Histone Methyltransferase KMT1A Restrains Entry of Alveolar Rhabdomyosarcoma Cells into a Myogenic Differentiated State

Min-Hyung Lee, Mathivanan Jothi, Andrei V. Gudkov, and Asoke K. Mal

Abstract

Alveolar rhabdomyosarcoma (ARMS) is an aggressive pediatric muscle cancer, which arrested during the process of skeletal muscle differentiation. In muscle myoblast cells, ectopic expression of the histone H3 lysine 9 (H3K9) methyltransferase KMT1A blocks differentiation by repressing a myogenic gene expression program. In this study, we tested the hypothesis that activation of a KMT1A-mediated program of transcriptional repression prevents ARMS cells from differentiating. We investigated whether KMT1A represses the expression of differentiation-associated genes in ARMS cells, thereby blocking muscle differentiation. Our results show that expression of KMT1A is induced in human ARMS cancer cell lines when cultured under differentiation-permissible conditions. shRNA-mediated knockdown of KMT1A decreased anchorage dependent and independent cell proliferation and tumor xenograft growth, increased expression of differentiation-associated genes, and promoted the appearance of a terminally differentiated-like phenotype. Finally, shRNA-directed KMT1A knockdown restored the impaired transcriptional activity of the myogenic regulator MyoD. Together, our results suggested that high levels of KMT1A in ARMS cells under differentiation conditions impairs MyoD function, thereby arresting myogenic differentiation in these tumor cells. Thus, targeting KMT1A may be a novel strategy for the treatment of this disease. Cancer Res; 71(11); 3921–31. ©2011 AACR.

Introduction

Rhabdomyosarcoma (RMS) is a childhood cancer of skeletal muscle (1). RMS is thought to arise from muscle progenitors that have arrested during the process of myogenic differentiation (2, 3). RMS consists of 2 major subtypes, embryonal (ERMS) and alveolar (ARMS) that differ in genetic alterations and clinical prognosis (2, 3). Although most RMS harbors oncogenic PAX3/FKHR genetic signature, ERMS is less genetically defined (2, 5). Genome-wide expression profile studies show an ample difference between ARMS and ERMS (5–7). Despite the significant difference between RMS subtypes, multimodality therapies for a particular subtype have not yet emerged. In particular, survival of metastatic ARMS is dismal and has been largely unchanged for decades. Therefore, new molecular targeted therapeutic strategies are needed for ARMS.

The lack of terminal myogenic differentiation is one of the hallmarks of ARMS (2). In normal muscle development, the activation of differentiation-associated genes is mediated by the combined activity of 2 key myogenic regulators of differentiation, MyoD and its target myogenin (8). In ARMS, myogenic gene activation is dysfunctional despite the expression of both these proteins (9, 10). This suggests that a repressor might circumvent MyoD activity thereby preventing differentiation. We have shown in C2C12 differentiation model system that the histone H3 lysine 9 (H3K9) methyltransferase Suv39h1 (herein referred to as KMT1A according to the new nomenclature; ref. 11) maintains the undifferentiated state in myoblasts by repressing myogenic genes expression mediated by MyoD (12). KMT1A belongs to the family of SET-domain containing histone lysine methyltransferases (HMTases; refs. 13, 14). Histone lysine methylation, which has predominantly been found within the histone H3/H4 tail, is associated with distinct transcriptional readouts (15). One of the best-studied histone lysine methylation is on H3K9 and is associated with transcriptionally repressed chromatin (13, 16). Although many H3K9-specific HMTases have been characterized, the best studied is KMT1A (13). KMT1A directs transcriptional repression by generating trimethylation of H3K9 (H3K9me3) that acts as universal repressive mark of transcription (17, 18). KMT1A overexpression blocks myoblasts differentiation (12) and its overexpression has been reported in different cancers (19–21). Based on these data, we examined KMT1A’s function in the failure of ARMS to differentiate. We found...
KMT1A overexpression in ARMS cells under differentiation-
permissive conditions. Conversely, KMT1A expression is
decreased in myoblasts under the same growth conditions
(12, 22). Here, we show that KMT1A knockdown arrests
proliferation and induces terminal differentiation of ARMS
cells. Furthermore, KMT1A downmodulation restores MyoD
transcriptional activation, including the induction of myo-
genin required for terminal differentiation (8, 23, 24). Finally,
we show that KMT1A-depleted ARMS cells are incapable of
forming tumors in mice. Together, our data show that
KMT1A overexpression acts as a repressive barrier for
MyoD-directed myogenic differentiation of ARMS cells.

Materials and Methods

Cell culture and retro-/lenti-viral transduction

The human ARMS cell lines: Rh28, Rh30, and Rh41 were
provided by Peter Houghton ( Nationwide Children’s Hospital,
Ohio) and Rh3 was obtained from Charles Keller ( Oregon
Health & Science University, Oregon). 293FT, human skeletal
myoblast (HSMM), human RD ERMS cells were purchased
from Invitrogen, Lonza and American Type Culture Collection,
respectively. After received/purchased, all cell lines were
immediately expanded and frozen down such that they could
be restarted from the same batch of cells. 293FT, HSMM, and
RD cells were characterized and authenticate by the respective
vendor. The ARMS cells were authenticated by PAX3-FKHR
protein expression by western blot, a hallmark of these ARMS
cells. Phoenix-ampho, HeLa, and C2C12 cells have used previously (12). Cell lines used throughout this study were not used for more than 2 months and/or 15 passages. Except C2C12 and
HSMM cells, all cells were grown in D-MEM supplemented with
10% FBS (growth medium [GM] 10% FBS) medium. C2C12
myoblasts were cultured in growth (GM, 20% FBS) and induced to
differentiate by incubating in differentiation-permissible (DM)
medium (25). HSMM cells were cultured in GM (SKGM-2 BulletKit, Lonza) and induced to differentiate by
switching to DM. Cells with inhibited MyoD and KMT1A
expression were generated by transducing retrovirus or lenti-
ivirus expressing shRNA of MyoD in pBabe-shMyoD (retro),
KMT1A in pLV-shKMT1A (lenti) vectors or scrambled shRNA
in pLV-shScramble vector (lenti). MyoD-responsive reporter
(4RE-luc) cells were established by lentiviral transduction with
the 4RE-luciferase reporter gene in pLA-4RE-luc vector. All cells
were grown at 37°C, 5% CO2 in a humidified atmosphere.

Retro-/lenti-viral production and transduction

 Stocks of retro-/lenti-virus were generated as described
previously (12). Virus supernatants harvested 48 hours post-
transfection were transduced into the target cells 3 con-
secutive times followed by appropriate antibiotic selection. Viral
titers were assessed by green fluorescent protein (GFP) fluores-
cence in HeLa cells transduced with serial dilutions of GFP
expressing retro-/lenti-viral stock.

Retro-/lenti-viral vector and antibodies

 Vectors. pLA-4RE-luc was generated by replacing β-galacto-
sidase (LacZ) with luciferase (luc) into pLA-4RE-LacZ (12).
shRNA expression (pBabe-shRNA) or (pLV-shRNA) and GFP
expression (pBabe-GFP) or (pLV-GFP) vectors were previously
reported (12,26). To obtain the vectors expressing shRNA against
MyoD (NM_002478) and KMT1A (NM_003173) the following
oligos representing 19bp of the mRNA were cloned into respective
vector. For MyoD, 5′-CCGCCAGGATATGGAGCA-3′; and
for KMT1A, 5′-GGGTATCGGATATGCACCC-3′.

 Antibodies used in this study. MyoD, p21<sup>WAF1</sup> and normal
rabbit IgG (NRIgG; Santa Cruz Biotechnology); KMT1A (Cell
Signaling, Millipore, and Santa Cruz Biotechnology); Myo-
genin (BD Bioscience); tri-methylated H3K9 and acetylated
H3K9 (Millipore); MHC (MF20; Developmental Hybridoma
Bank); GAPDH (BIODESIGN) and β-actin (Sigma-Aldrich).

Gene expression analysis

Total RNA was extracted from cells using TRizol Reagent
(Invitrogen) followed by cDNA synthesis using SuperScript
First-Strand Synthesis System (Invitrogen).

Semiquantitative PCR. cDNA was PCR amplified using the
following primers: For KMT1A, KMT1A-F: 5′-GCA-
CAAGTTTGCCCTACAAC-3′ and KMT1A-R: 5′-CAGGTGTCAAA-
GAGGTAGGTG-3′; β-actin, β-actin-F: 5′-CACACTGTCGCCCA-
TCTAGC-3′ and β-actin-R: 5′-TGCTTGGCTGATCCACATC-3′.
To ensure the semiquantitative nature of the results, optimal
PCR cycles for each pair were determined by several different
amplifications of the PCR products. PCR products was
resolved on agarose gel, and stained with ethidium bromide.
Analysis of the PCR products was performed using Image-
Quant software (Molecular Dynamics) and relative expression
was calculated by normalizing with control β-actin gene
expression in the same sample.

Quantitative real-time PCR. Real-time PCR was per-
formed using FastStart Universal SYBR Green Master (Roche)
in 7900HT Fast Real-Time PCR system (Applied Biosystems).
Gene expression changes were quantified using the delta-
delta Ct method. All reactions were performed in triplicate.

In vitro proliferation assays

Cells transduced with lentivirus and/or retrovirus shRNA
expressing either scrambled, KMT1A and MyoD were seeded in 12-well plates (1.4 × 10<sup>5</sup> cells per
well) and grown to a monolayer. Untransduced, control cells
were processed in parallel. Cell proliferation was evaluated by
counting trypsinized cultures at 24-hour intervals.

Anchorage-independent cell growth assay

Scrambled or KMT1A shRNA-transduced cells (2 × 10<sup>5</sup>
cells) were suspended in 0.3% Nobel agar in GM and plated on top of 0.5% bottom agar and cultured at 37°C with 5% CO<sub>2</sub>. GM was replaced every 2 days. After 30 days, plates were
stained with 0.005% of crystal violet and colonies >100 μm in
diameter were counted.

Protein purification, cell extracts, immunoprecipitation, immunoblotting, histone methyltransferase activity, and reporter assays

 All these methods were carried out as described in our
previous study (12).
Immunofluorescence

Cells expressing scrambled shRNA or KMT1A shRNA were cultured in DM, fixed with 4% paraformaldehyde, permeabilized in 3% BSA followed by incubation with MF20 primary antibody. Alexa 488 conjugated secondary antibodies (Molecular Probes) were used to visualize the staining and MHC-positive cells were counted from ≥6 randomly chosen fields.

ChIP assay

ChIP assays were performed as described previously (12) using ChIP DNA that was PCR amplified using primers for the myogenin promoter (27). Relative enrichment was calculated as the amount of the amplified DNA normalized to input.

Tumorigenicity in mice

All animal procedures were performed in accordance with IACUC-approved protocol. Rh28 cells were transduced with lentivirus expressing shRNA of scrambled control or KMT1A followed by selection against puromycin for 2 days. A total of $2.5 \times 10^6$ cells were injected subcutaneously into the right flank of ten 4- to 5-week-old athymic $nu/nu$ female mice (Taconic). Tumor diameter was measured with an electronic caliper every 3 days on onset of nodule formation and tumor volume was calculated using the following formula: $V = \frac{1}{2} \pi \times D_l \times D_s^2$, where $D_l$ and $D_s$ represent the largest and smallest diameter, respectively. At the end-point of the experiment (when a single tumor reached a volume of approximately 450 mm$^3$), tumors were photographed.

Results

KMT1A is overexpressed in ARMS cells under differentiation-permissive conditions

KMT1A expression is downregulated in C2C12 myoblasts under differentiation-permissive conditions (DM; refs. 12, 22). In contrast, we found a significant increase in KMT1A mRNA (Fig. 1A) and protein levels (Fig 1B) in Rh28 and Rh30 ARMS cells grown under the same growth conditions. Quantitative real-time PCR analysis confirmed the increased KMT1A transcription in Rh30 and 2 additional ARMS cell lines (Rh3...
and Rh41) cultured in DM (Supplementary Fig. S1A). We asked whether this expression pattern was also reflected in ERMS subtype and found that its expression is not induced in RD ERMS cells (Supplementary Fig. S1B). These results indicate KMT1A’s induced expression in these ARMS cells cultured in DM. We then evaluated whether the induced KMT1A expression in ARMS cells correlated with its HMTase activity. The data showed an increased KMT1A HMTase activity in ARMS cells grown in DM, which reflected the increased KMT1A protein levels (Fig. 1C).

**KMT1A knockdown inhibits proliferation and blocks anchorage-independent growth of ARMS cells**

Growth arrest is a prerequisite for terminal myogenic differentiation (28). Because ARMS cells are defective in this process (2, 10), we speculated that KMT1A overexpression in ARMS cells promoted cell proliferation in DM. Therefore, we examined whether KMT1A silencing affected ARMS cells growth. As illustrated in Supplementary Figure S2, KMT1A, but not scrambled, shRNA significantly reduced KMT1A levels in ARMS cells. Although scrambled shRNA had minimal effects on the growth of ARMS cells compared to their untransduced counterpart, KMT1A depletion caused >70% growth inhibition in cells grown in GM (Fig. 2A) and >90% growth inhibition when these cells were cultured in DM (Fig. 2B). Furthermore, KMT1A depletion decreased the ability of ARMS cells to grow in soft agar (Fig. 2C). These in vitro results suggest that KMT1A overexpression blocks the growth arrest that is required for terminal differentiation of ARMS cells.
KMT1A knockdown prevents ARMS tumor growth in vivo

To further verify that KMT1A overexpression blocks the growth arrest of ARMS cells, we evaluated the effect of KMT1A downmodulation on the ability of ARMS cells to form tumors. Hence, athymic nude mice were inoculated with Rh28 cells that had been transduced with lentivirus expressing scrambled shRNA or KMT1A shRNA. Tumor size was monitored for a period of 3 weeks. Whereas mice injected with cells transduced with scrambled shRNA expressing virus developed tumors, no tumor development was detected in any of the mice that received cells transduced with KMT1A shRNA expressing virus (Fig. 2D). The presence and absence of tumor mass at the experimental endpoint for 3 mice from scrambled shRNA and KMT1A shRNA transduced groups, respectively, is depicted in Figure 2E. Altogether, the data shows that KMT1A overexpression sustains the proliferative capacity of ARMS cells.

KMT1A knockdown restores MyoD-dependent growth suppression and gene activation in ARMS cells

Studies have indicated that the loss of MyoD-induced growth arrest alters the growth of RMS cells cultured in DM (29, 30). Our data indicates that despite MyoD expression, growth of ARMS cells is unaffected by changing from GM to DM (Supplementary Fig. S3A and B). To determine whether MyoD was required for the growth arrest in KMT1A-depleted ARMS cells, KMT1A and MyoD were depleted individually or concurrently by their respective shRNAs in Rh28 cells (Fig. 3A) followed by growth assessment of these cells in DM. As anticipated, cells expressing only KMT1A shRNA showed growth inhibition whereas those expressing MyoD shRNA alone continued to grow (Fig. 3B). However, simultaneous depletion of both MyoD and KMT1A prevented growth arrest, indicating that the KMT1A depletion-induced growth arrest in Rh28 cells requires MyoD. Similar results were obtained using Rh30 cells (data not shown). Thus, KMT1A knockdown restores MyoD-mediated growth arrest in ARMS cells grown in differentiating conditions.

Dysregulation of growth arrest in RMS cells is coupled to the repression of MyoD-mediated transcriptional activation (10, 29–31). Because MyoD is essential for KMT1A depletion-induced growth arrest of ARMS cells (Figs. 2 and 3B), we tested whether KMT1A depletion alleviates the repression of MyoD-dependent transcription in these cells. Loss of MyoD-dependent gene activation was verified in Rh30-generated MyoD-responsive reporter Rh30-4RE-luc cells cultured in DM using C2C12-generated readout C2-4RE-luc cells as positive control (Supplementary Fig. S4). We found that transduction of KMT1A shRNA only, but not MyoD shRNA or combination of both shRNAs, led to the reactivation of the reporter activity in the Rh30-4RE-luc cells (Fig. 3C), confirming that reactivation of the reporter after KMT1A depletion is mediated through MyoD. Immunoblotting of extracts used for reporter assay authenticated the depletion of MyoD and KMT1A (Fig. 3D). Together, the data supports the hypothesis that KMT1A prevents MyoD-mediated growth arrest and MyoD-driven transcriptional activation in ARMS cells.

Figure 3. KMT1A downregulation restores MyoD-dependent growth inhibition and transcriptional activation in ARMS cells. A, immunoblot of extracts from indicated shRNA-transduced Rh28 cells grown in GM, probed for KMT1A, MyoD, and β-actin. B, growth of indicated shRNA-transduced Rh28 cells was evaluated for a period of 3 days grown in DM. Cell numbers at each time point are expressed as ±SEM (n = 3). C, luciferase activity was evaluated in indicated shRNA-transduced Rh30-4RE-luc cells cultured in DM for 2 days. Values were expressed after protein normalization. Error bars, ± SEM (n = 3). D, immunoblot of extracts used in C, probed for KMT1A, MyoD, and β-actin.
Downregulation of KMT1A rescues the differentiation defect of ARMS cells

Consistent with earlier reports in RMS cells (10, 30–32), we found the abrogation of MyoD induced p21cip1, myogenin and myosin heavy chain (MHC) expression in ARMS cells but not in C2C12 or HSMM cells cultured in DM (Supplementary Fig. S5A and B). Furthermore, we showed reduced KMT1A expression in both HSMM and C2C12 cells cultured in DM (Supplementary Fig. S5C; ref. 12), which is opposite to that of ARMS cells grown in the same condition (Fig. 1A and B). Note that although myogenin expression is reduced in Rh28 cells, it is somewhat induced in Rh30 cells cultured in DM, as previously reported (32). In addition, we found even more myogenin expression in MyoD-depleted Rh30 cells cultured in DM (Supplementary Fig. S5D and E), suggesting MyoD-independent myogenin induction in these cells grown under differentiation conditions. Because KMT1A downregulation resumes MyoD-mediated growth arrest and transcriptional activation in ARMS cells (Fig. 3), we speculated that it would rescue the differentiation defect in these cells. Indeed, we observed differentiation-induced p21cip1, myogenin, and MyoD expression in KMT1A, but not scrambled, shRNA transduced ARMS cells cultured in DM (Fig. 4A). KMT1A depletion was verified by immunoblotting (Fig. 4B). The data also showed that KMT1A depletion caused a >50-fold increase of MHC, a cellular marker of terminal differentiation (Fig. 4C) and the appearance of the elongated, differentiated-like phenotype (Fig. 4D). Although Rh30 elongated cells occasionally fused into multinucleated cells but such a fusion event is absent in Rh28 cells. Together, the results show that KMT1A depletion overcomes the defect in terminal myogenic differentiation in ARMS cells. The data also indicate that KMT1A overexpression is engaged in the maintenance of the undifferentiated phenotype of ARMS cells in differentiation conditions.

![Western blot and Immunofluorescence images](image_url)

**Figure 4.** Induction of myogenic differentiation in ARMS cells by KMT1A knockdown. A, immunoblot of extracts of ARMS cells expressing indicated shRNA cultured in DM for 2 days, probed for p21cip1, myogenin, MyoD, and GAPDH. B, immunoblot of extracts used in A, probed for KMT1A and β-actin. C, immunofluorescence image of MHC in ARMS cells expressing indicated shRNA cultured in DM for 7 and 10 days for Rh30 and Rh28 cells, respectively. The graph showed % MHC-positive cells and is represented as the mean from triplicate plates. D, cell morphology of indicated shRNA expressing ARMS cells visualized by phase-contrast microscopy.
Increased association of KMT1A with MyoD in ARMS cells under differentiation conditions

The results presented thus far underscore that KMT1A requires MyoD as a downstream effector for its antimyogenic activity in ARMS cells. Thus, we examined the complex formed between KMT1A and MyoD in ARMS cells cultured in either GM or DM and found that KMT1A does associate with MyoD in ARMS cells by detecting MyoD in anti-KMT1A immunoprecipitates (Fig. 5A) and KMT1A in anti-MyoD immunoprecipitates (Fig. 5B). Subsequently, we evaluated whether there is also HMTase activity associated with MyoD in these cells cultured either in GM or DM. The data showed that an HMTase activity is associated with MyoD in ARMS cells under both conditions with this activity was enriched in cells in DM (Fig. 5C). Immunoblotting verified that the observed discrepancy of MyoD-associated HMTase activity was not due to a discrepancy in the levels of immunoprecipitated MyoD. Therefore, this increased MyoD-associated HMTase activity is presumably due to the increased level of KMT1A and its complex with MyoD to a greater extent in ARMS grown in DM (Figs. 1 and 5A and B). Hence, MyoD-associated HMTase activity was evaluated in KMT1A shRNA or scramble shRNA expressing cells grown in DM. We found a significant reduction of MyoD-associated HMTase activity in cells transduced with only KMT1A shRNA (Fig. 5D), suggesting that KMT1A is apparently the primary HMT associated with MyoD in differentiation conditions. To investigate that other than KMT1A is mediated the residual MyoD-associated HMTase activity observed in KMT1A shRNA-transduced ARMS cells, MyoD-associated this activity was reevaluated in these cells after confirming the complete depletion of KMT1A by its shRNA. The data showed the absence of MyoD-associated HMTase activity in completely KMT1A-depleted Rh28 (Supplementary Fig. S6A and B) and Rh30 (data not shown) cells, documenting that MyoD-associated this activity is exclusively contributed by KMT1A in these ARMS cells.

Elevated KMT1A occupancy on the myogenin promoter is functionally coupled with its confined expression in ARMS cells grown in differentiation conditions

MyoD-directed myogenin induction above basal levels is attenuated in ARMS cells growth in DM (Supplementary Fig. S5; refs. 10, 32). Therefore, we investigated by ChIP whether KMT1A occupies the myogenin promoter in ARMS cells and, if so, whether its level at this promoter was associated with a block in myogenin induction in cells grown in DM. As illustrated in Figure 6A, KMT1A was observed on the myogenin promoter in ARMS cells grown either in GM or DM. Furthermore, the level of KMT1A at the myogenin promoter is robustly increased in cells grown in DM. Because KMT1A recruitment at the myogenin promoter is mediated via its association with MyoD bound to this promoter (12), we

Figure 5. Increased KMT1A HMTase activity associated with MyoD in ARMS cells under differentiation conditions. A, NRIgG or KMT1A immunoprecipitates from extracts of indicated ARMS cells cultured in GM or DM for 2 days were immunoblotted with MyoD antibodies and reprobed for KMT1A. B, indicated immunoprecipitates from Rh30 cell extracts used in A were immunoblotted with KMT1A antibodies and reprobed for MyoD. C, indicated immunoprecipitates from extracts as in A were divided equally into 2 parts. One part was subjected to HMTase activity assay and processed as in Figure 1C, and another part was immunoblotted with MyoD-specific antibody. D, NRIgG or MyoD immunoprecipitates from extracts of ARMS cells expressing indicated shRNA cultured in DM for 2 days were subjected to HMT activity assay and processed as in Figure 1C.
evaluated MyoD occupancy on the myogenin promoter in ARMS cells. In contrast to KMT1A, the level of MyoD was similar for cells grown in either GM or DM, suggesting that MyoD can recruit more KMT1A to the myogenin promoter in ARMS cells under differentiation conditions. We next determined whether the variation of KMT1A levels on the myogenin promoter in ARMS cells was reflected in KMT1A activity-directed methylation (H3K9me3) at this gene promoter. This analysis revealed a significant increase in H3K9me3 levels on the myogenin promoter in cells cultured in DM that was consistent with the increased KMT1A presence at this gene promoter. The enrichment of H3K9me3 at the myogenin promoter was also observed by real-time PCR in Rh30 cells cultured in DM (Fig. 6B). Therefore, it appears that the increased level of KMT1A mediates the amplification of repressive H3K9me3 methylation at the myogenin promoter thereby blocking its induction in ARMS cells grown in DM. Therefore, the restoration of MyoD-mediated myogenin induction after KMT1A depletion in ARMS cells grown in DM (Fig. 4A) may result from reduced H3K9me3 at this gene promoter. Indeed, ChIP experiment performed in ARMS cells transduced with lentivirus expressing scrambled shRNA or KMT1A shRNA showed a reduction of H3K9me3 levels at the myogenin promoter only in KMT1A shRNA transduced cells in DM (Fig. 6C). ChIP results further showed an enrichment of gene-activation associated H3K9 acetylation (H3K9ac) at the myogenin promoter only in KMT1A shRNA transduced ARMS cells in DM. Together, the ChIP data indicates that increased KMT1A, presumably in association with MyoD, at the myogenin promoter causes increased H3K9me3 methylation of this gene promoter that blocks myogenin induction in ARMS cells grown under differentiation conditions.

Discussion

The arrest of myogenic differentiation is a common feature between ARMS and ERMS (2). Studies have identified diverse individual pathways that block myogenic differentiation in these 2 major RMS subtypes (32–36). Yet, the molecular
mechanism(s) that specifically contribute to the blockade of differentiation in ARMS is largely unknown. The importance of oncogenic PAX3/7-FKHR transcription factor has been attributed as a molecular candidate in ARMS development (37). We found KMT1A overexpression in PAX3-FKHR-positive ARMS cells but not PAX3-FKHR negative ERMS-RD cells on switching to differentiation conditions, suggesting that KMT1A induction may be regulated by PAX3-FKHR in ARMS cells, which we plan to pursue. Because PAX3/7-FKHR fusion scenario occurs in about 80% of ARMS (38), we also plan to investigate whether KMT1A overexpression is a common scenario of PAX3/7-FKHR-positive ARMS.

KMT1A overexpression has been reported in cancer cells of different tissue origins (19–21). Knockdown of KMT1A in prostate cancer PC3 and immortalized/transformed bronchoepithelial cells inhibited the growth of these cells (21, 39). Here, we show that KMT1A overexpression in ARMS cells occurs only under differentiation conditions, which promotes a switch from proliferation to terminal differentiation of muscle cells. Similarly, ectopic KMT1A expression in C2C12 myoblasts blocks growth arrest and the ability of these cells to differentiate (12). Conversely, our present data showed reduced KMT1A expression in differentiated HSMM and C2C12 myoblasts. Together, these data suggest that KMT1A overexpression in ARMS cells prevents growth arrest, a prerequisite step that is essential for myogenic differentiation (28, 40). In fact, the growth of ARMS cells is unchanged regardless of the growing conditions (i.e., GM vs. DM). One may strongly argue that insensitivity of ARMS cells to differentiation signals is due to various growth promoting autocrine/paracrine pathways utilized by these cells. Indeed, several autocrine loops have been reported to sustaining RMS proliferation under differentiation conditions (31, 41). However, our findings show that KMT1A depletion causes growth inhibition of ARMS cells grown in differentiation conditions. Furthermore, KMT1A depletion significantly impaired the anchorage-independent growth of ARMS cells and severely inhibited the ability of these cells to form tumors in nude mice. Together, these findings strongly support our hypothesis that KMT1A overexpression itself permits the growth of ARMS cells even in the presence of differentiation signals.

Studies have established that ARMS cells retain the ability to grow under differentiation conditions due to a functional abnormality in the MyoD-induced growth arrest pathway (10, 29, 30, 32, 42). However, MyoD’s inability to induce growth arrest is unique to RMS cells as it retains growth inhibitory activity in normal muscle cells induced to differentiate (28–30, 40) and when expressed ectopically in a variety of nonmuscle origin tumor cells (29, 30). Our data shows KMT1A overexpression in ARMS cells and that its downregulation induces MyoD-mediated growth arrest in these cells. The differentiation defect in ARMS cells has been correlated with a lack of MyoD-mediated transcriptional activation of its growth arrest and muscle-specific gene targets (10, 29, 30, 32, 43, 44). From our data presented here, KMT1A overexpression causes such a phenotype, which can be overcome using KMT1A-specific shRNA. This provides proof-of-principle evidence that targeting KMT1A in ARMS should reactivate MyoD function in these cells, leading to differentiation. However, a previous study emphasized that MyoD can induce growth arrest independent of differentiation and that the MyoD’s growth inhibitory domain mapped to that essential for the induction of muscle differentiation (29). Based on our work, growth arrest and differentiation are not independent events but rather growth arrest precedes and is required for terminal differentiation. We found that KMT1A depletion induced both MyoD target growth arrest p21<sup>cip1</sup> (28, 30, 40) and muscle differentiation mediator myogenin genes (8) in ARMS cells. Moreover, KMT1A-depleted ARMS cells expressed late muscle phenotypic marker, MHC, also displayed the elongated differentiated-like morphology, confirming that these were terminally differentiated cells. Although, terminally differentiated Rh30 cells occasionally fused into multinucleated cells, Rh28 cells did not. It is unclear at present the reason behind this fusion discrepancy between these 2 cell lines, however, it might arises due to distinct site of tumor origin-established cell lines of 2 individual patients (45). In this regard, MyoD transcriptional activation as a result of KMT1A depletion appears to be critical in orchestrating double duty function in ARMS cells to terminally differentiate: MyoD-mediated induction of p21<sup>cip1</sup> in growth arrest (28, 30, 40) and myogenin (8, 23, 24) in cooperation with MyoD for mediating terminal differentiation (8).

The data thus far suggests that KMT1A overexpression blocks both roles of MyoD in ARMS cells, namely growth arrest and terminal differentiation. This mechanism appears to involve increased association of KMT1A with MyoD when it is bound to the promoters of target genes, principally at myogenin, in the presence of differentiation signals. At present, it is unclear what causes KMT1A to remain associated with MyoD in ARMS cells under differentiation conditions, a phenomenon that does not occur in normal myoblasts (12). In this regard, we are investigating whether this mechanism involves hyperphosphorylated MyoD or altered promyogenic signaling pathway(s) in ARMS cells (43). Our results in C2C12 cells have established that KMT1A in association with MyoD represses the myogenin promoter by H3K9me3 methylation (12). Here, we show that the same is true for ARMS cells in differentiation conditions. Moreover, decreased KMT1A levels at the myogenin promoter in KMT1A-depleted ARMS cells correlates with the reduction of H3K9me3 and increased transcription-active H3K9ac mark at this promoter in ARMS cells grown in differentiation conditions.

Taken together, our results identify that KMT1A overexpression promotes sustained myogenic undifferentiated features in ARMS cells by arresting MyoD-directed gene expression signatures that define the terminal differentiation phenotype. We provide the first evidence that complex differentiation inhibitory mechanisms present in ARMS cells can be rescued by eradication of KMT1A in these cells. Clearly, our data indicates that the loss of KMT1A from ARMS cells abolishes the tumorigenicity of the ARMS cells. Thus, we propose that KMT1A may serve as a druggable target for the development of novel therapeutics for the treatment of ARMS, an aggressive childhood cancer.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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