5-Azacytidine inhibits human rhabdomyosarcoma cell growth by downregulating insulin-like growth factor 2 expression and reactivating the *H19* gene product miR-675, which negatively affects insulin-like growth factors and insulin signaling

MACIEJ TARNOWSKI[1], MARTA TKACZ[1], MICHAŁ CZEREWATY[1], AGATA PONIEWIERSKA-BARAN[1], KATARZYNA GRYMUŁA[1] and MARIUSZ Z. RATAJCZAK[1,2,3]

[1]Department of Physiology Pomeranian Medical University, Szczecin, Poland; [2]Stem Cell Biology Program at the James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA; [3]Department of Regenerative Medicine Medical University, Warsaw, Poland

Received December 12, 2014; Accepted December 29, 2014

DOI: 10.3892/ijo.2015.2906

Abstract. Insulin-like growth factor 2 (IGF2) and 1 (IGF1) and insulin (INS) promote proliferation of rhabdomyosarcoma (RMS) cells by interacting with the insulin-like growth factor 1 receptor (IGF1R) and the insulin receptor (INSR). Loss of imprinting (LOI) by DNA hypermethylation at the differentially methylated region (DMR) for the IGF2-H19 locus is commonly observed in RMS cells and results in an increase in the expression of proliferation-promoting IGF2 and downregulation of proliferation-inhibiting non-coding H19 miRNAs. One of these miRNAs, miR-675, has been reported in murine cells to be a negative regulator of IGF1R expression. To better address the role of IGF2 and 1, as well as INS signaling in the pathogenesis of RMS and the involvement of LOI at the IGF2-H19 locus, we employed the DNA demethylating agent 5-azacytidine (AzaC). We observed that AzaC-mediated demethylation of the DMR at the IGF2-H19 locus resulted in downregulation of IGF2 and an increase in the expression of H19. This epigenetic change resulted in a decrease in RMS proliferation due to downregulation of IGF2 and, IGF1R expression in an miR-675-dependent manner. Interestingly, we observed that miR-675 not only inhibited the expression of IGF1R in a similar manner in human and murine cells, but we also observed its negative effect on the expression of the INSR. These results confirm the crucial role of LOI at the IGF2-H19 DMR in the pathogenesis of RMS and are relevant to the development of new treatment strategies.

Correspondence to: Professor Mariusz Z. Ratajczak, Stem Cell Biology Program at the James Graham Brown Cancer Center, University of Louisville, 500 S. Floyd Street, Rm. 107, Louisville, KY 40202, USA
E-mail: mzrata01@louisville.edu

Dr Maciej Tarnowski, Department of Physiology Pomeranian Medical University, al. Powstańców Wielkopolskich 72, 70-111 Szczecin, Poland
E-mail: maciejt@pum.edu.pl

Key words: rhabdomyosarcoma, IGF2, H19, miR-675, AzaC

Introduction

Rhabdomyosarcoma (RMS) is a tumor that derives from early skeletal muscle cells and displays characteristics of muscle differentiation (1-6). There are two major histologic subtypes of RMS: alveolar (A)RMS and embryonal (E)RMS (7). Clinical evidence indicates that ARMS is more aggressive and has a significantly worse outcome than ERMS (8). It is well known that RMS cells, particularly ARMS cells, can infiltrate the bone marrow (BM) and, because they can resemble hematologic blasts, are sometimes misdiagnosed as acute leukemia blasts.

Genetic characterization of RMS has identified markers that show a correlation with histologic subtype. Specifically, ARMS is characterized by the translocation t(2;13)(q35;q14) in 70% of cases or the variant t(1;13)(p36;q14) in a smaller percentage of cases (9). These translocations disrupt the paired box 3 (*PAX3*) and 7 (*PAX7*) genes on chromosome 2 and 1, respectively, and the forkhead (*FKHR*) gene on chromosome 13, which leads to generation of *PAX3-FKHR* and *PAX7-FKHR* fusion genes in RMS (10-13). These fusion genes encode the fusion proteins PAX3-FKHR and PAX7-FKHR, which are involved in cell survival and dysregulation of the cell cycle in ARMS cells (14).

Moreover, both ARMS and ERMS are characterized by important epigenetic mutations and an epigenetic imbalance that may affect some important genes. One of the most common epigenetic changes is loss of imprinting (LOI) at chromosome locus 11p15.5, which alters the expression of the tandem gene *IGF2-H19* (15-18). Encoded by the insulin-like growth factor 2 (*IGF2*) gene, IGF2 is a growth factor essential for normal embryonic and neonatal growth and development, while *H19* gives rise to a non-coding mRNA that has the opposite effect on cell proliferation.

IGF2 signals through the tyrosine kinase insulin-like growth factor 1 receptor (IGF1R), which is frequently overexpressed in human RMS (19-25) and a number of other cancers (25). The

function of *H19*-derived non-coding RNA (ncRNA) is largely unknown and still under debate. *H19* is maternally expressed and may act either as a tumor suppressor or as an oncogene, depending on cancer type (26,27). It spans >2 kb and encodes short non-coding microRNAs, including miR-675, which was recently shown to be a negative regulator of IGF1R expression in mice (28). LOI in RMS cells due to hypermethylation of a differentially methylated region (DMR) that controls IGF2-H19 expression leads to formation of a potent autocrine loop (IGF2-IGF1R) and, at the same time, decreases the level of growth-suppressing *H19*-derived miRNAs.

The methylation of genomic DNA in cells is catalyzed by DNA methyltransferases (DNMTs). Recent studies have shown that application of methyltransferase inhibitors, such as 5-azacitidine (AzaC), may become a clinically relevant strategy for tumor growth inhibition (29,30). DNMT inhibitors, which are usually analogues of the cytidine nucleoside, bind to DNA methyltransferase 1, which results in DNA hypomethylation. As a result of DNA hypomethylation, some silenced tumor-suppressor genes like *H19* may become activated.

In this study we evaluated the effect of the DNMT inhibitor AzaC on the tumorigenic potential of RMS cells. We found that DNA hypomethylation led to inhibition of RMS growth. This inhibition correlated with reactivation of *H19* and upregulation of miR-675, which, as expected, negatively affected the expression of not only IGF1R (28) but also the insulin receptor (INSR), which we show for the first time. Thus, the downregulation of both receptors inhibits signaling of IGF2, insulin-like growth factor 1 (IGF1), and insulin (INS), which are all potent growth factors in RMS cells.

Materials and methods

Cell lines. We used human RMS cell lines (a gift of Dr Peter Houghton, World Children's Research Hospital, Columbus, OH, USA), including the RH30 ARMS cell line and the RD ERMS cell line. RMS cells used for experiments were cultured in Roswell Park Memorial Institute medium (RPMI)-1640 (Sigma, St. Louis, MO, USA), supplemented with 100 IU/ml penicillin, 10 μg/ml streptomycin, and 50 μg/ml neomycin (Life Technologies, Grand Island, NY, USA) in the presence of 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies). The cells were cultured in a humidified atmosphere at 37˚C in 5% CO_2 at an initial cell density of $2.5x10^4$ cells/flask (Corning, Cambridge, MA, USA), and the medium was changed every 48 h.

Flow cytometry analysis of receptor expression. The expression of IGF1R, and INSR in RMS cell lines was evaluated by flow cytometry analysis as previously described (31). The antigens were detected with anti-human/mouse INS R/CD220 conjugated with APC (R&D Systems, Minneapolis, MN, USA); and phycoerythrin (PE)-anti-IGF1R monoclonal antibody, clone no. 33255 (R&D Systems). Briefly, the cells were stained in phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free) supplemented with 2% bovine calf serum (BCS) (HyClone Laboratories, Inc., Logan, UT, USA). After the final wash, cells were resuspended in PBS and analyzed by FACS using the Navios Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Quantitative reverse transcription-PCR (RT-qPCR). Total RNA was isolated from cells treated with 5-azacitidine (AzaC), 2'-deoxy-5-azacytidine (DAC), or trichostatin A (TsA) (all from Sigma) and from cell controls with the RNeasy kit (Qiagen, Valencia, CA, USA). The RNA was reverse-transcribed with MultiScribe Reverse Transcriptase and oligo-dT primers (Applied Biosystems, Foster City, CA, USA). Quantitative assessment of mRNA levels was performed by RT-qPCR on an ABI 7500 instrument with Power SYBR-Green PCR Master Mix reagent. Real-time conditions were as follows: 95˚C (15 sec), 40 cycles at 95˚C (15 sec), and 60˚C (1 min). According to melting point analysis, only one PCR product was amplified under these conditions. The relative quantity of a target, normalized to the endogenous control β-2 microglobulin gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where Ct is the threshold cycle, ΔCt = (Ct of target genes) - (Ct of endogenous control gene, β-2 microglobulin), and $\Delta\Delta Ct$ = (ΔCt of samples for target gene) - (ΔCt of calibrator for the target gene). The following primer pairs were used: IGF2 forward, CCATGTCCT CCTCGCATCTC and reverse, CGTGGCAGAGCTGGTG AAG; H19 forward, GGCTCTGGAAGGTGAAGCTAGA and reverse, GCGGGCGCTGCTGTT.

Methylation studies using a combined bisulfite restriction analysis (COBRA) assay and bisulfite sequencing. DNA was isolated with the DNeasy Blood and Tissue kit (Qiagen), and exactly 100 ng of genomic DNA prepared from the indicated cells were used for bisulfite modification with the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. Bisulfite-treated DNA was subjected to nested PCR with methylation-specific primers for the human *H19-IGF2* DMR (32): 1st outer primer hH19O forward, AGG TGT TTT AGT TTT ATG GAT GAT GG and reverse, TCC TAT AAA TAT CCT ATT CCC AAA TAA CC; 2nd inner primer hH19I forward, TGT ATA GTA TAT GGG TAT TTT TGG AGG TTT and hH19-DMR-I reverse, same as hH19-DMR-O reverse. Next, the PCR products were cut with BstUI restriction enzyme and visualized on an agarose gel. In parallel after agarose gel electrophoresis, the PCR products were eluted using the QIAquick Gel Extraction kit (Qiagen). The eluted amplicons were subsequently ligated into the pCR2.1-TOPO vector and transformed into TOP10 bacteria using the TOPO TA Cloning kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The plasmids were prepared using the QIAprep Spin Miniprep kit (Qiagen), sequenced with M13 forward and reverse primers, and the methylation pattern visualized using CpGviewer software. All experiments were conducted with three independent isolations of all cell populations.

miR-675 transfection and H19 gene cloning. Full-length *H19* and Exon1 complementary DNA (GenBank: AF087017.1) was PCR-amplified and cloned into the pcDNA3.1 expression vector, and the vector was introduced into RH30 cells using Lipofectamine 2000 (both from Invitrogen Life Technologies). Cells were positively selected with G418 (200 μg/ml) for 2 weeks. The level of H19 overexpression was assessed by RT-qPCR, and the cells were subjected to proliferation assays. Enhanced expression of miR-675 was performed by transfecting RD and RH30 cells with miR-675-3p, -5p, or

both (Sigma). Cells were seeded into 24-well plates ($3x10^4$) and transfected with Lipofectamine 2000 (Invitrogen Life Technologies) and 40 μM microRNA for 24 h. In parallel, cells were transfected with MISSION miRNA Negative Control (Sigma). Next, cells were replated for proliferation assays or receptor expression analysis by flow cytometry.

Cell cycle analysis. The cells were incubated with or without 0.5, 1, 2, 5, 10, 20, or 50 μM AzaC (Sigma). After 96 h, the cells were collected, washed with PBS, centrifuged at 1,200 rpm for 8 min, and resuspended in 1 ml RPMI-1640 medium supplemented with 10% FBS at a concentration of 10^6 cells/ml. Then, 2 μl of Vybrant DyeCycle Orange Stain (Invitrogen Life Technologies) was added to the cells, which were gently vortexed. Samples were kept at 37˚C for 30 min in the dark and were analyzed using a flow cytometer (Navios, Beckman Coulter, Inc.).

Annexin V/propidium iodide (PI) assays for apoptosis. For Annexin V/PI assays, cells were stained with Annexin V-FITC and PI and evaluated for apoptosis by flow cytometry according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Briefly, $1x10^6$ cells were washed twice with PBS and stained with 5 μl of Annexin V-FITC and 10 μl of PI (5 μg/ml) in 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. The apoptotic cells were determined using a Navios flow cytometer (Beckman Coulter, Inc.). Annexin V^+ PI^- cells represented the early apoptotic populations, while Annexin V^+ PI^+ cells represented either late apoptotic or secondary necrotic populations.

Chemotaxis assay. Polycarbonate membranes (8-μm) were covered with 50 μl of 0.5% gelatin. The cells were detached with 0.5 mmol/l ethylenediaminetetraacetic acid (EDTA), washed in RPMI-1640, resuspended in RPMI-1640 with 0.5% bovine serum albumin (BSA), and seeded at a density of $3x10^4$ in 120 μl into the upper chambers of Transwell inserts (Corning). The lower chambers were filled with IGF1 (100 ng/ml), IGF2 (100 ng/ml), INS (10 ng/ml), or 0.5% BSA in RPMI-1640 (control). After 24 h, the inserts were removed from the Transwell supports. Cells remaining in the upper chambers were scraped off with cotton wool, and cells that had transmigrated were stained by HEMA 3 according to the manufacturer's instructions (Thermo Fisher Scientific, Pittsburgh, PA, USA) and counted either on the lower side of the membrane or on the bottom of the Transwell chamber.

Colony-formation assay. Cells were incubated with or without 1 or 5 μM AzaC for 72 h, collected, counted, mixed in 0.35% top agar (in RPMI-1640 medium supplemented with 10% FBS) and plated at 1,250 cells/well onto 24-well plates containing a solidified bottom layer (0.5% base agar in the same growth medium). Every 3 days, colonies were fed with 250 μl/well culture medium with 10% FBS. After 21 days, unstained colonies were counted.

Cell proliferation. Cells were plated in culture flasks at an initial density of 10^4 cells/cm^2 in the presence or absence of AzaC and miR-675-3p or -5p or both. The cell number was calculated at 24, 48, 72, and 96 h after culture initiation. At the indicated time points, the cells were harvested from the culture flasks by trypsinization, and the number of cells was determined using a Navios cytometer.

Phosphorylation of intracellular pathway proteins and western blotting. Western blotting was performed on extracts prepared from RMS cell lines ($2x10^6$ cells) that were kept in RPMI medium containing low levels of BSA (0.5%) to render the cells quiescent, as previously described (33). The cells were divided and stimulated with optimal doses of IGF1 (100 ng/ml), IGF2 (100 ng/ml), INS (10 ng/ml) for 5 min at 37˚C and then lysed (for 10 min) on ice in RIPA buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), containing protease and phosphatase inhibitors (Roche Diagnostics Corp., Indianapolis, IN, USA). Subsequently, the extracted proteins were separated by either 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the fractionated proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Phosphorylation of the intracellular kinases, p42/44 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) was detected using commercial mouse phospho-specific mAb (p42/44) polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Inc.). Equal loading in the lanes was evaluated with appropriate mAb: p42/44 anti-MAPK clone no. 9102 (Cell Signaling Technology, Inc.). The membranes were developed with an enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Little Chalfont, UK).

Statistical analysis. All results are presented as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using the non-parametric Mann-Whitney test or Student's t-test, with $p<0.05$ considered significant.

Results

Hypermethylation at the DMR for the IGF2-H19 tandem gene is erased after exposure of RMS cells to AzaC. Hypermethylation of the DMR at the IGF2-H19 locus (known as LOI) is an important epigenetic change occurring in ARMS and ERMS cells and leads to overexpression of IGF2 and downregulation of H19. As mentioned above, while IGF2 is an autocrine factor for RMS cells that stimulates proliferation of these cells after binding to the IGF1R and INSR, *H19*-derived ncRNA gives rise to several miRNAs that negatively regulate cell proliferation. One of these miRNAs, miR-675, downregulates IGF1R, which in turn binds IGF2 and IGF1 in murine placental cells (28).

To better address the LOI phenomenon at the DMR for the IGF2-H19 locus and the role of IGF2 and *H19*-derived miR-675 in the proliferation of RMS cells, we employed the DNMT inhibitor AzaC to reverse hypermethylation of the DMR at the IGF2-H19 locus (Fig. 1A and B). We found that exposure of the RH30 ARMS cell line and the RD ERMS cell line to 5 μM AzaC resulted in demethylation of the hypermethylated DMR for this tandem gene from ~76 to 25% and from ~80 to 29%, respectively.

The erasure of methylation at the DMR for the IGF2-H19 locus in response to increasing doses of AzaC resulted, as predicted, in upregulation of H19 and downregulation of IGF2

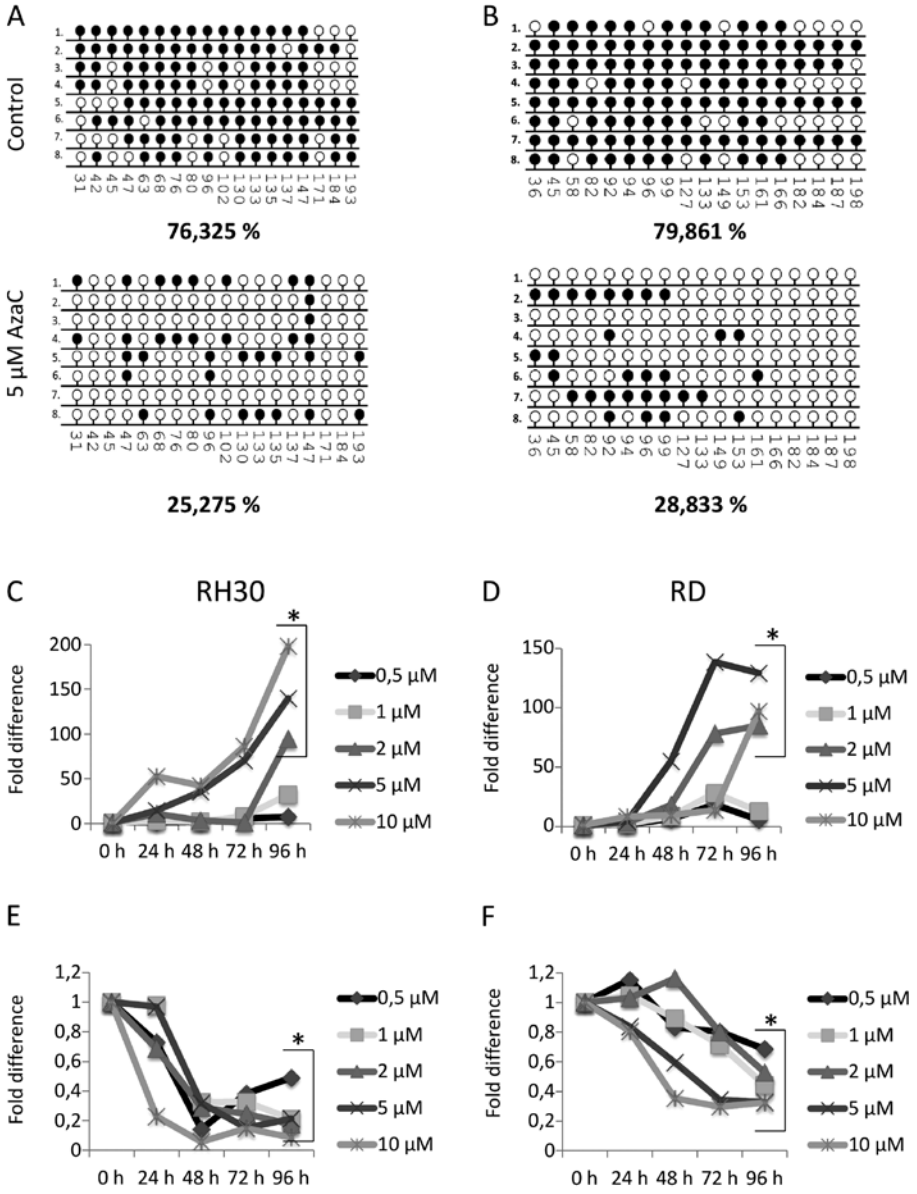


Figure 1. The effect of 5-azacytidine (AzaC) on the methylation state of the differentially methylated region (DMR) at the IGF2-H19 locus and expression of this tandem gene. After 72 h of AzaC treatment, the DMR for the IGF2-H19 locus became demethylated in (A) RH30 and (B) RD cells as evaluated by bisulfite modification of DNA followed by sequencing. (C-F) Time-lapse expression of H19 and insulin-like growth factor 2 (IGF2) mRNA by quantitative reverse transcription-PCR (RT-qPCR) in cells exposed to different doses of AzaC. Increase in expression of H19 mRNA and a decrease in expression of IGF2 mRNA was noted in (C and E) RH30 and (D and F) RD cells. *P<0.05.

expression at the mRNA level (Fig. 1C-F). Similar results were obtained by employing another DNMT inhibitor, decitabine (data not shown). At the same time, TsA, which is a potent deacetylating agent, did not change the IGF2/H19 mRNA ratio in RMS cells (data not shown).

Downregulation of IGF2 after AzaC treatment was subsequently confirmed at the protein level in RH30- and RD-derived conditioned media by employing a sensitive ELISA (data not shown).

Exposure to AzaC inhibits proliferation of RMS cells. Next, we performed *in vitro* assays to see whether AzaC, by downregulating IGF2 and upregulating H19, affects proliferation of RMS cells. First, to exclude a non-specific toxic effect of AzaC treatment on RMS cells, we evaluated the effect of increasing doses of AzaC on Annexin V binding in RH30 and RD cells (data not shown). By employing PI staining and Annexin V binding, we found that AzaC does not significantly affect cell survival at concentrations <10 μM.

Next, by employing an anchorage-dependent proliferation assay in plastic dishes (Fig. 2A and C) and an anchorage-independent colony-forming assay in soft agar (Fig. 2B and D), we observed an AzaC dose-dependent inhibition of RH30 (Fig. 2A and B) and RD (Fig. 2C and D) cell proliferation. The effect of AzaC on proliferation of RMS cells was subsequently evaluated by employing FACS-based time-lapse monitoring of the cell cycle (Fig. 3). We found that AzaC at a dose of 5 μM inhibits cell proliferation in G2/M phase and reduces the number of cells in G1 phase in both RH30 (Fig. 3A) and RD (Fig. 3B) cells.

We are aware that this effect is most likely the result of a combined increase in H19 expression as well as epigenetic

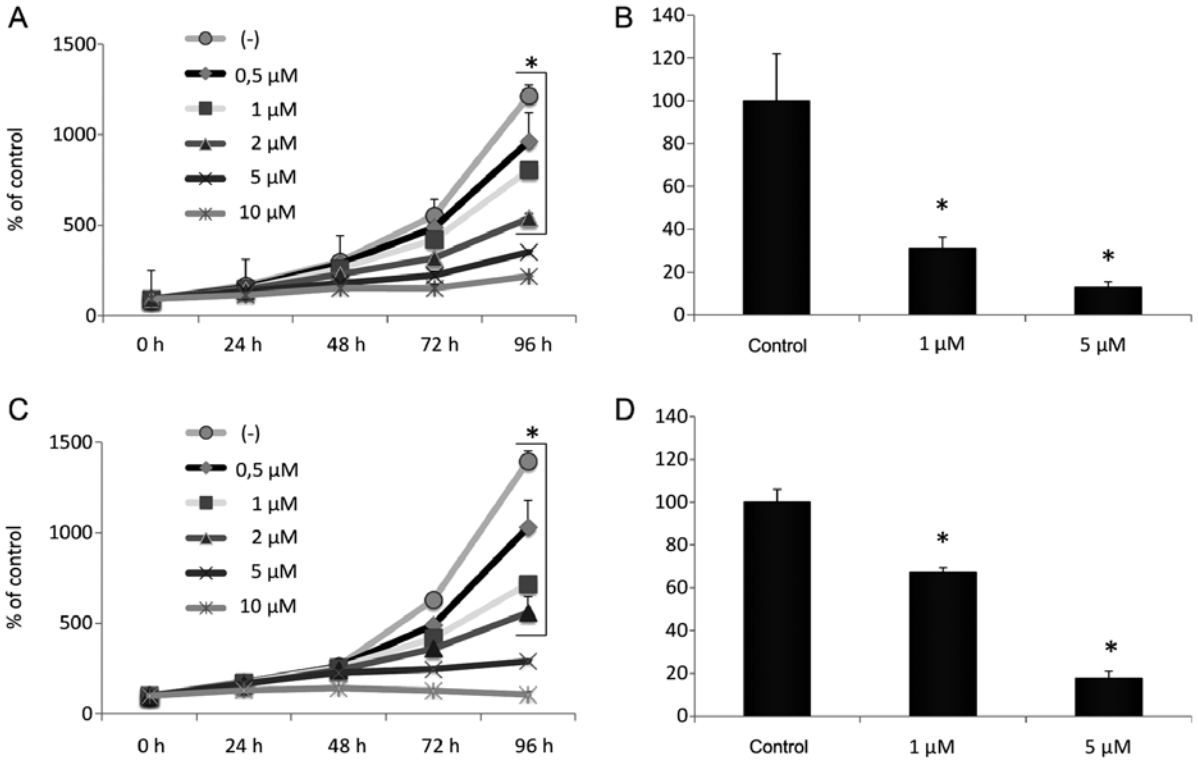
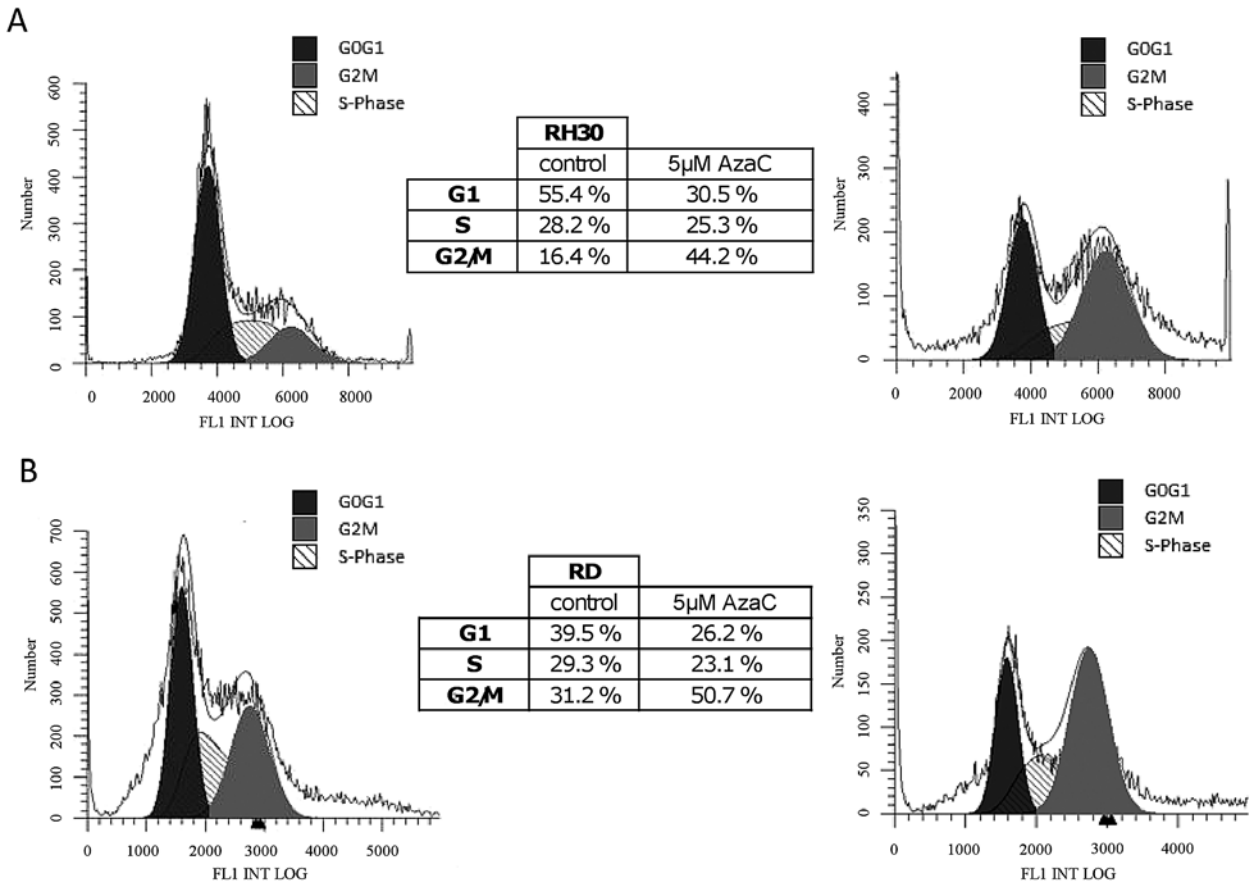


Figure 2. 5-Azacytidine (AzaC) affects anchorage-dependent and -independent cell proliferation. (A and B) RH30 and (C and D) RD cells were treated with increasing doses of AzaC (0-10 μM), and their proliferative capacity was measured in cultures in (A and C) anchorage-dependent plastic dishes and (B and D) anchorage-independent soft agar cultures. Combined data from three independent experiments are shown. *P<0.05.



	RH30	
	control	5µM AzaC
G1	55.4 %	30.5 %
S	28.2 %	25.3 %
G2/M	16.4 %	44.2 %

	RD	
	control	5µM AzaC
G1	39.5 %	26.2 %
S	29.3 %	23.1 %
G2/M	31.2 %	50.7 %

Figure 3. Cell cycle analysis of rhabdomyosarcoma (RMS) cells exposed to 5-azacytidine (AzaC). AzaC inhibits cell proliferation in G2/M phase and decreases the number of cells in G1 phase. Data were collected by flow cytometry and analyzed by ModFit software. (A) RH30 cells, (B) RD cells. A representative analysis out of three independent experiments is shown.

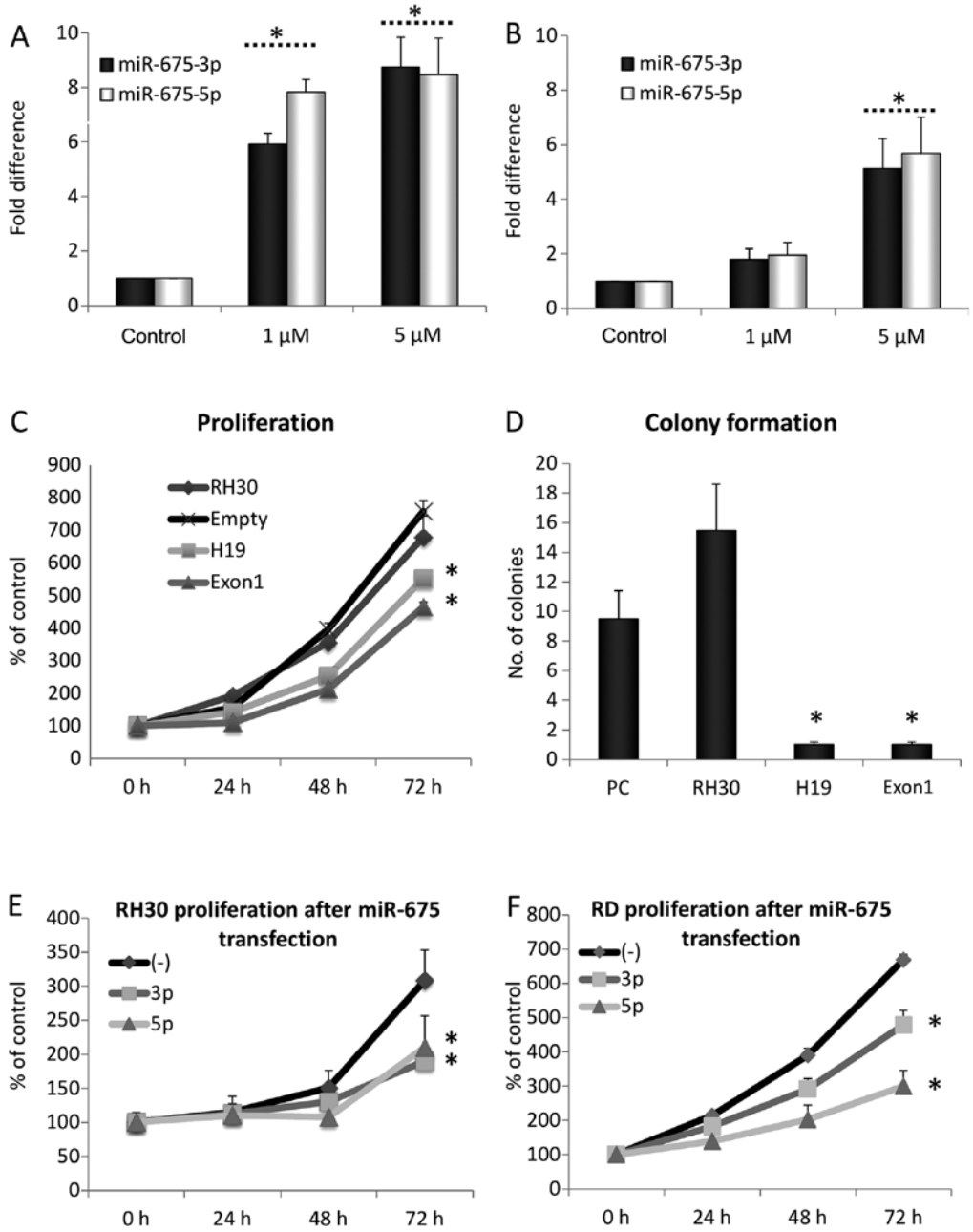


Figure 4. The *H19*-derived miR-675 effect on proliferation of rhabdomyosarcoma (RMS) cells. (A and B) Increase in *H19*-derived miR-675 expression in (A) RH30 and (B) RD cells treated by increasing doses of 5-azacytidine (AzaC). miR-675 expression was evaluated by quantitative reverse transcription-PCR (RT-qPCR), and U6RNA served as internal control. (C and D) Effect of *H19* and *H19* Exon1 cDNA cloned into the pcDNA3.1 vector on proliferation of RH30 cells in (C) plastic dishes and (D) a soft agar colony-forming assay. (E and F) Effect of *H19*-encoded miR-675 (-3p and -5p) on proliferation of (E) RH30 and (F) RD cells. All experiments were repeated three times. *P<0.05.

changes in other genes that regulate the cell cycle. At this point, however, we observed that AzaC treatment did not reactivate other epigenetically regulated suppressor genes, such as *p16*^{INK4A} and *p14*, and did not increase the expression of *p21*^{waf1} and *p53* (data not shown). Furthermore, despite the fact that IGF2 is downregulated in RMS cells after AzaC treatment, addition of this growth factor to RMS cells did not abrogate AzaC-mediated G2/M inhibition, which again indicates major involvement of *H19*-derived miR-675 in expression of the IGF2 signaling receptor, IGF1R.

The negative effect of H19 expression on RMS cell proliferation may be partially explained by miR-675-mediated downregulation of IGF1R expression. As mentioned above, *H19*-derived miR-675 downregulates the expression of IGF1R in murine placental cells (24). To address whether miR-675 is involved in regulation of IGF1R expression in human RMS cells, we measured the expression of two isoforms of this miRNA (miR-675-3p and -5p) in RH30 and RD cells, unexposed and exposed to AzaC (Fig. 4A and B). We observed an AzaC dose-dependent increase in both miR-675 isoforms in these cells.

Next, to shed more light on the role of *H19* and miR-675 on proliferation of RMS cells we cloned the entire *H19* gene and *H19* Exon1 (E1.1) into the pcDNA3.1 expression vector and transfected them into RH30 cells. We observed an inhibitory

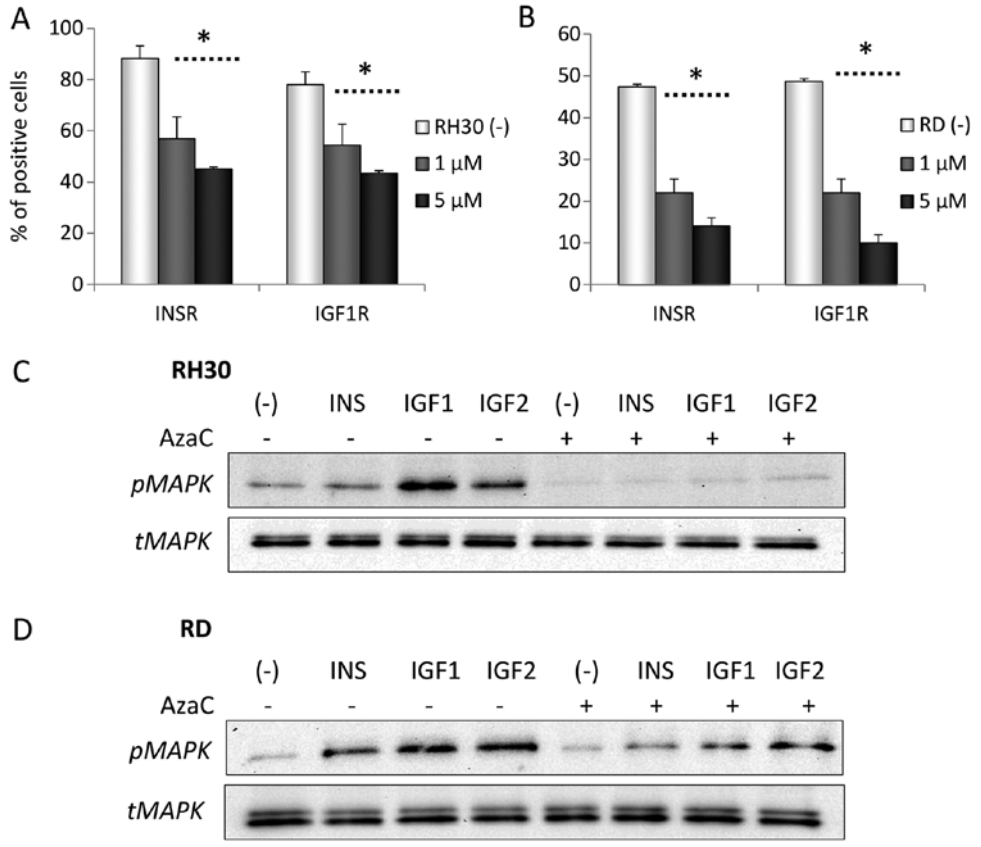


Figure 5. Effects of 5-azacytidine (AzaC) on the expression of the insulin receptor (INSR) and the insulin-like growth factor 1 receptor (IGF1R). (A) RH30 and (B) RD cells were exposed to increasing doses of AzaC, and after 48 h the receptor expression of the INSR and the IGF1R was assessed by flow cytometry. *P<0.05. (C and D) Effect of AzaC treatment on insulin (INS), insulin-like growth factor 1 (IGF1) and 2 (IGF2) signaling in rhabdomyosarcoma (RMS) cells. (C) RH30 and (D) RD cells, untreated or AzaC-treated for 72 h, were stimulated by INS (10 ng/ml), IGF1 (100 ng/ml), or IGF2 (100 ng/ml) for 5 min, and phosphorylation was assessed by western blotting. The experiment was repeated three times with similar results.

effect of *H19* and *H19* Exon1 (E1.1) ncRNA on proliferation of RMS cells, both in plastic dishes (Fig. 4C) and in a soft agar colony-forming assay (Fig. 4D). To better address the potential involvement of *H19*-encoded miR-675, we transfected RH30 and RD cells with miR-675-3p or -5p and observed the expected decrease in proliferation of RH30 and RD cells (Fig. 4E and F).

Effect of AzaC on the expression of the IGF1R and INSR. Our results described above demonstrate an inhibitory effect of AzaC on the proliferation of RMS cells due to upregulation of miR-675 and downregulation of IGF1R expression. However, it is known that, in addition to IGF2, IGF1 and INS also affect the proliferation of RMS cells (19-25). While IGF2 binds to IGF1R, both IGF1 and INS interact also with the INSR. Since we did not see a positive effect of IGF1 or INS as a replacement for IGF2 in AzaC-treated cells, we asked whether miR-675 also downregulates the INSR.

In order to clarify this issue, we applied a bioinformatic tool (rna22v1.0) to search for potential miR-675 targets in the INSR mRNA. As with IGF1R, we observed the presence of miR-675-3p and/or -5p sites in the 3'UTR and coding sequence of the *INSR* gene (data available upon request).

To confirm whether AzaC affects the expression of the INSR, we first evaluated the expression of the INSR in parallel with the expression of IGF1R in RH30 and RD cells by FACS analysis (Fig. 5A and B). We found that exposure of RMS cells to AzaC decreases the expression of IGF2-, IGF1-, and INS-binding receptors in a dose-dependent manner and, moreover, attenuates activation of MAPK p42/44 in RMS cells stimulated with INS, IGF1 and 2 and exposed to AzaC (Fig. 5C and D).

This effect of AzaC on INSR and IGF1R signaling was correlated with a decrease in the chemotactic responsiveness of RH30 and RD cells to INS, IGF1 and 2 gradients (Fig. 6A and B). Importantly, as was the case for the IGF1R (Fig. 5A and B), downregulation of the INSR in RH30 and RD cells transfected with miR-675-3p and -5p isoforms was observed by FACS analysis (Fig. 6C and D).

Discussion

Changes in DNA methylation are among the most common molecular alterations in human neoplasia (34-37). The best characterized are related to hypermethylation of DNA in the DMRs of genes that regulate cell proliferation, such as the *IGF2-H19* tandem gene (38).

The IGF2-H19 locus is part of a cluster of imprinted genes on human chromosome 11p15, which also includes *KCNQ1* (39). In normal tissues, methylation of the DMR for this gene on the paternal chromosome prevents binding of the CTCF insulator protein to the DMR, which allows the distal enhancer to

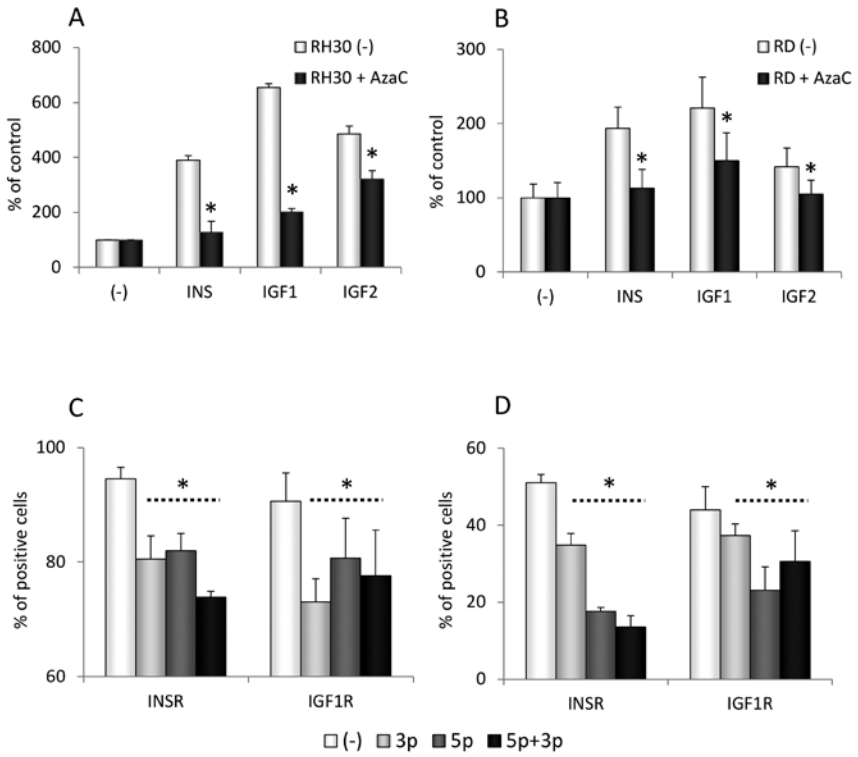


Figure 6. Chemotaxis of rhabdomyosarcoma (RMS) cells exposed to 5-azacytidine (AzaC) in response to insulin (INS), insulin-like growth factor 1 (IGF1), and 2 (IGF2). (A) RH30 and (B) RD cells unexposed (white bars) or exposed to a chemotactic gradient of INS (10 ng/ml), IGF1 (100 ng/ml), or IGF2 (100 ng/ml). (C and D) Effect of miR-675-3p and -5p employed alone or in combination with expression of insulin receptor (INSR) and insulin-like growth factor 1 receptor (IGF1R) in (C) RH30 and (D) RD cells according to FACS analysis. All experiments were repeated three times with similar results. *P<0.05.

stimulate the expression of IGF2 from the paternal chromosome. On the maternal chromosome, the DMR for IGF2-H19 is demethylated and CTCF binds to this region of DNA, with the result that the distal enhancer activates transcription of only the *H19* gene (40). Thus, in normal somatic cells a balance is established between transcription of IGF2 and H19 from paternally and maternally derived chromosomes, respectively. This balance is perturbed in several malignancies in which DMRs on both maternally and paternally derived chromosomes are hypermethylated, and a distal enhancer stimulates transcription of the *IGF2* gene from both chromosomes (41). This situation is known as LOI for this particular DMR.

LOI at the IGF2-H19 locus is observed in patients suffering from Beckwith-Wiedemann syndrome, in which neonates suffer from organomegaly and frequently develop pediatric sarcomas, including RMS (42). However, LOI at the IGF2-H19 locus that develops independently of Beckwith-Wiedemann syndrome is also observed in RMS (15,16) as well as in several other malignancies in mice and humans. In all these cases, the proliferation of malignant cells is driven by overexpression of IGF2. These cells also have higher expression of IGF1R and INSR because of the lack of H19-derived miR-675, which downregulates the expression of IGF1R (28). What is most important in this report, we provide for the first time evidence that miR-675 also downregulates the expression of INSR.

Thus, *in toto*, LOI at the IGF2-H19 locus leads to enhanced stimulation of cancer cells by the family of insulin-like growth factors and INS due to upregulation of autocrine IGF2 and, as a result of H19 downregulation, to the high expression of IGF1R and INSR, which bind IGF2 and 1, as well as INS.

Based on these findings, it was tempting to postulate that demethylation of the DMR at the IGF2-H19 locus would have the opposite effect, leading to attenuation of INS and insulin-like growth factor signaling in RMS cells. To test this hypothesis, we employed the DNMT inhibitor AzaC to widely demethylate the DNA in RMS cells. We were aware that this non-specific approach would also lead to the demethylation of several other genes. To our surprise, however, exposure of RMS cells to AzaC resulted in inhibition of cell proliferation that was paralleled by a decrease in IGF2 expression, both at the mRNA and protein levels, as well as an increase in H19-derived miR-675, which led to downregulation of IGF1R and INSR expression. The inhibitory effect of miR-675 on the expression of IGF1R and also the INSR (as is shown here) in human RMS cells was also accomplished by exposure of RMS cells to an H19 expression vector or by transducing cells with miR-675.

We are aware that AzaC leads to demethylation of several other genes, but our data tend to confirm a crucial role of the IGF2-H19 locus in driving the pro-proliferative effect of INS and insulin-like growth factor signaling in human RMS cells. Moreover, a similar result was obtained with human ARMS and ERMS cell lines. In RMS cells, AzaC at non-cytotoxic doses strongly inhibited anchor-dependent cell proliferation, caused cell cycle arrest in G2 phase, as well as decreased anchor-independent growth in a colony-formation assay in soft agar in plastic dishes. Most importantly, the effect of AzaC on the methylation state of the DMR at the IGF2-H19 locus was confirmed in our study by DNA bisulfite exposure and subsequent sequencing.

Our results are in accordance with previously published study in which AzaC efficiently reactivated the expression of silenced H19 by demethylation of the DMR within the IGF2-H19 locus (43). By contrast, a potent deacetylating agent, TsA, did not influence the expression from IGF2-H19. This result is in agreement with results obtained with murine cells in which the epigenetic state of the IGF2-H19 locus was regulated by methylation and not by acetylation (44,45).

AzaC is a chemical analogue of the cytosine nucleoside, which is present in DNA and RNA. AzaC induces antineoplastic activity by employing two mechanisms (46,47). At low doses, it inhibits DNMT activity, causing hypomethylation and synthesis of DNA. As a ribonucleoside, at high doses AzaC incorporates into RNA, which leads to the disassembly of polyribosomes and defective methylation and acceptor function of transfer RNA and finally results in the inhibition of protein synthesis. AzaC is employed in some clinical settings, for example, as an antineoplastic drug to inhibit HIV and HTLV infection in leukemia or to induce γ-globin synthesis in thalassemia patients (48-52). Our results indicate that AzaC could also be employed as a drug for RMS patients. This, however, will require further study in animal xenotransplant models of RMS.

In conclusion, we have demonstrated for the first time the *in vitro* efficacy of AzaC as a novel potential drug for treatment of RMS. Our data also confirm a pivotal role for LOI at the IGF2-H19 locus in the proliferation and migration state of RMS cells. We also show that IGF1R is regulated by miR-675 in human cells, as has been reported for murine cells, and for the first time that miR-675 also regulates the expression of human INSR. Further studies are needed to assess whether changes in the methylation state of other genes also contribute to the observed effects of AzaC.

Acknowledgements

Supported by Maestro grant 2011/02/A/NZ4/00035 and Innovative Economy Operational Programme POIG.01.01.02-00-109/09 to M.Z.R., and FNP 'Homing PLUS' programme co-financed from European Union, Regional Development Fund to M.T.

References

1. Barr FG, Galili N, Holick J, Biegel JA, Rovera G and Emanuel BS: Rearrangement of the PAX3 paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. Nat Genet 3: 113-117, 1993.
2. Collins MH, Zhao H, Womer RB and Barr FG: Proliferative and apoptotic differences between alveolar rhabdomyosarcoma subtypes: a comparative study of tumors containing PAX3-FKHR or PAX7-FKHR gene fusions. Med Pediatr Oncol 37: 83-89, 2001.
3. Hazelton BJ, Houghton JA, Parham DM, Douglass EC, Torrance PM, Holt H and Houghton PJ: Characterization of cell lines derived from xenografts of childhood rhabdomyosarcoma. Cancer Res 47: 4501-4507, 1987.
4. Kelly KM, Womer RB and Barr FG: PAX3-FKHR and PAX7-FKHR gene fusions in rhabdomyosarcoma. J Pediatr Hematol Oncol 20: 517-518, 1998.
5. Sandberg AA, Stone JF, Czarnecki L and Cohen JD: Hematologic masquerade of rhabdomyosarcoma. Am J Hematol 68: 51-57, 2001.
6. Sharp R, Recio JA, Jhappan C, *et al*: Synergism between INK4a/ARF inactivation and aberrant HGF/SF signaling in rhabdomyosarcomagenesis. Nat Med 8: 1276-1280, 2002.
7. Gordon T, McManus A, Anderson J, Min T, Swansbury J, Pritchard-Jones K, Shipley J; United kingdom Children's Cancer Study Group; United Kingdom Cancer Cytogenetics Group: Cytogenetic abnormalities in 42 rhabdomyosarcoma: a United Kingdom Cancer Cytogenetics Group Study. Med Pediatr Oncol 36: 259-267, 2001.
8. Gurney JG, Severson RK, Davis S and Robison LL: Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer 75: 2186-2195, 1995.
9. Davis RJ, D'Cruz CM, Lovell MA, Biegel JA and Barr FG: Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res 54: 2869-2872, 1994.
10. Galili N, Davis RJ, Fredericks WJ, Mukhopadhyay S, Rauscher FJ 3rd, Emanuel BS, Rovera G and Barr FG: Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet 5: 230-235, 1993.
11. Bennicelli JL, Advani S, Schäfer BW and Barr FG: PAX3 and PAX7 exhibit conserved cis-acting transcription repression domains and utilize a common gain of function mechanism in alveolar rhabdomyosarcoma. Oncogene 18: 4348-4356, 1999.
12. Barr FG, Nauta LE, Davis RJ, Schäfer BW, Nycum LM and Biegel JA: In vivo amplification of the PAX3-FKHR and PAX7-FKHR fusion genes in alveolar rhabdomyosarcoma. Hum Mol Genet 5: 15-21, 1996.
13. Anderson J, Gordon A, Pritchard-Jones K and Shipley J: Genes, chromosomes, and rhabdomyosarcoma. Genes Chromosomes Cancer 26: 275-285, 1999.
14. Barr FG: Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. Oncogene 20: 5736-5746, 2001.
15. Anderson J, Gordon A, McManus A, Shipley J and Pritchard-Jones K: Disruption of imprinted genes at chromosome region 11p15.5 in paediatric rhabdomyosarcoma. Neoplasia 1: 340-348, 1999.
16. Casola S, Pedone PV, Cavazzana AO, Basso G, Luksch R, d'Amore ES, Carli M, Bruni CB and Riccio A: Expression and parental imprinting of the H19 gene in human rhabdomyosarcoma. Oncogene 14: 1503-1510, 1997.
17. Zhan S, Shapiro DN and Helman LJ: Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma. J Clin Invest 94: 445-448, 1994.
18. Schneider G, Bowser MJ, Shin DM, Barr FG and Ratajczak MZ: The paternally imprinted DLK1-GTL2 locus is differentially methylated in embryonal and alveolar rhabdomyosarcomas. Int J Oncol 44: 295-300, 2014.
19. El-Badry OM, Minniti C, Kohn EC, Houghton PJ, Daughaday WH and Helman LJ: Insulin-like growth factor II acts as an autocrine growth and motility factor in human rhabdomyosarcoma tumors. Cell Growth Differ 1: 325-331, 1990.
20. Wang W, Kumar P, Wang W, Epstein J, Helman L, Moore JV and Kumar S: Insulin-like growth factor II and PAX3-FKHR cooperate in the oncogenesis of rhabdomyosarcoma. Cancer Res 58: 4426-4433, 1998.
21. Hahn H, Wojnowski L, Specht K, *et al*: Patched target Igf2 is indispensable for the formation of medulloblastoma and rhabdomyosarcoma. J Biol Chem 275: 28341-28344, 2000.
22. Makawita S, Ho M, Durbin AD, Thorner PS, Malkin D and Somers GR: Expression of insulin-like growth factor pathway proteins in rhabdomyosarcoma: IGF-2 expression is associated with translocation-negative tumors. Pediatr Dev Pathol 12: 127-135, 2009.
23. Rikhof B, de Jong S, Suurmeijer AJ, Meijer C and van der Graaf WT: The insulin-like growth factor system and sarcomas. J Pathol 217: 469-482, 2009.
24. Martins AS, Olmos D, Missiaglia E and Shipley J: Targeting the insulin-like growth factor pathway in rhabdomyosarcomas: rationale and future perspectives. Sarcoma 2011: 209736, 2011.
25. Gallagher EJ and LeRoith D: The proliferating role of insulin and insulin-like growth factors in cancer. Trends Endocrinol Metab 21: 610-618, 2010.
26. Yoshimizu T, Miroglio A, Ripoche MA, *et al*: The H19 locus acts in vivo as a tumor suppressor. Proc Natl Acad Sci USA 105: 12417-12422, 2008.
27. Lottin S, Adriaenssens E, Dupressoir T, Berteaux N, Montpellier C, Coll J, Dugimont T and Curgy JJ: Overexpression of an ectopic H19 gene enhances the tumorigenic properties of breast cancer cells. Carcinogenesis 23: 1885-1895, 2002.
28. Keniry A, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G and Reik W: The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nat Cell Biol 14: 659-665, 2012.

29. Kimura S, Kuramoto K, Homan J, Naruoka H, Ego T, Nogawa M, Sugahara S and Naito H: Antiproliferative and antitumor effects of azacitidine against the human myelodysplastic syndrome cell line SKM-1. Anticancer Res 32: 795-798, 2012.
30. Thakur S, Feng X, Qiao Shi Z, Ganapathy A, Kumar Mishra M, Atadja P, Morris D and Riabowol K: ING1 and 5-azacytidine act synergistically to block breast cancer cell growth. PLoS One 7: e43671, 2012.
31. Grymula K, Tarnowski M, Wysoczynski M, Drukala J, Barr FG, Ratajczak J, Kucia M and Ratajczak MZ: Overlapping and distinct role of CXCR7-SDF-1/ITAC and CXCR4-SDF-1 axes in regulating metastatic behavior of human rhabdomyosarcomas. Int J Cancer 127: 2554-2568, 2010.
32. Kerjean A, Dupont JM, Vasseur C, Le Tessier D, Cuisset L, Pàldi A, Jouannet P and Jeanpierre M: Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. Hum Mol Genet 9: 2183-2187, 2000.
33. Tarnowski M, Schneider G, Amann G, Clark G, Houghton P, Barr FG, Kenner L, Ratajczak MZ and Kucia M: RasGRF1 regulates proliferation and metastatic behavior of human alveolar rhabdomyosarcomas. Int J Oncol 41: 995-1004, 2012.
34. Baylin SB, Herman JG, Graff JR, Vertino PM and Issa JP: Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72: 141-196, 1998.
35. Bird A: The essentials of DNA methylation. Cell 70: 5-8, 1992.
36. Leonhardt H and Cardoso MC: DNA methylation, nuclear structure, gene expression and cancer. J Cell Biochem (Suppl 35): 78-83, 2000.
37. Esteller M: Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv Exp Med Biol 532: 39-49, 2003.
38. Dammann RH, Kirsch S, Schagdarsurengin U, Dansranjavin T, Gradhand E, Schmitt WD and Hauptmann S: Frequent aberrant methylation of the imprinted IGF2/H19 locus and LINE1 hypomethylation in ovarian carcinoma. Int J Oncol 36: 171-179, 2010.
39. Smilinich NJ, Day CD, Fitzpatrick GV, *et al*: A maternally methylated CpG island in KvLQT1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. Proc Natl Acad Sci USA 96: 8064-8069, 1999.
40. Bell AC and Felsenfeld G: Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405: 482-485, 2000.
41. Feinberg AP: Phenotypic plasticity and the epigenetics of human disease. Nature 447: 433-440, 2007.
42. Feinberg AP: The two-domain hypothesis in Beckwith-Wiedemann syndrome. J Clin Invest 106: 739-740, 2000.
43. Lynch CA, Tycko B, Bestor TH and Walsh CP: Reactivation of a silenced H19 gene in human rhabdomyosarcoma by demethylation of DNA but not by histone hyperacetylation. Mol Cancer 1: 2, 2002.
44. Grandjean V, O'Neill L, Sado T, Turner B and Ferguson-Smith A: Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. FEBS Lett 488: 165-169, 2001.
45. Svensson K, Mattsson R, James TC, Wentzel P, Pilartz M, MacLaughlin J, Miller SJ, Olsson T, Eriksson UJ and Ohlsson R: The paternal allele of the H19 gene is progressively silenced during early mouse development: the acetylation status of histones may be involved in the generation of variegated expression patterns. Development 125: 61-69, 1998.
46. Jones PA, Taylor SM and Wilson VL: Inhibition of DNA methylation by 5-azacytidine. Recent Results Cancer Res 84: 202-211, 1983.
47. Taylor SM and Jones PA: Mechanism of action of eukaryotic DNA methyltransferase. Use of 5-azacytosine-containing DNA. J Mol Biol 162: 679-692, 1982.
48. Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, Krushel L, Aukerman SL, Heise C and MacBeth KJ: A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One 5: e9001, 2010.
49. Greggs WM 3rd, Clouser CL, Patterson SE and Mansky LM: Discovery of drugs that possess activity against feline leukemia virus. J Gen Virol 93: 900-905, 2012.
50. Dapp MJ, Clouser CL, Patterson S and Mansky LM: 5-Azacytidine can induce lethal mutagenesis in human immunodeficiency virus type 1. J Virol 83: 11950-11958, 2009.
51. Clouser CL, Patterson SE and Mansky LM: Exploiting drug repositioning for discovery of a novel HIV combination therapy. J Virol 84: 9301-9309, 2010.
52. Ley TJ, DeSimone J, Anagnou NP, Keller GH, Humphries RK, Turner PH, Young NS, Keller P and Nienhuis AW: 5-Azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia. N Engl J Med 307: 1469-1475, 1982.