3 hours prior to euthanasia and measured cell proliferation by staining tumor sections with an antibody directed against BrdU. Counting of stained cells provides an indicator of cells in S phase. Using this proliferation assay, no significant differences were observed between tumors with and without MMP13 shRNA expression, regardless of amplicon status (Figure 4A).

To compare rates of apoptosis in the transplanted tumors with and without MMP13 knockdown, we performed a TUNEL assay for apoptotic cells on tumor sections of the various tumors. Counting of stained apoptotic cells in microscopic fields from multiple tumor sections revealed that both A+ and A- transplanted MMP13 shRNA vector-transduced osteosarcomas displayed significantly higher levels of apoptosis than their empty vector-transduced counterparts (Figure 4B). Thus, reduction of MMP13 in the transplanted osteosarcomas increases rates of tumor cell apoptosis, leading to decreased overall tumor growth rates.

Histopathological examination of the transplanted tumors revealed an additional effect of MMP13 knockdown. The stained sections of both A+ and A- tumors transduced with MMP13 shRNA vectors showed reduced cellular density and increased osteoid component compared to control vector transduced tumors, suggesting a higher level of differentiation (Figure 4C). In osteosarcomas, differentiation has been considered as antithetical to the oncogenic process, and loss of differentiation has powerful prognostic significance (40). To quantitatively determine whether bone matrix and tumor density were increased when MMP13 expression was reduced, computed tomography (CT) scans were performed, and the tumor density was measured three-dimensionally by Amira® and converted into Hounsfield units. Both A+ and A- shMMP13-expressing tumors were denser than their vector-expressing controls ($p < 0.05$) (Figure 4D, Figure S5). Since differentiated osteosarcoma cells secrete more osteoid than proliferating cells, and mineralized osteoid is the major component of the bone matrix, these results indicate that reduction of MMP13 induces a more differentiated tumor cell state.

Reduction of Birc2 and Birc3 enhances apoptosis and reduces tumor growth rates in amplicon positive osteosarcomas

The anti-apoptotic genes *Birc2* (*cIAP1*) and *Birc3* (*cIAP2*) are located near the epicenter of the chromosome 9A1 amplicon. Real-time PCR and RNA microarray analysis of the p53+/- osteosarcomas and rhabdomyosarcomas showed high expression of Birc2 and Birc3 mRNA expression in virtually all tumors (Figure 5A, Figure S6). Moreover, high Birc3 expression was observed in most human osteosarcomas (Figure 5A, lower right panel). To determine whether Birc2 and/or Birc3 influences osteosarcoma growth, lentiviral vectors expressing shRNAs against Birc2 or Birc3 (or empty vectors) were transduced into clonal A+ and A- osteosarcoma cell lines and stably transduced lines were selected in puromycin (lentiviral vectors in these experiments encoded puromycin resistance genes). After allografting into nude mice, tumor growth was monitored. Birc2 or Birc3 shRNA-expressing A+ tumors grew significantly slower than empty vector-transduced A+ tumors (Figure 5B, upper panels). In contrast, Birc2 and Birc3 shRNA did not significantly affect the growth rate of the A- osteosarcoma cells (Figure 5B, lower panels). TUNEL experiments showed that Birc2 or Birc3 shRNA-expressing A+ osteosarcoma sections contained significantly increased apoptotic cell

percentages (Figure 5C), while no significant differences in apoptotic cell percentages were noted in shRNA vector transduced A[−] osteosarcoma sections. These results indicated that high *Birc2* and high *Birc3* expression contributed to tumor growth and reduced apoptosis only in osteosarcomas with the chromosome 9A1 amplicon.

Yap1 knockdown does not affect osteosarcoma growth rate

The *Yap1* gene is directly adjacent to *Birc2* and *Birc3* and is co-amplified with these anti-apoptotic genes in the chromosome 9A1 amplicon containing osteosarcomas. Because *Yap1* has been shown to be oncogenic in some contexts (12,23), we analyzed *Yap1* RNA expression by real time RT-PCR and microarray analyses. We found that *Yap1* mRNA was highly expressed only in the mouse p53^{+/−} osteosarcomas that contained the chromosome 9A amplicon (Figure 6A, Figure S7). Osteosarcomas without this amplification event and rhabdomyosarcomas generally did not show elevated expression of this gene. In human osteosarcomas, *Yap1* expression was consistently downregulated relative to osteoblasts (Figure 6B). We then transduced A⁺ and A[−] osteosarcoma cells with a *Yap1* shRNA lentiviral vector (or control vector), selected stable transductants and performed allograft studies. No significant changes in tumor growth rate between *Yap1* shRNA and empty vector transduced tumors, either A⁺ or A[−], were observed (Figure 6C,D). However, despite reduction of *Yap1* RNA levels by the shRNA vectors in the osteosarcoma cells (Figure S8), western blot analyses of the tumors indicated that *Yap1* protein in all tumors was undetectable (data not shown). These results argue that *Yap1* expression is not significant in altering osteosarcoma cell growth in either the A⁺ or A[−] transplanted osteosarcomas.

Discussion

The initial goal of this study was to identify genes that cooperate with p53 deficiency in promoting osteosarcoma progression, using the osteosarcoma prone p53^{+/−} mice as a starting point. We employed a multi-dimensional approach that integrated four major methods: (i) scanning of the p53^{+/−} mouse osteosarcoma genomes for frequent copy number changes; (ii) global expression analyses of the osteosarcoma transcriptomes (supplemented by real time PCR for candidate genes); (iii) validation of mouse osteosarcoma candidate gene expression in human osteosarcomas; and (iv) functional analyses to determine the effects of altering candidate gene expression in an *in vivo* osteosarcoma model. By combining these methods in a systematic way, we hoped to identify cooperating oncogenes, tumor suppressors, or tumor modifiers. This report represents a proof of principle study for a set of genes localized to a single amplicon in our mouse osteosarcoma model.

Given the association of p53 deficiency with increased genomic instability and the high genomic instability noted in human osteosarcomas (4,8,41), we anticipated that large numbers of copy number changes would be observed in the mouse osteosarcomas. Surprisingly, this was not the case, as only seven regions of frequent copy number gain or loss were identified. This is in contrast to what Man *et al.* (4) had reported in a human osteosarcoma array-CGH study. Amplifications and deletions were frequent in human osteosarcoma, including amplification at 6p21.1 (45%), 1p36.32 (43%), 6p21.1 (another BAC clone) (37.5%), 6p12.3 (37.5%) and 16p13.3 (37.5%) and deletion at 17q12 (27%), 6q27 (22.9%), 13q12.2 (20.8%), 13q22.1 (20.8%), 6q16.3 (20.8%), and 7q35 (20.8%) (4). We did not observe any amplifications or deletions in the mouse osteosarcomas that were syntenic with those observed in human osteosarcomas. We were encouraged by the three regions of copy number gain, two of which contained known oncogenes or plausible oncogene candidates. Seven of 41 p53^{+/−} osteosarcomas contained an amplicon with *c-myc* at its epicenter, consistent with similar observations in human osteosarcomas (21,22). We focused our studies on the chromosome 9A1 amplicon, which contained a number of interesting genes, some with oncogenic potential.

Moreover, this particular amplicon is frequently observed in a number of human tumor types as well as in a mouse hepatocellular carcinoma model (12–17). Zender et al. (12) recently demonstrated that *Birc2* and *Yap1*, near the epicenter of the chromosome 9A1 amplicon in mouse liver carcinomas, were oncogenic in functional assays.

Once the chromosome 9A1 amplicon was delineated, we used a three part screening approach to identify those amplicon genes likely to be contributing to osteosarcomagenesis. First, using microarray expression analysis and real time RT-PCR methods, we examined gene expression of each of the amplicon genes. In the p53^{+/-} mouse osteosarcomas, MMP13, *Birc2*, *Birc3*, and *Yap1* were expressed at high levels relative to osteoblasts, though *Yap1* was only overexpressed in osteosarcomas with the chromosome 9A1 amplicon. The expression of these genes was further examined in human osteosarcomas. MMP13 and *Birc3* retained relatively high expression in most human osteosarcomas, while *Yap1* was expressed at low levels.

The final assessment of the candidate genes was functional in nature and addressed oncogenicity. We used lentiviral shRNA vectors to downregulate expression of the candidate genes in clonal osteosarcoma cell lines (with and without chromosome 9A1 amplification) that could reconstitute intact osteosarcomas when transplanted into recipient immunodeficient mice. MMP13 RNA downregulation inhibited tumor growth rates of both amplicon-containing and amplicon-negative tumors in our transplantation assay, through increased induction of apoptosis in tumor cells. This indicated that high MMP13 expression is important for osteosarcoma cell survival and tumor growth. Interestingly, MMP13 downregulation also enhanced the differentiated phenotypes of the osteosarcomas, as manifested by increased osteoid deposition and increased tumor density. Because tumors without the 9A1 amplicon had high MMP13 expression and were similarly affected by MMP13 downregulation, it seems unlikely that increased MMP13 expression was a driving force for selection of tumor cells containing this amplicon. It is also unlikely that increased *Yap1* expression was a driver because its expression level, while high in the amplicon positive osteosarcomas, was low in mouse and human osteosarcomas, and the shRNA downregulation experiments did not result in any effects on tumor growth. This result was different from that observed by Zender et al. (12) in studies with the mouse hepatocellular carcinomas with similar chromosome 9A1 amplifications. Here *Yap1* was shown to be oncogenic and contributory to hepatocarcinogenesis by both *in vitro* and *in vivo* assays in collaboration with *Birc2*. In addition, a mouse mammary tumor model exhibited focal amplification and overexpression of the *Yap1* gene alone, and *Yap1* was capable of inducing parameters of transformation in mammary epithelial cells (42). Thus, different tumor types can have similar amplification events, but the relevant oncogenic drivers within the amplicon may vary.

The anti-apoptotic genes *Birc2* and *Birc3* showed elevated expression in both A+ and A- osteosarcomas and *Birc3* expression was elevated in a majority of human tumors. Interestingly, the shRNA vector downregulation studies showed differential effects on tumor growth. A+ osteosarcomas were inhibited in growth by knockdown of *Birc2* and *Birc3* expression, while A- osteosarcoma growth was not dependent on *Birc2* and *Birc3* expression levels. As expected for these anti-apoptotic genes, apoptosis was significantly increased in the A+ tumors with *Birc2* and *Birc3* knockdown (but not in A- tumors). These results indicate that high *Birc2* and *Birc3* expression provide a selective cell survival advantage to those tumors with the 9A1 amplicon while no such advantage is provided to the amplicon negative tumors. We hypothesize that the formation of the 9A1 amplicon results in increased *Birc2* and *Birc3* expression during early stages of osteosarcoma evolution. This increase in expression in turn provides a cell survival advantage and the tumor remains dependent on high *Birc2* and *Birc3* during subsequent stages of tumor evolution. Because these tumors are dependent on high *Birc2* and *Birc3* levels, downregulating these genes through shRNA approaches will have profound effects on tumor growth rates. In amplicon negative tumors, *Birc2* and *Birc3*

expression, while elevated, are not as high as in the amplicon positive tumors (see Figure 5A). These tumors may activate other anti-apoptotic genes that enhance cell survival and may depend on such genes during further tumor progression. Thus, when subjected to Birc2 and Birc3 shRNA, the amplicon negative tumors, dependent on other anti-apoptotic genes for tumor cell survival, will not be affected. We believe that Birc2 and Birc3 are potential oncogenic drivers of selection for the chromosome 9A1 amplicon and are required for A+ osteosarcoma progression, but upregulation of these two genes is not universally required for osteosarcoma progression.

The mechanisms by which Birc2 and Birc3 may enhance cancer cell survival have been discussed (31,32,43,44). Birc2 and Birc3 may block apoptosis by impeding caspase-8 dependent autocrine TNF- α signaling. In addition to amplification in cancers (12–17), their upregulation at the mRNA and/or protein level has been shown in many human cancer types. Birc2 mRNA is upregulated in human myeloid leukemia and higher levels of Birc2 protein were associated with resistance to anticancer drugs (45). Overexpression of Birc2 and Birc3 has been observed in esophageal cancer (46), chronic neutrophilic leukemia (47), renal cell carcinomas (48), B-cell lymphomas (49), and pancreatic cancer (50). Taken together, amplification or upregulation of antiapoptotic factors Birc2 and/or Birc3 occurs with some frequency during cancer progression, suggesting that treatments targeting these two gene products may provide useful therapeutic options.

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References

1. Huvos, A. Bone Tumors: Diagnosis, Treatment and Prognosis. Philadelphia: WB Saunders; 1991.
2. Klein MJ, Siegal GP. Osteosarcoma: anatomic and histologic variants. *Am J Clin Pathol* 2006;125:555–581. [PubMed: 16627266]
3. Wang LL. Biology of osteogenic sarcoma. *Cancer J* 2005;11:294–305. [PubMed: 16197719]
4. Man TK, Lu XY, Jaewon K, et al. Genome-wide array comparative genomic hybridization analysis reveals distinct amplifications in osteosarcoma. *BMC Cancer* 2004;4:45. [PubMed: 15298715]

5. Gokgoz N, Wunder JS, Mousses S, Eskandarian S, Bell RS, Andrulis IL. Comparison of p53 mutations in patients with localized osteosarcoma and metastatic osteosarcoma. *Cancer* 2001;92:2181–2189. [PubMed: 11596036]
6. Lane DP. Cancer. p53, guardian of the genome. *Nature* 1992;358:15–16. [PubMed: 1614522]
7. Miller CW, Aslo A, Won A, Tan M, Lampkin B, Koeffler HP. Alterations of the p53, Rb and MDM2 genes in osteosarcoma. *J Cancer Res Clin Oncol* 1996;122:559–565. [PubMed: 8781571]
8. Overholtzer M, Rao PH, Favis R, et al. The presence of p53 mutations in human osteosarcomas correlates with high levels of genomic instability. *Proc Natl Acad Sci U S A* 2003;100:11547–11552. [PubMed: 12972634]
9. Michiels L, Merregaert J. Retroviruses and oncogenes associated with osteosarcomas. *Cancer Treat Res* 1993;62:7–18. [PubMed: 8096761]
10. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992;356:215–221. [PubMed: 1552940]
11. Lengner CJ, Steinman HA, Gagnon J, et al. Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. *J Cell Biol* 2006;172:909–921. [PubMed: 16533949]
12. Zender L, Spector MS, Xue W, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* 2006;125:1253–1267. [PubMed: 16814713]
13. Dai Z, Zhu WG, Morrison CD, et al. A comprehensive search for DNA amplification in lung cancer identifies inhibitors of apoptosis cIAP1 and cIAP2 as candidate oncogenes. *Hum Mol Genet* 2003;12:791–801. [PubMed: 12651874]
14. Imoto I, Yang ZQ, Pimkhaokham A, et al. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. *Cancer Res* 2001;61:6629–6634. [PubMed: 11559525]
15. Snijders AM, Schmidt BL, Fridlyand J, et al. Rare amplicons implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma. *Oncogene* 2005;24:4232–4242. [PubMed: 15824737]
16. Reardon DA, Michalkiewicz E, Boyett JM, et al. Extensive genomic abnormalities in childhood medulloblastoma by comparative genomic hybridization. *Cancer Res* 1997;57:4042–4047. [PubMed: 9307291]
17. Weber RG, Sommer C, Albert FK, Kiessling M, Cremer T. Clinically distinct subgroups of glioblastoma multiforme studied by comparative genomic hybridization. *Lab Invest* 1996;74:108–119. [PubMed: 8569172]
18. Bashyam MD, Bair R, Kim YH, et al. Array-based comparative genomic hybridization identifies localized DNA amplifications and homozygous deletions in pancreatic cancer. *Neoplasia* 2005;7:556–562. [PubMed: 16036106]
19. Cai WW, Mao JH, Chow CW, Damani S, Balmain A, Bradley A. Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays. *Nat Biotechnol* 2002;20:393–396. [PubMed: 11923847]
20. Qin XF, An DS, Chen IS, Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* 2003;100:183–188. [PubMed: 12518064]
21. Barrios C, Castresana JS, Ruiz J, Kreicbergs A. Amplification of c-myc oncogene and absence of c-Ha-ras point mutation in human bone sarcoma. *J Orthop Res* 1993;11:556–563. [PubMed: 8101872]
22. Squire JA, Pei J, Marrano P, et al. High-resolution mapping of amplifications and deletions in pediatric osteosarcoma by use of CGH analysis of cDNA microarrays. *Genes Chromosomes Cancer* 2003;38:215–225. [PubMed: 14506695]
23. Huang J, Wu S, Barrera J, Matthews K, Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* 2005;122:421–434. [PubMed: 16096061]
24. Overall CM, Kleinfeld O. Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 2006;6:227–239. [PubMed: 16498445]
25. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161–174. [PubMed: 11990853]

26. Leeman MF, Curran S, Murray GI. New insights into the roles of matrix metalloproteinases in colorectal cancer development and progression. *J athol* 2003;201:528–534.
27. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002;295:2387–2392. [PubMed: 11923519]
28. Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. *Oncogene* 2003;22:8568–8580. [PubMed: 14634619]
29. Li X, Yang Y, Ashwell JD. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature* 2002;416:345–347. [PubMed: 11907583]
30. Lee JS, Hong US, Lee TH, Yoon SK, Yoon JB. Mass spectrometric analysis of tumor necrosis factor receptor-associated factor 1 ubiquitination mediated by cellular inhibitor of apoptosis 2. *Proteomics* 2004;4:3376–3382. [PubMed: 15468071]
31. Petersen SL, Wang L, Yalcin-Chin A, et al. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell* 2007;12:445–456. [PubMed: 17996648]
32. Varfolomeev E, Blankenship JW, Wayson SM, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007;131:669–681. [PubMed: 18022362]
33. Vince JE, Wong WW, Khan N, et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007;131:682–693. [PubMed: 18022363]
34. Hu S, Yang X. Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. *J Biol Chem* 2003;278:10055–10060. [PubMed: 12525502]
35. Basu S, Totty NF, Irwin MS, Sudol M, Downward J. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Mol Cell* 2003;11:11–23. [PubMed: 12535517]
36. Strano S, Monti O, Pediconi N, et al. The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA Damage. *Mol Cell* 2005;18:447–459. [PubMed: 15893728]
37. Venkatachalam S, Shi YP, Jones SN, et al. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *Embo J* 1998;17:4657–4667. [PubMed: 9707425]
38. Stahle-Backdahl M, Sandstedt B, Bruce K, et al. Collagenase-3 (MMP-13) is expressed during human fetal ossification and re-expressed in postnatal bone remodeling and in rheumatoid arthritis. *Lab Invest* 1997;76:717–728. [PubMed: 9166290]
39. Ala-aho R, Ahonen M, George SJ, et al. Targeted inhibition of human collagenase-3 (MMP-13) expression inhibits squamous cell carcinoma growth in vivo. *Oncogene* 2004;23:5111–5123. [PubMed: 15094779]
40. Aubin JE. Bone stem cells. *J Cell Biochem Suppl* 1998;30–31:73–82.
41. Tarkkanen M, Karhu R, Kallioniemi A, et al. Gains and Losses of DNA Sequences in Osteosarcomas by Comparative Genomic Hybridization. *Cancer Res* 1995;55:1334–1338. [PubMed: 7882332]
42. Overholtzer M, Zhang J, Smolen GA, et al. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc Natl Acad Sci U S A* 2006;103:12405–12410. [PubMed: 16894141]
43. Gaither A, Porter D, Yao Y, et al. A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. *Cancer Res* 2007;67:11493–11498. [PubMed: 18089776]
44. Bertrand MJ, Milutinovic S, Dickson KM, et al. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell* 2008;30:689–700. [PubMed: 18570872]
45. Tamm I, Kornblau SM, Segall H, et al. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin Cancer Res* 2000;6:1796–1803. [PubMed: 10815900]
46. Nemoto T, Kitagawa M, Hasegawa M, et al. Expression of IAP family proteins in esophageal cancer. *Exp Mol Pathol* 2004;76:253–259. [PubMed: 15126108]
47. Hasegawa T, Suzuki K, Sakamoto C, et al. Expression of the inhibitor of apoptosis (IAP) family members in human neutrophils: up-regulation of cIAP2 by granulocyte colony-stimulating factor

- and overexpression of cIAP2 in chronic neutrophilic leukemia. *Blood* 2003;101:1164–1171. [PubMed: 12393423]
48. Kempkensteffen C, Hinz S, Christoph F, et al. Expression of the apoptosis inhibitor livin in renal cell carcinomas: correlations with pathology and outcome. *Tumour Biol* 2007;28:132–138. [PubMed: 17519534]
49. Akyurek N, Ren Y, Rassidakis GZ, Schlette EJ, Medeiros LJ. Expression of inhibitor of apoptosis proteins in B-cell non-Hodgkin and Hodgkin lymphomas. *Cancer* 2006;107:1844–1851. [PubMed: 16983704]
50. Esposito I, Kleeff J, Abiatari I, et al. Overexpression of cellular inhibitor of apoptosis protein 2 is an early event in the progression of pancreatic cancer. *J Clin Pathol* 2007;60:885–895. [PubMed: 16775116]

Figure 1. BAC array CGH results show recurrent copy number gains and losses in 41 $p53^{+/-}$ mouse osteosarcomas

(A) Representative array CGH results showing copy number gain in a $p53^{+/-}$ osteosarcoma on mouse chromosome 15. The *c-myc* gene lies at the epicenter of this amplicon. The Y axis is a log scale indicating the relative copy number gain (or loss), with 0 representing a normal diploid copy number. The X axis shows the DNA sequence position in 100 kb units along the chromosome beginning from the proximal telomere (using the Ensembl genome browser). (B and C) Representative copy number gain in two $p53^{+/-}$ osteosarcomas (Het192 and Het249) on mouse chromosome 9 (Band A1). (D) Higher resolution view of the chromosome 9A1 amplicon. The region shown is position 4.4~12.1 Mb on chromosome 9. The positions of the relevant genes within this amplicon are indicated.

Figure 2. Expression of MMPs in mouse and human osteosarcomas

(A) Summary of MMP expression in mouse and human osteosarcomas. Mouse data was obtained by real-time PCR analyses, and human data was obtained by RNA microarray analyses. Among the mouse MMPs, MMP12, MMP13 and MMP9 are over-expressed in osteosarcomas. Samples are listed in the order of their relative positions on the chromosome, except MMP9 and 19 (marked with *) which are not on the chromosome 9A1 amplicon. (B) Real-time PCR showing that MMP13 is overexpressed in mouse osteosarcomas, but not in rhabdomyosarcomas. Each bar represents a single tumor and the Y axis measures the log₂ ratio of tumor expression relative to mc3T3 osteoblast expression. OS, A+: osteosarcomas with chromosome 9 amplicon. OS, A-: osteosarcomas without chromosome 9 amplicon. RS: rhabdomyosarcomas. (C) RNA microarray analyses of human osteosarcomas showed that MMP13 is also overexpressed in the majority of 40 human osteosarcomas relative to normal human osteoblasts. Each bar represents a single tumor and the Y axis measures the log₂ ratio of tumor expression relative to human osteoblasts. (D) MMP13 immunohistochemistry on tissue sections from three murine osteosarcoma samples and one murine rhabdomyosarcoma sample (lower right panel) shows that MMP13 protein is expressed at high levels specifically in osteosarcomas.

Figure 3. Downregulation of MMP13 expression in osteosarcoma cells reduces tumor growth rates *in vivo*

(A and C) Tumor growth curves of allograft studies with chromosome 9A amplicon-positive (A+) (A) and 9A-amplicon-negative (A-) (C) clonal osteosarcoma cells transduced with lentiviral shRNA vectors. Two different shRNA vectors against MMP13 were used (sh1 and sh2). 2×10^6 of A+ or 0.5×10^6 of A- cells were subcutaneously injected into the dorsal flanks of immunodeficient nude mice and tumor volumes measured at four day intervals. $n=5$ for each group. Error bars represent $\pm$ SD. (B and D) Western blots against MMP13 in lysates from harvested transplant tumors confirm knock down of MMP13 expression. MMP13 protein is normalized to beta actin control.

Figure 4. Downregulation of MMP13 in osteosarcoma cells enhances apoptosis and differentiation, but does not affect proliferation

(A) Proliferation of osteosarcoma cells in vivo is unaffected by shRNA mediated MMP13 knockdown. BrdU immunohistochemistry of either A+ or A- transplanted tumor sections (left panel) with or without shMMP13 show no difference in staining frequency (for cells in S phase). Quantitation of multiple sections for frequency of cells with BrdU staining confirms no significant differences with or without MMP13 knockdown (right panel). (B) Apoptosis in osteosarcoma cells is increased following MMP13 knockdown. TUNEL staining of A+ or A- transplanted tumor sections with or without shMMP13 knockdown shows increased TUNEL staining in shMMP13 transduced cells (left panel). Quantitation of staining in multiple sections confirmed that MMP13 downregulation significantly increased apoptosis in the transplanted

tumors (right panel). (C) Increased osteoid deposition in osteosarcomas with reduced MMP13 expression. Hematoxylin and eosin staining of either A+ or A- tumor sections with or without shMMP13 expression shows increased osteoid deposition (pink staining matrix between cells) in shMMP13 expressing osteosarcomas (left panel). (D) Increased density of transplanted osteosarcomas expressing MMP13 shRNA. Quantitation by CT scanning indicated a significantly higher density (measured by Hounsfield units) in the A+ and Atransplanted osteosarcomas with MMP13 knockdown. The scans were quantified three-dimensionally. n=5 for each group. p<0.05 by t-test.

Figure 5. Birc2 and Birc3 expression are increased in mouse and human osteosarcomas and are required for robust growth of osteosarcomas with chromosome 9A1 amplification

(A) Birc2 (left) and Birc3 (right) mRNA expression in mouse tumors. OS: osteosarcoma. RS: rhabdomyosarcoma. A+: chromosome 9A amplicon-positive. A-: chromosome 9A amplicon-negative. The mouse data was from real-time PCR analyses, and the human Birc3 expression data (lower right panel) was obtained from RNA microarray analyses. (B) Birc2 and Birc3 knockdown result in reduced tumor growth rates in A+ osteosarcoma cells, but not A- osteosarcoma cells after subcutaneous transplant in nude mice. Left panels: A+ or A- cell line tumor growth curves with Birc2 or Birc3 knockdown. n=5 for each group. Error bars represent $\pm$ SD. Right panels: Western blot of transplanted tumor lysates using antibodies to Birc2,

Birc3, and actin (normalization control). (C) Birc2 and Birc3 knockdown increases tumor apoptosis rates in A+ osteosarcoma cells, but not A- osteosarcoma cells after transplantation in nude mice. Representative TUNEL assays are shown in the left panels and quantitation of TUNEL fluorescence in multiple microscope fields for five mice for each vector/cell type category are shown in the two graphs at right. Asterisks represent $p < 0.001$ by t-test.

Figure 6. Yap1 expression is generally low in mouse and human osteosarcomas and shRNA mediated Yap1 knockdown does not affect osteosarcoma growth
 (A) Yap1 mRNA expression in mouse tumors by real-time PCR. OS: osteosarcoma. RS: rhabdomyosarcoma. A+: chromosome 9A amplicon-positive. A-: chromosome 9A amplicon-negative. (B) Yap1 mRNA expression in 40 human osteosarcomas by RNA microarray. (C) and (D) Tumor growth curves of Yap1 shRNA-expressing or vector-only A+ (C) or A- (D) osteosarcoma cells after transplantation into nude mice.