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

Ou Ma,1,2 Wei-Wen Cai,2,3 Lars Zender,8 Tajhal Dayaram,1,4 Jianhe Shen,5 Alan J. Herron,6 Scott W. Lowe,4 Tsz-Kwong Man,2,5 Ching C. Lau,2,5 and Lawrence A. Donehower1,2,4,8

1Department of Molecular Virology and Microbiology, Dan L. Duncan Cancer Center, 2Department of Molecular and Human Genetics, Program in Cell and Molecular Biology, 3Department of Pediatrics, 4Dan L. Duncan Cancer Center, Department of Pediatrics, 5Texas Children’s Cancer Center, Department of Pediatrics, 6Center for Comparative Medicine and Department of Pathology, and 7Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas and 8Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

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 several human tumor types. Three genes on this amplicon, the matrix metalloproteinase gene MMP13 and the antiapoptotic 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 short hairpin RNA vectors to show that down-regulation 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 antiapoptotic genes Birc2 and Birc3 are potential oncogenic drivers in the chromosome 9A1 amplicon.

Introduction
Osteosarcoma is the most common malignant bone tumor in children and young adults and accounts for ~60% of malignant bone tumors in the first 2 decades of life (1). About 900 cases are diagnosed yearly in the United States. With adjuvant chemotherapy and surgery, the 5-year survival rate has been improved to 50% to 65% (2, 3). However, 25% to 50% of patients with initial nonmetastatic disease later develop metastatic disease, which is generally fatal. Osteosarcomas do not have molecular genetic abnormalities that serve as a tumor-specific marker (3), although they exhibit high frequencies of genome instability (4). Osteosarcomas are also associated with inherited cancer syndromes (1). For example, Li-Fraumeni syndrome patients with a defective germ-line p53 allele develop soft tissue sarcomas and osteosarcomas, considered signature tumors (5). p53 is a prototypical tumor suppressor, mediating cellular stress responses and preserving genome integrity (6). p53 mutations occur in 15% to 30% of human osteosarcomas (7), and the presence of p53 mutations correlates with increased genome instability (8).

Sporadic osteosarcomas in inbred mouse strains are rare (9). However, in the p53-deficient mouse model generated by our laboratory, 38% of p53−/− mice developed osteosarcomas (10). p53−/− mice develop early thymic lymphomas rather than osteosarcomas, but recent studies of conditional p53 knockout models, in which both p53 alleles are deleted specifically in osteoblasts, show early osteosarcoma development (11). The propensity of humans and mice with germ-line p53 mutations to develop osteosarcomas indicates that p53 deficiency may affect aspects of osteoblast signaling that make these cells particularly susceptible to oncogenic transformation.

Here, we describe strategies to identify genes with functional relevance for osteosarcoma development. We analyzed an archive of p53−/− mouse osteosarcomas by DNA array-based comparative genomic hybridization (array CGH). Array CGH analyses performed on 41 p53−/− osteosarcomas revealed seven frequent regions of copy number gain or loss. One region of copy number gain on mouse chromosome 9 contained a cluster of 10 matrix metalloproteinase (MMP) genes and two antiapoptotic genes, Birc2 (cIAP1) and Birc3 (cIAP2). DNA amplification centered on Birc2 and Birc3 has also been observed in mouse liver cancers (12) and human lung cancers (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. Functional assays determined whether alteration of candidate gene expression levels affected in vivo tumor growth rates. We found that the MMP gene MMP13 and the antiapoptotic genes Birc2 (cIAP1) and Birc3 (cIAP2) are promoters of osteosarcomagenesis.

Materials and Methods
Array-based CGH. The mouse bacterial artificial chromosome (BAC) arrays contained 19,000 BACs covering the mouse genome. BAC array CGH
was performed on 41 p53+/− osteosarcomas and 10 p53−/− rhabdomyosarcomas as previously described (19). Each sample/control pair was done twice by reciprocal labeling of sample or control to remove ratio artifacts. CGH data were analyzed as described by Cai and colleagues (19). Hybridizations comparing wild-type C57BL/6 and wild-type 129/SvEv genomic DNA were performed to filter out strain-specific polymorphisms.

**Tumor cell culture.** Tumor fragments were collected from p53−/− mice, washed twice with sterile PBS, minced, and incubated with 0.25% trypsin solution for two consecutive 15-min incubations at 37°C with shaking. Cells were filtered through a 70-μm cell strainer (BD Falcon), and the filtrate was centrifuged at 1,500 rpm for 5 min at 4°C. Cells were collected and cultured in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO2.

**Immunohistochemistry.** Tumor sections were stained with antibodies against BrdUrd, c-Myc, cIAP1, cIAP2, c-Myc, and p53, as previously described (12, 23). The cluster of MMP genes within chromosome 9 amplicon about 1 Mb downstream of the MMP gene, oncogenic in some contexts (12, 23), is also located in the chromosome 9A1 amplicon and is a potential oncoprotein that may be important for osteosarcoma cell invasion if overexpressed (24–27).

**Results**

**Array CGH identifies genome instability in p53−/− osteosarcomas.** To identify copy number changes in p53−/− sarcoma genomes, we performed array CGH analysis on genomic DNA isolated from 41 p53−/− osteosarcomas and 10 rhabdomyosarcomas. For the 41 osteosarcomas, seven frequent regions of copy number gain or loss were observed (Supplementary Table S1). Among these, three were regions of copy number gain and four were regions of copy number loss. Analysis of the regions of copy number losses revealed no obvious tumor suppressor candidates. However, two regions of copy number gain (on chromosomes 9 and 15) had candidate oncogenes. Seven of 41 osteosarcomas had amplifications on chromosome 15 with the c-myc oncogene at the ampiclon epicenter (Fig. 1A). This was consistent with observations that human osteosarcomas also exhibit amplification 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 MMP genes as well as the antiapoptotic Birc2 (cIAP1) and Birc3 (cIAP2) genes (Fig. 1B–D). DNA amplification centered on Birc2 and Birc3 has been observed in multiple human tumor types (12–17). Finally, the Yap1 gene, oncogenic in some contexts (12, 23), is also located in this amplicon.

Because of its previous association with human tumors, we focused on the chromosome 9A1 amplicon at chromosomal position 5 to 9 Mb (Fig. 1D). The cluster of MMP genes within the amplicon is a family of extracellular endopeptidases that collectively cleave all the components of the extracellular matrix that may be important for osteosarcoma cell invasion if overexpressed (24–27). Birc2, Birc3, and Yap1 are located in the chromosome 9 amplicon about 1 Mb downstream of the MMP cluster. Birc proteins, or the baculoviral inhibitor of apoptosis protein repeat-containing proteins, are inhibitors of apoptosis (12, 28–34). Importantly, Birc2 acts as an oncogene in a mouse hepatocellular cancer model containing a similar chromosome 9A1 amplicon as described here (12). Yap1 also resides on the chromosome 9A1 amplicon and is a potential oncoprotein (12, 23, 35, 36). Yap1 has been shown to exhibit oncogenic activity in mouse hepatocellular cancers with similar chromosome 9A1 amplicons to those described here (12). We examined the roles of MMP13, Birc2, Birc3, and Yap1 in osteosarcomagenesis.

---

9 http://www.bcm.edu/mcfweb/
10 http://www.genesifter.net
Southern blot hybridization of osteosarcoma DNAs with MMP13 and MMP7 probes confirmed copy number gain in those tumors with chromosome 9A1 amplification measured by array CGH (Supplementary Fig. S1). Because these tumors were derived from p53+/- mice, we assessed p53 allele status and found that the wild-type p53 allele was retained in some tumors but was lost in cell lines derived from these tumors (Supplementary Fig. S1). About half of all p53+/- tumors retain the wild-type p53 allele and about half undergo loss of the wild-type allele (37).

MMP13 is overexpressed in osteosarcomas. To determine whether a particular gene in the chromosome 9A1 amplicon could drive osteosarcomagenesis, we analyzed RNA expression of all genes within the chromosome 9A1 amplicon in A+ and A/- osteosarcomas by microarray analysis and real-time PCR. MMP genes were first analyzed. A murine osteoblast cell line, mc3T3, as well as normal bone were used as controls. 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 (Supplementary Figs. S2 and S3). About half of all p53+/- tumors retain the wild-type p53 allele and about half undergo loss of the wild-type allele (37).

MMP13 is overexpressed in osteosarcomas. To determine whether a particular gene in the chromosome 9A1 amplicon could drive osteosarcomagenesis, we analyzed RNA expression of all genes within the chromosome 9A1 amplicon in A+ and A/- osteosarcomas by microarray analysis and real-time PCR. MMP genes were first analyzed. A murine osteoblast cell line, mc3T3, as well as normal bone were used as controls. 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 (Supplementary Figs. S2 and S3). However, within the chromosome 9A1 amplicon, MMP1, MMP12, MMP8, MMP10, or MMP20 did not exhibit elevated RNA expression. This was confirmed by RNA microarray analysis of all MMP genes (Supplementary Figs. S2 and S3). Furthermore, MMP expression in the mouse osteosarcomas was consistent with corresponding gene expression in human osteosarcomas (except MMP19; Supplementary Fig. S3).

These results suggested that overexpression of the chromosome 9A1 amplicon-associated MMP13 gene might facilitate osteosarcoma progression. MMP13 expression is most abundant in adult bone compared with other tissues (38), and MMP13 knockdown increases apoptosis in squamous cell carcinomas (39). Real-time reverse transcription-PCR (RT-PCR) analysis of MMP13 gene expression in 15 p53+/- osteosarcomas showed that virtually all osteosarcomas exhibited high RNA expression relative to osteoblast cells (Fig. 2A). High MMP13 RNA expression was not dependent on gene amplification, as most tumors were diploid at the MMP13 locus (Fig. 2A). High MMP13 RNA expression was also observed by microarray analyses on 40 human osteosarcoma samples relative to normal osteoblasts (Fig. 2B). In contrast, six rhabdomyosarcomas exhibited low MMP13 expression (Fig. 2A). The RNA expression patterns were confirmed at the protein level by immunohistochemical staining of osteosarcoma and rhabdomyosarcoma sections (Fig. 2C). No MMP13 staining was evident in rhabdomyosarcoma sections, whereas osteosarcoma sections showed cytoplasmic and cell surface MMP13 staining. Other MMP genes did not show elevated expression in human osteosarcomas (Supplementary Fig. S3).

Reduction of MMP13 expression decreases osteosarcoma growth rate. Before 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 s.c. into immunodeficient BALB/c-nu/nu mice, formed osteosarcomas histopathologically indistinguishable from primary p53+/- osteosarcomas several weeks following injection (Supplementary Fig. S4). Lentiviral vectors expressing two MMP13 short hairpin RNAs (shRNA) were constructed and transduced into A+ and A/- osteosarcoma lines. Stably transduced cells were selected by flow cytometric cell sorting for GFP fluorescence. MMP13 mRNA levels in transduced osteosarcoma cells were reduced over 80% compared with empty vector–transduced cells (data not shown). Equal

**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 relative copy number gain or loss, with 0 representing diploid copy number. The X axis shows DNA sequence position in 100-kb units along the chromosome. 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 (4.4–12.1 Mb on chromosome 9).
numbers of MMP13 shRNA–transduced or empty vector–transduced cells were s.c. 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 with their empty vector–transduced counterparts, indicating that MMP13 plays a role in osteosarcoma progression (Fig. 3A and C; Supplementary Fig. S5). Western blots confirmed reduced MMP13 protein expression in the shRNA-expressing tumor allografts compared with empty vector–containing tumors (Fig. 3B and D, top).

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 BrdUrd for 3 hours before euthanasia and measured cell proliferation by staining tumor sections with a BrdUrd antibody. Staining provides an indicator of S-phase cells. Using this proliferation assay, no significant differences were observed between tumors with and without MMP13 shRNA expression, regardless of amplicon status (Fig. 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. Counting of stained apoptotic cells in microscopic fields from multiple tumor sections revealed that A+ and A− transplanted MMP13 shRNA vector–transduced osteosarcomas displayed significantly higher levels of apoptosis than their empty vector–transduced counterparts (Fig. 4B). Thus, reduction of MMP13 in the transplanted osteosarcomas increases rates of tumor cell apoptosis.

Histopathologic examination of the transplanted tumors revealed an additional effect of MMP13 knockdown. The sections of A+ and A− tumors transduced with MMP13 shRNA vectors reduced cellular density and increased osteoid component compared with control vector–transduced tumors, suggesting a higher level of differentiation (Fig. 4C). In osteosarcomas, differentiation is considered antithetical to the oncogenic process (40). To quantitatively determine whether bone matrix and tumor density were increased by MMP13 reduction, CT scans were performed. Tumor density was measured three dimensionally by Amira and converted into Hounsfield units. A+ and A− shMMP13-expressing tumors were denser than vector-expressing controls (P < 0.05; Fig. 4D; Supplementary Fig. S5). These results suggest that MMP13 reduction induces a more differentiated tumor cell state.

Birc2 and Birc3 knockdown enhances apoptosis and reduces tumor growth in amplicon-positive osteosarcomas. The antiapoptotic 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 (Fig. 5A; Supplementary Fig. S6). Moreover, high Birc3 expression was observed in most human osteosarcomas (Fig. 5A). To determine whether Birc2 and Birc3 influence 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. 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 (Fig. 5B, top). In contrast, Birc2 and Birc3 shRNA did not significantly affect the growth rate of the A− osteosarcoma cells (Fig. 5B, bottom). TUNEL experiments showed that Birc2 or Birc3 shRNA-expressing A+ osteosarcoma sections contained significantly increased apoptotic cell percentages (Fig. 5C), whereas 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 amplified in the chromosome 9A1 amplicon-containing osteosarcomas. Because Yap1 is oncogenic in some contexts (12, 23), we analyzed Yap1 RNA expression by real-time RT-PCR and microarray analyses. Yap1 mRNA was highly expressed only in the mouse p53+/− osteosarcomas that contained the chromosome 9A amplicon (Fig. 6A; Supplementary Fig. S7). In human osteosarcomas, Yap1 expression was down-regulated relative to osteoblasts (Fig. 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 changes in tumor growth rate between Yap1 shRNA–transduced and empty vector–transduced tumors were observed (Fig. 6C and D). Despite reduction of Yap1 RNA levels by shRNA vectors in the osteosarcoma cells (Supplementary Fig. 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 A+ or A− transplanted osteosarcomas.

**Discussion**

The goal of this study was to identify genes that cooperate with p53 deficiency in promoting osteosarcoma progression using the osteosarcoma-prone p53+/− mouse model. We used a multidimensional approach integrating four major methods: (a) scanning of the p53+/− mouse osteosarcoma genomes for frequent copy number changes, (b) global analyses of the osteosarcoma transcriptomes, (c) validation of mouse osteosarcoma candidate gene expression in human osteosarcomas, and (d) functional analyses to determine the effects of altering candidate gene expression in an in vivo osteosarcoma model. 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 high genomic instability in human osteosarcomas (4, 8, 41), we anticipated that many copy number changes would be observed in the mouse osteosarcomas. Surprisingly, only seven regions of frequent copy number gain or loss were identified. This contrasts to the frequent amplifications observed by Man and
colleagues (4) in human osteosarcomas. We did not observe any amplifications or deletions in the mouse osteosarcomas that were syntenic with those observed in human osteosarcomas. We noted three regions of copy number gain, two of which contained known or likely oncogenes. We focused our studies on the chromosome 9A1 amplicon. This particular amplicon is observed in numerous human tumor types as well as a mouse hepatocellular carcinoma model (12–17). Zender and colleagues (12) recently showed 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 analysis and real-time RT-PCR methods, we examined expression of each amplicon gene. In the p53+/− mouse osteosarcomas, MMP13, Birc2, Birc3, and Yap1 were expressed at high levels relative to osteoblasts, although Yap1 was only overexpressed in osteosarcomas with the chromosome 9A1 amplicon. MMP13 and Birc3 retained relatively high expression in most human osteosarcomas, whereas Yap1 was expressed at low levels.

The final assessment of the candidate genes functionally addressed oncogenicity. We used lentiviral shRNA vectors to down-regulate expression of the candidate genes in clonal osteosarcoma cell lines that could reconstitute intact osteosarcomas when transplanted into immunodeficient mice. MMP13 RNA

Figure 4. MMP13 down-regulation in osteosarcomas enhances apoptosis and differentiation but does not affect proliferation. A, osteosarcoma cell proliferation in vivo is unaffected by MMP13 shRNA knockdown. BrdUrd immunohistochemistry of either A+ or A− transplanted tumor sections (left) with or without shMMP13 show no difference in staining frequency. Right, quantitation of BrdUrd staining confirms no significant differences with or without MMP13 knockdown. B, apoptosis in osteosarcoma cells is increased following MMP13 knockdown. Left, TUNEL staining of A+ or A− transplanted tumor sections shows increased TUNEL staining in shMMP13-transduced cells; right, quantitation confirmed that MMP13 down-regulation significantly increased apoptosis in transplanted tumors. C, increased osteoid deposition in osteosarcomas with reduced MMP13 expression. Left, H&E staining of A+ and A− tumor sections with or without shMMP13 expression shows increased osteoid deposition (pink staining matrix between cells) in shMMP13-expressing osteosarcomas. D, increased density of transplanted osteosarcomas expressing MMP13 shRNA. Quantitation by CT scanning indicated significantly higher density (measured by Hounsfield units) in A+ and A− transplanted osteosarcomas with MMP13 knockdown. n = 5 for each group. P < 0.05, 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. Mouse data were from real-time PCR analyses, and human Birc3 expression data (bottom right) were obtained from RNA microarray analyses. B, Birc2 and Birc3 knockdown reduces tumor growth rates in A+ osteosarcoma cells but not in A− osteosarcomas. Left, A+ or A− cell line tumor growth curves with Birc2 or Birc3 knockdown. n = 5 for each group. Right, Western blot of transplanted tumor lysates using antibodies to Birc2, Birc3, and actin. C, Birc2 and Birc3 knockdown increases tumor apoptosis rates in A+ osteosarcoma cells but not A− osteosarcoma cells after transplantation in nude mice. Left, representative TUNEL assays; right, quantitation of TUNEL fluorescence for five mice for each vector/cell type category. *, P < 0.001, t test.
down-regulation inhibited tumor growth rates of A+ and A− allograft tumors through increased induction of apoptosis in tumor cells. This indicated that high MMP13 expression is important for osteosarcoma cell survival. Because tumors without the 9A1 amplicon had high MMP13 expression and were similarly affected by MMP13 down-regulation, it seems unlikely that increased MMP13 expression is a driving force for selection of tumor cells containing this amplicon. It is also unlikely that increased Yap1 expression was a driver because shRNA down-regulation experiments did not result in any effects on tumor growth. This result contrasted with that observed by Zender and colleagues (12) studying mouse hepatocellular carcinomas with similar chromosome 9A1 amplifications. Here, Yap1 was shown to contribute to hepatocarcinogenesis in collaboration with Birc2. In addition, a mouse mammary tumor model exhibited focal amplification and overexpression of the Yap1 gene alone, and Yap1 was transforming 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 antiapoptotic genes Birc2 and Birc3 showed elevated expression in A+ and A− osteosarcomas and Birc3 expression was elevated in most human osteosarcomas. The shRNA vector studies showed differential effects on tumor growth. A+ osteosarcomas were growth inhibited by knockdown of Birc2 and Birc3 expression, whereas A− osteosarcoma growth was not dependent on Birc2 and Birc3 expression levels. Apoptosis was significantly increased in the A+ tumors with Birc2 and Birc3 knockdown, but not in A− tumors, indicating that high Birc2 and Birc3 expression provide a selective cell survival advantage to the A+ tumors, but not the A1 tumors. We hypothesize that formation of the 9A1 amplicon results in increased Birc2 and Birc3 expression during early stages of osteosarcoma evolution. This increase in expression 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, down-regulating these genes through shRNA approaches will have profound effects on tumor growth rates. In A− tumors, Birc2 and Birc3 expression, although elevated, are not as high as in the amplicon-positive tumors (see Fig. 5A). These tumors may activate other antiapoptotic genes during tumor progression. When subjected to Birc2 and Birc3 shRNA, the A− tumors, dependent on other antiapoptotic genes for cell survival, will be unaffected. Thus, Birc2 and Birc3 are oncogenic drivers of selection for the chromosome 9A1 amplicon and are required for A+ osteosarcoma progression, but up-regulation 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). In addition to amplification in cancers (12–17), their up-regulation at the mRNA and/or protein level has been shown in many human cancer types, including myeloid and neutrophilic leukemias, B-cell lymphomas, esophageal carcinomas, and renal and pancreatic cancers (45–50). Taken together, amplification or up-regulation 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.

Disclosure of Potential Conflicts of Interest

L.A. Donehower: ownership interest in p53 knockout mouse patent; licensing income, Taconic. The other authors disclosed no potential conflicts of interest.
Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank laboratory members C. Wu, C. Gatzia, L. Liles, and L. White (BCM Microarray Core); D. Townley and M. Mancini (BCM Integrated Microscopy Core); J. Santoussou, I. Hu, and R. Belinda (BCM Mouse Phenotyping Core); A. Rice and X. Qin for lentiviral vectors; D. Burton (BCM B4 Barrier Facility); and T. Triche and R. Gorlick for human sarcoma tissues.

References


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

Ou Ma, Wei-Wen Cai, Lars Zender, et al.