HHS Public Access

Author manuscript

J Neuropathol Exp Neurol. Author manuscript; available in PMC 2016 February 01.

Published in final edited form as:

J Neuropathol Exp Neurol. 2015 February ; 74(2): 177–185. doi:10.1097/NEN.0000000000000161.

The Chromatin Modifying Protein HMGA2 Promotes Atypical Teratoid Rhabdoid Cell Tumorigenicity

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Abstract

Atypical teratoid/rhabdoid tumor (AT/RT) is an aggressive pediatric central nervous system tumor. The poor prognosis of AT/RT warrants identification of novel therapeutic targets and strategies. High mobility group A2 (HMGA2) is a developmentally important chromatin modifying protein that positively regulates tumor growth, self-renewal and invasion in other cancer types. HMGA2 was recently identified as being upregulated in AT/RT tissue, but the role of HMGA2 in brain tumors remains unknown. We used lentiviral short hairpin RNA to suppress HMGA2 in AT/RT cell lines and found that loss of HMGA2 led to decreased cell growth, proliferation, colony formation and increased apoptosis. We also found that suppression of HMGA2 negatively affected in vivo orthotopic xenograft tumor growth, more than doubling median survival of the mice from 58 days to 153 days. Our results indicate a role for HMGA2 in AT/RT in vitro and in vivo and demonstrate that HMGA2 is a potential therapeutic target in these lethal pediatric tumors.

Keywords

SNF5; Atypical teratoid/rhabdoid tumor; High motility group AT-hook 2; INI1; *let-7*; Malignant rhabdoid tumor; Pediatric brain tumor

INTRODUCTION

Atypical teratoid/rhabdoid tumors (AT/RTs) are highly aggressive and deadly pediatric brain tumors with a very poor prognosis (1). Current therapies of surgery, radiation, and intense chemotherapy allow only a very small subset of children to survive (2). Exposing

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Authors report no conflict of interest.

infants and young children to very aggressive therapy also leads to lifelong cognitive, behavioral and growth deficits in survivors (3).

The sole consistently identified genomic alteration in AT/RTs is in the chromatin remodeling complex gene *SMARCB1/INI1/SNF5* (4, 5). Loss of the tumor suppressor INI1 blocks proper differentiation of neural stem and progenitor cells and is believed to be critical for the development of AT/RTs (6). Therapeutic failure in aggressive brain tumors such as AT/RTs is due to the lack of potency of existing agents, the impermeability of the blood-brain barrier, intratumoral and intertumoral heterogeneity, and activation of anti-apoptotic and metabolic programs that allow tumor cells to survive treatment (7, 8). Identification and validation of novel targets is essential to develop better therapies and improve the dismal prognosis of this lethal pediatric tumor.

AT/RTs share many characteristics with stem cells, including an ability to differentiate into cells with neuronal and “rhabdoid” features, as well as resistance to chemotherapy and radiation (1, 9). AT/RTs express multiple stem cell factors, including SOX2, NANOG, KLF4, and high mobility group A2 (HMGA2) (10, 11). HMGA2 is a chromatin-architectural protein that is highly expressed during embryogenesis with little to no expression in normal adult tissues (12–16). Increased expression of HMGA2 is associated with a poor prognosis in multiple adult cancer types, including lung, gastric, pancreatic and ovarian carcinomas, and leukemia (11, 17–26). HMGA2 promotes tumor cell growth, invasion and clonogenic potential in cancer cells (13, 14, 17–21, 27–33). Reduction of HMGA2 in a kidney rhabdoid tumor cell line decreased proliferation and colony formation (11) but the functional significance of HMGA2 in central nervous system (CNS) AT/RTs and the role of HMGA2 in CNS AT/RT tumor formation in vivo are unknown.

We here show that HMGA2 is expressed in CNS AT/RT cell lines derived from pediatric patients. Short hairpin (shRNA)-mediated knockdown of HMGA2 in these AT/RT cell lines suppressed growth, proliferation and colony formation in vitro. Knockdown of HMGA2 increased apoptosis in vitro and increased tumor latency in vivo. Our studies demonstrate the functional importance of HMGA2 in regulating multiple transformed properties of AT/RTs and suggest that targeting HMGA2 may be a valid therapeutic approach in this aggressive tumor.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The BT37 AT/RT cell line was derived from a human xenograft that originated at St. Jude Children’s Research Hospital (Memphis, TN) and was passaged serially in immunodeficient mice. The tumor tissue was minced and suspended in RPMI-1640 medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 20% fetal bovine serum (FBS). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 4 to 5 days. Upon reaching the confluent state, the monolayers were treated with trypsin and the dispersed cells were transferred into new culture flasks. Cells were acclimated to growth as semi-adherent cells in 10% FBS/RPMI-1640, 1% penicillin/streptomycin, 1% L-glutamine. CHLA-02-ATRT, CHLA-04-ATRT, CHLA-05-ATRT and

CHLA-06-ATRT cell lines were generated from pediatric AT/RT tumor samples obtained at Children's Hospital of Los Angeles (Los Angeles, CA). Tumor tissue was prepared within 30 to 60 minutes as described (34). Cells were initially cultured as neurospheres in modified Neurobasal medium consisting of 1:1 DMEM:F12 containing 15 mM HEPES, 110 mg/L sodium pyruvate, 1.2 g/L sodium bicarbonate, B27 supplement (Gibco, Grand Island, NY), 20 ng/mL epidermal growth factor (Peprotech, Inc., Rocky Hill, NJ), 20 ng/mL basic fibroblast growth factor (Peprotech) and 25 μg/ml gentamicin (Gibco). Gentamicin was removed after the first 2 weeks of culture. Passaging was at ratio of 1:2–3 with 25% (v/v) conditioned medium in the new flask. CHLA-05-AT/RT and CHLA-06-AT/RT were originally described in (35). Details of the cell lines are described in Supplementary Table 1. All the AT/RT cell lines were authenticated using short tandem repeat profiling using StemElite kit (Promega, Madison, WI), at the Genetic Resources Core Facility in The Johns Hopkins University. Eight short tandem repeat loci along with a gender-determining marker, Amelogenin, were used to authenticate the BT37 cell line (Supplementary Table 2). CHLA-02 and CHLA-04 are available from American Type Culture Collection ([ATCC] Manassas, VA). BT-12 is available from the Children's Oncology Group cell line and xenograft repository (36, 37). All of the AT/RT cell lines used in this manuscript were authenticated to be human and did not match the short tandem repeat profile of any other cell line in the established databases (ATCC, DSMZ, and JCRB). 293T cells were used for the production of lentiviral particles and were cultured in DMEM containing 10% FBS and were obtained through ATCC. SF188 cell line was used as a positive control for the expression of INI1 and was cultured in DMEM/F12 supplemented with 10% FBS (38).

Lentiviral Knockdown of HMGA2

Two lentiviral MISSION TRC (Human) shRNA constructs were used to reduce HMGA2 mRNA expression: TRCN0000342671 (targets the coding region, denoted as shHMGA2#2) and TRCN0000342673 (targets the 3'-UTR, denoted as shHMGA2#1). MISSION® pLKO. 1-puro Empty Vector Control was used as control shRNA (denoted as shCTL) in all experiments. For producing lentiviruses, 293T cells were transfected with 0.3 μg, 2.5 μg and 3 μg of VSVG envelope plasmid, delta 8.9 gag/pol plasmid and the lentiviral plasmid of interest, respectively, using Fugene 6 (Promega). Viral supernatants were pooled after 48, 72, 96 and 120 hours post-transfection and filtered through a 0.45-μm filter. Virus was concentrated using 5% PEG-8000 and 0.15M NaCl and resuspended in 500 μl of DMEM. Small aliquots of concentrated virus were stored at −80°C until further use. For knocking down HMGA2, concentrated virus was added to confluent cultures of BT37 and CHLA-06 cells. After 48 hours, transduced cells were selected using puromycin (1–2 μg/ml, Sigma-Aldrich, St. Louis, MO) for 5 days. Reduced expression of HMGA2 in puromycin-selected cells was confirmed using immunoblotting. All experiments were performed within 14 days of infecting AT/RT cells with shRNA lentivirus.

Immunoblotting

Cells were harvested and washed in ice-cold 1X phosphate-buffered saline (Life Technologies, Grand Island, NY). Cell pellets were lysed, separated on polyacrylamide gels and immunoblotted, as described (39). INI1, HMGA2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and cleaved poly (ADP-ribose) polymerase (PARP)

expression were analyzed using the antibodies to the following: INI1 (Sigma-Aldrich), HMGA2 (Cell Signaling Technology, Inc., Beverly, MA), GAPDH (Research Diagnostics, Inc., Flanders, NJ), β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX), and cleaved PARP (Cell Signaling Technology, Inc.). Primary antibodies were detected using goat anti-mouse and goat anti-rabbit antibodies conjugated to horseradish peroxidase (KPL, Gaithersburg, MD). Secondary antibodies were detected using Western Lightning Plus ECL (PerkinElmer, Waltham, MA). Densitometry analysis was performed using Image J software (40).

Cell Growth and Proliferation Assays

For assessing differences in cell growth, 2500 transduced single cells were plated in triplicate in 96-well plates. Cell growth was measured on day 1 (day of plating cells), day 3 and day 5 using the colorimetric CellTiter 96®AQueous One Solution Assay (MTS, Promega). For measuring cell proliferation, transduced cells were pulsed with bromodeoxyuridine (BrdU) (Sigma-Aldrich) for 6 hours and then processed for immunofluorescence as described (39).

Apoptosis Assays

For measuring apoptosis, transduced and selected cells were cytospun onto positively charged slides and processed for immunofluorescence for detecting cleaved caspase-3. Immunoblotting was performed to detect cleaved PARP as described above.

Immunofluorescence

Immunofluorescence was performed as described previously (39) with primary antibodies to the following: BrdU (Sigma-Aldrich) and cleaved caspase-3 (Cell Signaling Technology).

Colony Formation Assay

Infected and selected cells were plated in soft agarose to form colonies, as previously described (39) with the following modifications: Media were changed every 2 days and the cultures were incubated for 2 weeks.

Intracranial Tumor Xenografts

Freshly infected BT37 cells expressing either control or HMGA2 shRNA were selected under puromycin for 5 days, and after knockdown was verified by immunoblotting, 100,000 viable cells were injected into the right striatum of nude mice, as previously described (39). Mice were monitored daily and were killed when they showed signs of tumor formation (neurologic impairment, weight loss, hunching) in accordance with IACUC accepted protocols.

Immunohistology

Brains were removed and placed into 10% formalin for 48 hours, after which they were embedded in paraffin and sectioned by the Johns Hopkins histology core facility.

Survival Analysis and Statistical Methods

Kaplan-Meier survival graphs and log-rank tests were generated for the in vivo experiments using GraphPad Prism (GraphPad software, Inc., La Jolla, CA). Statistical tests for all other experiments were performed using Excel (Microsoft). Results are shown for each experiment performed independently at least twice with concordant results. For in vivo experiments, data were analyzed from at least 4 animals injected per condition. Error bars denote SE of experiments performed independently at least twice. Significance of the experimental results was determined by an unpaired, 2-sided Student t-test, where $p < 0.05$ was considered to be significant.

RESULTS

The BT37 Cell Line Forms Aggressive Orthotopic Xenograft Tumors Pathologically and Clinically Similar to AT/RT

We transitioned the BT37 AT/RT serially passaged human xenograft tumor to a rapidly growing, semi-adherent cell line. Injection of BT37 into the brains of immunodeficient mice led to aggressive xenograft tumor formation, with most mice succumbing within 60 days of injection. Examination of orthotopic xenografts revealed the presence of multiple mitotic figures, apoptosis, and the presence of classic rhabdoid tumor cells (Fig. 1A, B).

AT/RT tumors are characterized by chromosomal mutations or deletions in the *hSNF5/INI1* gene leading to loss of INI1 protein expression (41, 42). BT37, along with other AT/RT cell lines, did not express the INI1 protein, as shown by immunoblotting (Fig. 1C).

AT/RT Cell Lines Express HMGA2

When we tested the expression of HMGA2 protein in our AT/RT cell lines by immunoblotting, we found that 5/6 AT/RT cell lines express HMGA2 (Fig. 1D) protein. To investigate the role of HMGA2 in AT/RT directly, we used shRNA to suppress HMGA2 expression. CHLA-06 and BT37 cell lines were infected with lentivirus encoding either control or HMGA2 shRNA. Infected cells were selected with puromycin for 5 days and tested for HMGA2 expression by immunoblotting (Fig. 1E). Densitometry analysis of immunoblots depicted an average reduction of 59% and 52% for shHMGA2#1 and #2 in CHLA06 and 77% and 59% for shHMGA2#1 and #2 in BT37, compared to control shRNA.

Knockdown of HMGA2 Reduces Growth and Proliferation of AT/RT Cells

AT/RT is a rapidly growing tumor of the CNS (1). Because expression of HMGA2 is associated with increased growth and proliferation (17, 18, 29, 32), we hypothesized that HMGA2 promotes the growth and proliferation of AT/RT cells. We found that shRNA knockdown of HMGA2 reduced growth of CHLA-06 and BT37 cells compared to control shRNA expressing cells (Fig. 2A, $p < 0.05$ for shHMGA2#1 for both cell lines, with concordant but not statistically significant results using shHMGA2#2). To investigate the mechanism of growth inhibition, we performed BrdU incorporation assay to determine if HMGA2 suppression would inhibit AT/RT proliferation. Reduction of HMGA2 significantly inhibited proliferation of CHLA-06 and BT37 cells compared to control

shRNA transduced cells, as measured by BrdU uptake (Fig. 2B, C, $p < 0.05$ for shHMGA2#1 and #2).

Suppression of HMGA2 Increases Apoptosis of AT/RT Cells

Another potential mechanism by which HMGA2 loss could affect AT/RT growth is by inducing apoptosis. HMGA2 is known to prevent apoptosis in multiple tumor types (43–45). During apoptosis, caspase-3 enzyme is activated due to proteolytic cleavage (46). Cleaved caspase-3 (CC3) then cleaves and activates PARP (47). CC3 and cleaved PARP are well-accepted markers to denote cells undergoing apoptosis. We determined the induction of CC3 by immunofluorescence in CHLA-06 and BT37 cells after lentiviral transduction with either control or HMGA2 shRNA. Knockdown of HMGA2 in CHLA-06 cells increased CC3 immunopositivity by an average of 2.6- and 2.2-fold for shHMGA2#1 and #2, respectively compared to control shRNA (Fig. 3A, B, $p < 0.05$ for shHMGA2#1 and #2). Knockdown of HMGA2 in BT37 led to a 9.1- and 9.8-fold average increase in CC3 immunopositivity for shHMGA2#1 and #2, respectively compared to control shRNA (Fig. 3A, B, $p < 0.05$ for shHMGA#1 and #2). Similarly, there was an increase in cleaved PARP expression in HMGA2 shRNA-transduced cells compared to control cells as assessed by immunoblotting (Fig. 3C). These results suggest that HMGA2 prevents apoptosis to promote growth of AT/RT cells in vitro.

HMGA2 Suppression Prevents Clonogenic Growth of AT/RT Cells

We plated single cells of CHLA-06 expressing either control or HMGA2 shRNA in soft agarose (Fig. 4A, B). After 2 weeks, cells expressing control shRNA formed an average of 82 ± 4 colonies. In comparison, cells expressing HMGA2 shRNA formed only 40 ± 4 (for shHMGA2#1, $p < 0.01$) and 62 ± 5 (for shHMGA2#2, $p < 0.05$) colonies (Fig. 4A, B).

Knockdown of HMGA2 Decreases Tumor Formation In Vivo

To explore whether HMGA2 is required for tumor formation in an in vivo, orthotopic environment, we injected BT37 cells expressing either control or HMGA2 shRNA into the right striatum of immunodeficient mice. Animals were monitored regularly for signs of an increasing tumor mass and sacrificed upon reaching a moribund state. Log rank analysis of Kaplan Meier survival curves showed that the cohort of mice receiving control cells ($n = 4$) survived for a median of 58 days whereas mice injected with HMGA2 deficient cells ($n = 8$) lived longer (median survival = 153 days, $p = 0.0004$) (Fig. 4C). These results indicate that knockdown of HMGA2 significantly increased the latency period of tumor formation and led to increased survival of orthotopically xenografted mice, signifying the importance of HMGA2 for AT/RT tumor growth in the brain environment. Histologic examination of tumors showed no clear differences between tumors that formed from HMGA2 deficient BT37 cells compared to control BT37 cells.

DISCUSSION

The hallmark alteration in AT/RT is the loss of the INI1 chromatin modifying protein, which may function to promote differentiation (5, 6). Maintenance of an undifferentiated state and persistence of factors that promote self-renewal, invasion, and proliferation likely contribute

to AT/RT tumorigenicity. HMGA2 is a regulator of normal neural stem cell renewal (30). Our findings that inhibition of HMGA2 suppresses AT/RT cell growth are concordant with those showing that HMGA2 loss blocks proliferation of malignant rhabdoid tumor cell lines (11). The suppression of AT/RT orthotopic xenograft formation after HMGA2 inhibition suggests that targeting HMGA2 directly may be a useful therapeutic modality in AT/RT and malignant rhabdoid tumor.

HMGA2 is a chromatin-modifying protein that binds to the minor grove of DNA at multiple sites in the genome (12, 14, 48–51). The exact mechanism by which HMGA2 promotes self-renewal, proliferation and invasion in multiple tumor types remains largely unknown. The wide range of promoters and enhancers that HMGA2 can bind to makes it an attractive driver of AT/RT tumorigenesis, because similar to INI1, HMGA2 can influence thousands of genes in the genome. In addition to binding directly to enhancers and activating or suppressing transcription, HMGA2 is also known to bind to and stabilize RNA molecules (24). Continued expression of HMGA2 in addition to INI1 may allow AT/RT tumor cells to regulate thousands of tumorigenic genes and provides an attractive mechanism to explain the aggressive nature of AT/RT in the absence of multiple recurrent activating genetic mutations.

Netropsin and other drugs that bind in the minor groove of DNA, including Hoechst 33258, inhibit the binding of AT-hook containing proteins to their consensus sites (52–55). Netropsin can suppress the growth of HMGA1 expressing medulloblastoma cells (56). The data presented here show that targeting HMGA2 may be therapeutically beneficial in AT/RT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Sabeen Zulfiqar Ali for technical assistance with immunofluorescence experiments and Dr. Peter Houghton for kind gift of the BT37 AT/RT xenograft.

Funding: Alex’s Lemonade Stand Foundation grant to Eric H. Raabe; National Cancer Institute Core Grant (P30 CA006973) to the Johns Hopkins SKCCC (CA91048) to Bernard E. Weissman; CHLA AT/RT cell lines were generated with generous support from Grayson's Gift and the Michael Hoefflin Foundation; Eric H. Raabe is a St. Baldrick’s Scholar.

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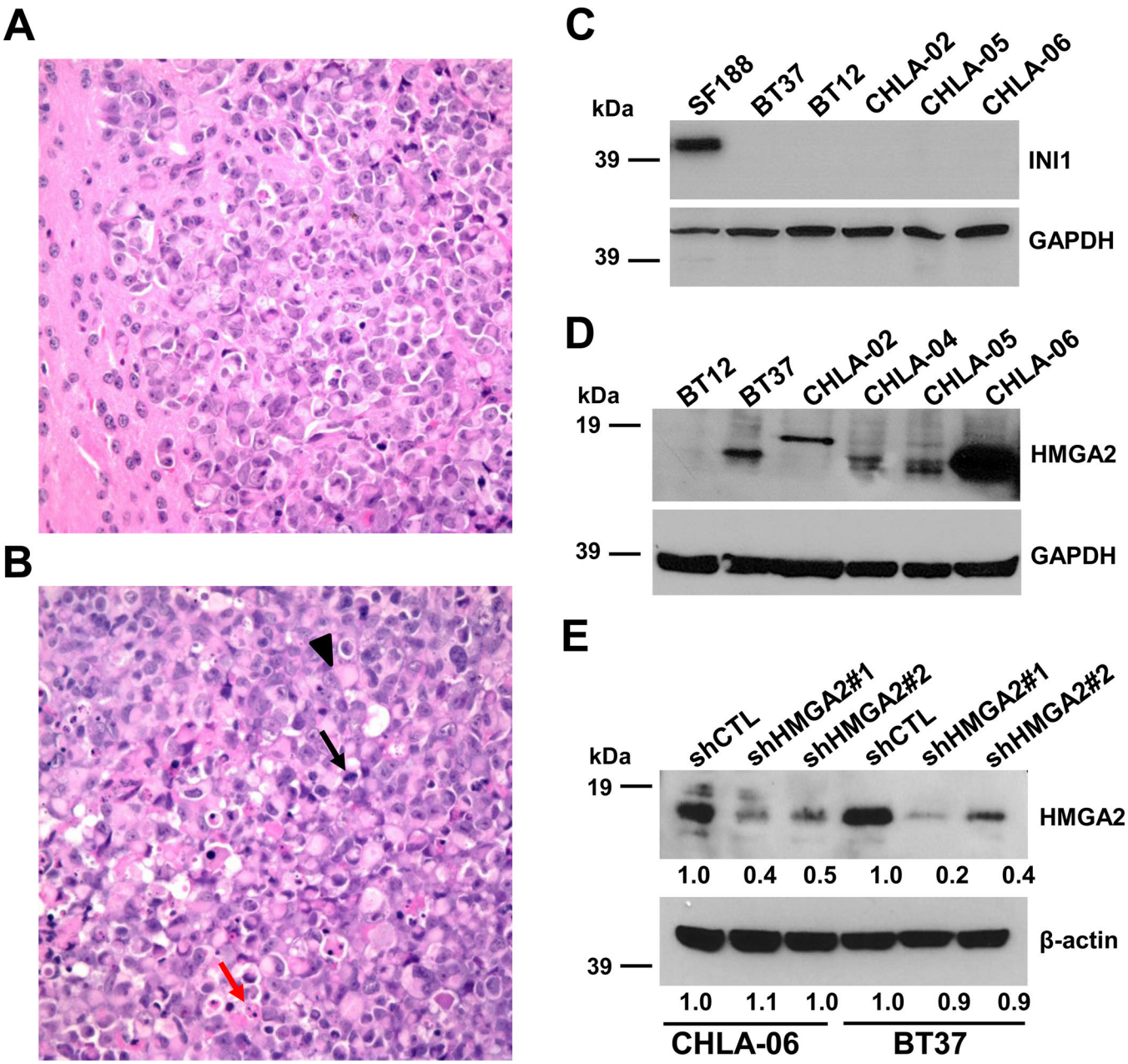

Figure 1.

The BT37 cell line formed aggressive orthotopic xenograft tumors with an atypical teratoid/rhabdoid tumor (AT/RT) phenotype. (**A**) 200× magnification showing BT37 xenograft (right) in a normal brain (left). (**B**) 200× view showing high-grade features such as multiple mitotic figures (black arrow), apoptotic bodies (red arrow) and “rhabdoid” cells, which are classic for AT/RT (black arrowhead). (**C**) Immunoblot for INI1 shows retained INI1 expression in the pediatric glioblastoma cell line SF188 and loss of INI1 in AT/RT cell lines. Immunoblots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Molecular weights are shown at left in kDa. (**D**) Immunoblot of AT/RT cell lines BT12, BT37, CHLA-02, CHLA-04, CHLA-05 and

CHLA-06 showing expression of high mobility group A2 protein (HMGA2). Immunoblots were stripped and reprobed for GAPDH as a loading control. (**E**) Immunoblot showing knockdown of HMGA2 by 2 different lentiviral short hairpin RNAs in BT37 and CHLA06 AT/RT cell lines. Control lentiviral short hairpin RNA (shCTL) or HMGA2 shRNA (shHMGA2#1 and shHMGA2#2) were analyzed for HMGA2 expression by immunoblotting; β-actin was used as a loading control. HMGA2 and β-actin protein levels were quantitated on the immunoblot using densitometry and values normalized to control (shCTL) for each cell line.

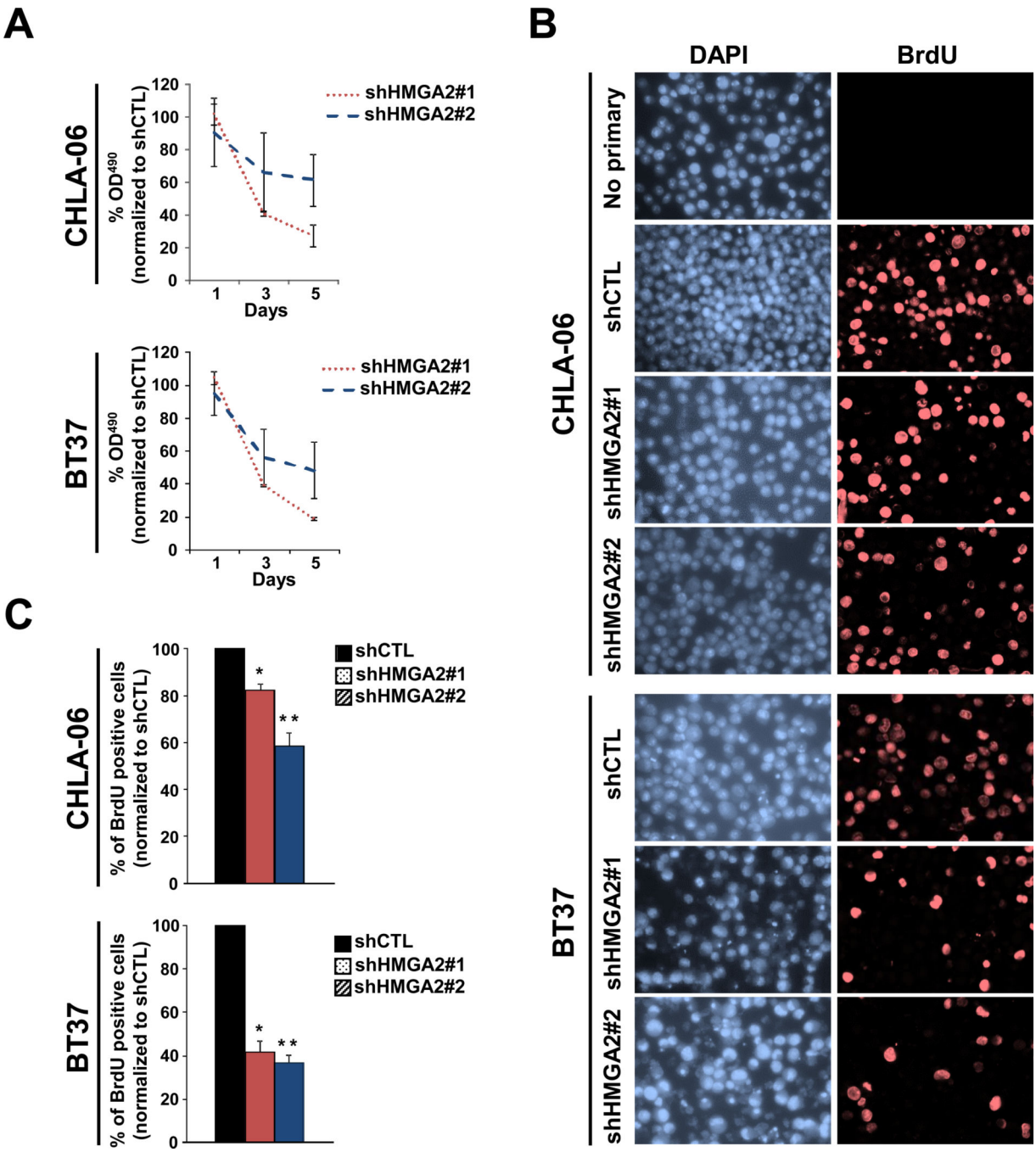

Figure 2.

Knockdown of high mobility group A2 protein (HMGA2) reduced growth and proliferation of atypical teratoid/rhabdoid tumor (AT/RT) cells in vitro. We infected AT/RT cell lines with lentiviral pLKO empty vector or HMGA2 targeting lentiviral short hairpin RNA (shRNA), selected cells with puromycin for 5 days, verified knockdown of HMGA2 by western blot, and performed assays of growth and proliferation. Control shRNA and HMGA2 shRNA are depicted as shCTL and shHMGA2 (#1 or #2), respectively. (**A**) HMGA2 knockdown inhibited growth of AT/RT cells. CHLA-06 and BT37 cells expressing

HMGA2 shRNA showed reduced growth at day 3 and 5 compared to control shRNA by MTS assay ($p < 0.05$ for shHMGA2#1 compared to shCTL, $n = 2$ for CHLA-06, $n = 3$ for BT37). (**B**) HMGA2 knockdown reduced proliferation of AT/RT cells. Representative photomicrographs (400x magnification) of HMGA2 shRNA expressing CHLA-06 and BT37 cells depicting reduced bromodeoxyuridine (BrdU) incorporation compared to control shRNA by immunofluorescence. DAPI was used to stain all nuclei. (**C**) Quantitation of BrdU incorporation. The percentage of BrdU positive nuclei in shHMGA2 compared to shCTL is depicted (* and **, $p < 0.05$ for shHMGA#1 and #2, compared to shCTL, for both CHLA-06 [$n = 2$] and BT37 [$n = 3$]).

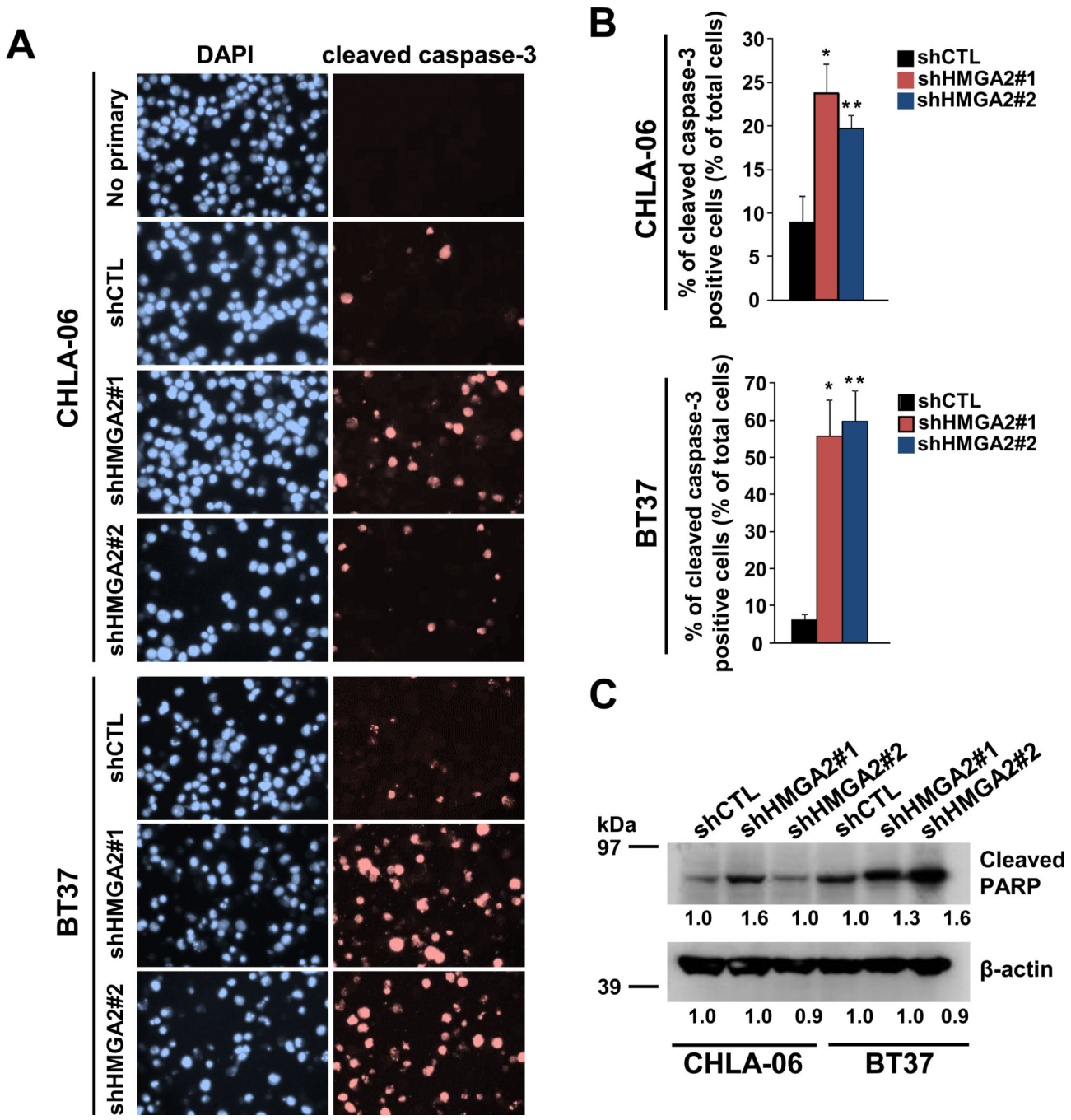

Figure 3.

Knockdown of high mobility group A2 protein (HMGA2) increased apoptosis of atypical teratoid/rhabdoid tumor (AT/RT) cells. (**A**) HMGA2 knockdown increased cleaved caspase-3. Representative photomicrographs (400× magnification) showing increased cleaved caspase-3 staining in CHLA-06 and BT37 cells infected with HMGA2 lentiviral short hairpin RNA (shRNA) compared to control shRNA by immunofluorescence. (**B**) Quantitation of cleaved caspase-3 immunopositivity after HMGA2 knockdown. The percentage of cells positive for cleaved caspase-3 out of the total number of cells (DAPI was

used to stain all nuclei) are shown (* and **, $p < 0.05$ for shHMGA#1 and #2, compared to shCTL, for both CHLA-06 [n = 3] and BT37 [n = 3]). (**C**) Knockdown of HMGA2 increased cleaved poly (ADP-ribose) polymerase (PARP) expression. CHLA-06 and BT37 cells infected with either control or HMGA2 shRNA were analyzed for cleaved PARP protein expression by immunoblotting. β-actin was used as a loading control. Cleaved PARP and β-actin protein levels were quantitated on the immunoblot using densitometry and values normalized to control shRNA (shCTL) for each cell line are depicted.

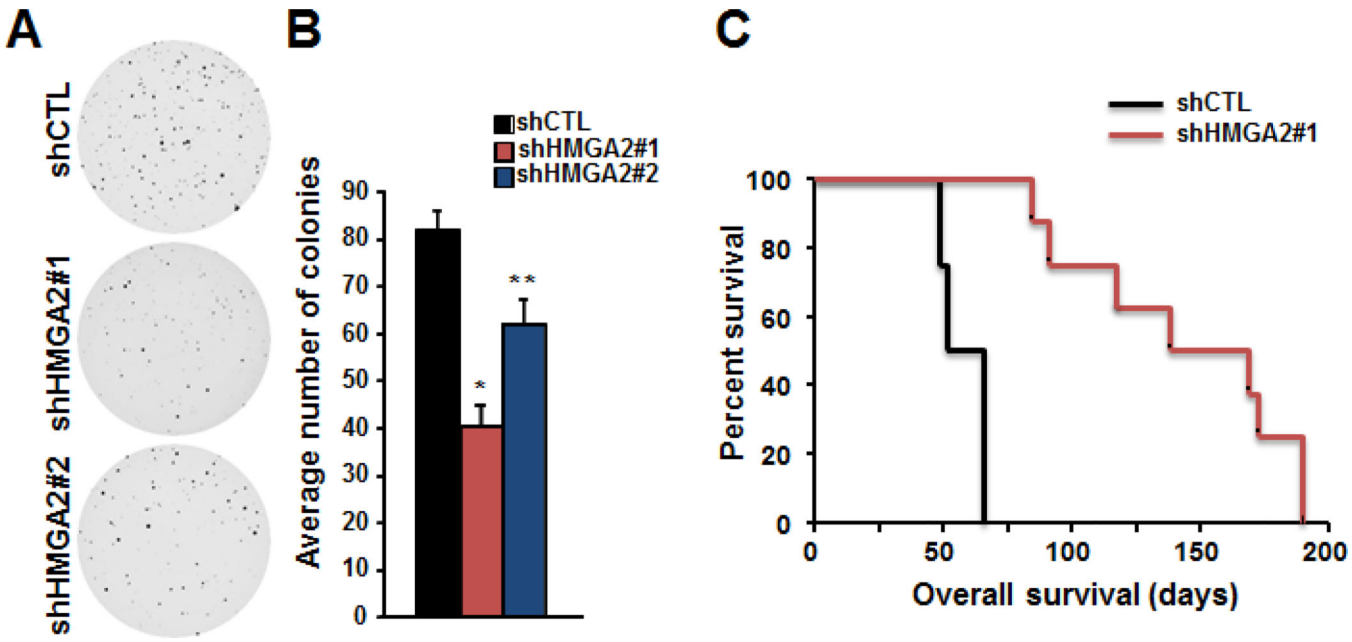

Figure 4.

High mobility group A2 protein (HMGA2) knockdown reduces colony formation and tumorigenicity of atypical teratoid/rhabdoid tumor (AT/RT) cells. (**A**) Representative photographs of nitroblue tetrazolium-stained colonies formed by CHLA-06 cells expressing control lentiviral short hairpin RNA (shRNA) or HMGA2 shRNA. (**B**) Quantitation of number of colonies depicted in (A), showing reduced colony formation in CHLA-06 cells infected with HMGA2 shRNA (#1 and #2) vs. control shRNA (*$p < 0.01$ for shHMGA#1 [$n = 3$] and **$p < 0.05$ for shHMGA2#2 [$n = 2$]). (**C**) Knockdown of HMGA2 increased survival. Kaplan-Meier survival curves showing increased survival of mice that were injected intrastriatally with HMGA2 shRNA infected BT37 cells compared to control infected cells ($p < 0.0004$, Log-rank test). Data were collected from at least 4 animals per condition ($n = 4$ for shCTL and $n = 8$ for shHMGA2#1).