

# Downregulation of microRNAs miR-1, -206 and -29 stabilizes PAX3 and CCND2 expression in rhabdomyosarcoma

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Elevated levels of PAX3 and cell proliferation genes are characteristic features of rhabdomyosarcoma (RMS). We hypothesize that the increased levels of these genes are stabilized due to downregulation of specific miRNAs. In this study, we show that downregulation of miR-1, -206 and -29 stabilizes the expression of PAX3 and CCND2 in both embryonal (ERMS) and alveolar (ARMS) RMS types. Ectopic expression of miR-1 and 206 in JR1, an ERMS cell line, show significant downregulation of PAX3 protein expression, whereas overexpression of these miRNAs in Rh30, an ARMS cell line, did not show any effect in PAX3 protein levels. In ARMS, PAX3 forms a fusion transcript with FOXO1 and the resultant loss of PAX3 3'UTR in the fusion transcript indicate an oncogenic mechanism to evade miRNA-mediated regulation of PAX3. Further, we show that miR-1, -206 and -29 can regulate the expression of CCND2, a cell cycle gene. In addition to CCND2, miR-29 also targets E2F7, another cell cycle regulator. Cell function analysis shows that overexpression of miR-29 downregulates the expression of these cell cycle genes, induces partial G1 arrest leading to decreased cell proliferation. Taken together our data suggest that the RMS state is stabilized by the deregulation of multiple miRNAs and their target genes, supporting a tumor suppressor role for these miRNA.

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Rhabdomyosarcoma (RMS) is a malignant striated muscle tumor that accounts for ~3% of all childhood cancers.<sup>1</sup> RMS arises from primitive muscle cells and tumors show varying degrees of skeletal muscle differentiation that serves to define their classification as either embryonal (ERMS) or alveolar (ARMS) types.<sup>2,3</sup> ERMS, the most common type has features of embryonic muscle and are generally associated with favorable prognosis. In contrast, ARMS display poor muscle differentiation and are associated with poor outcomes.<sup>4</sup> Most ARMS cases are characterized by chromosomal translocation t(2;13) or t(1;13) involving genes PAX3-FKHR or PAX7-FKHR, respectively.<sup>5</sup> Regulatory disruptions in growth and differentiation pathways of myogenic precursor cells have been implicated in RMS development. Genes involved with muscle cell differentiation and cell proliferation have been associated with RMS development and metastasis.<sup>6–11</sup> Gene expression profiles comparing PAX-FOXO1-positive ARMS vs translocation-negative ERMS have identified genes relevant to tumorigenic process of ARMS and ERMS.<sup>12</sup>

microRNAs (miRNAs) have been implicated in RMS.<sup>13–15</sup> miRNAs are small (18–22 nucleotides) evolutionarily conserved, non-coding RNAs that post-transcriptionally regulate gene expression through mRNA degradation, translation inhibition or chromatin-based silencing mechanisms.<sup>16</sup> Each miRNA can potentially regulate hundreds of targets either directly or indirectly. miRNAs have been shown to be deregulated in many types of cancers.<sup>17</sup> As a consequence, miRNAs from tumor tissue have been proposed for use in the diagnosis, classification and prognosis of tumors.<sup>18,19</sup> Previous work in our lab has shown that miRNA expression profiles of ARMS and ERMS samples can be used to identify a misdiagnosed case.<sup>13</sup>

miRNAs such as miR-1, -133, -206 and -29 that have been implicated in skeletal muscle proliferation and differentiation<sup>20,21</sup> are undergoing investigation about their roles in RMS. Transient transfection of miR-1/206 into cultured RMS cells led to a significant decrease in cell growth and migration.<sup>22</sup> Further, exogenous expression of miR-206 promoted

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myogenic differentiation and blocked RMS growth in a xenotransplant mice model.<sup>14</sup> In our recent work, we have showed that many miRNAs including miR-29 family are significantly downregulated in RMS compared with normal skeletal muscle (NSM),<sup>13</sup> and that miR-183 is upregulated as an oncogene by targeting transcription factor EGR1, which in turn may affect the activation of tumor suppressor gene, PTEN.<sup>23</sup> Although miRNAs have been extensively studied in the context of muscle differentiation, the miRNA expression patterns in RMS and their role in tumorigenesis is not completely understood.

In this study, using a large series of RMS and NSM tissue samples, we show that ARMS and ERMS are characterized by combined perturbation of miRNA and mRNA expression relative to NSM. Further, using *in vitro* approaches, we functionally evaluated the role of miR-1 and -206 in the direct regulation of PAX3 in RMS. Additionally, we show that miR-29 can directly regulate both *CCND2* and *E2F7*, genes implicated in cell cycle control, suggesting a potential tumor suppressor role for miR-29 in RMS. Our results support a model in which multiple perturbations of miRNA networks lead to deregulation of PAX3 and cell cycle genes involved in RMS tumorigenesis.

## MATERIALS AND METHODS

Detailed methods including constructs and primers are given as Supplementary Information.

### Tissue Samples

In all, 48 fresh frozen tumor and normal tissue samples (21 ARMS, 20 ERMS and 7 NSMs) were used in the analyses. RMS tumor tissues and normal muscle samples were obtained from the cooperative human tissue network and from the tissue procurement facility at the University of Minnesota, respectively. For three RMS cases (FT-261, -271 and -281), patient-matched normal muscle tissues were also available. The institutional review board approved this study. Basic clinical data of RMS samples are given in Supplementary Information 1.

### miRNA and mRNA Expression Analysis

Total RNA extraction and purification were followed as described previously.<sup>13</sup> miRNA expression profiles were generated for all the 48 RMS and NSM tissue samples (21 ARMS, 20 ERMS and 7 NSMs). We used Illumina Sentrix Array Matrix for miRNA expression profiling as previously described.<sup>24</sup> mRNA expression profiles of RMS and normal tissue samples were generated using Illumina human (HT-12) arrays, allowing high throughput expression profiling of 48 000 human RefSeq and UniGene annotated genes.<sup>25</sup>

### Statistical Analysis for miRNA and mRNA Profiling Data

miRNA and mRNA fluorescence values were obtained from the Illumina detection system without background subtraction and were quantile normalized using GeneData

Expressionist Software (Genedata, San Francisco, CA, USA). Principal component analyses (PCAs) were carried out directly on the quantile-normalized data. Both datasets were then further normalized to the average value obtained from the normal muscle tissue. Statistically significant genes were determined using two-group *t*-test. A *P*-value cutoff was required to be less than 0.001 and the data set was further filtered, and the ratio of the group median needed to be greater than two-fold for an RNA transcript to be included in the analyses. Ingenuity pathways analyses (<http://www.ingenuity.com/>) tool was used to determine functional enrichment and canonical pathway enrichment. B and H multiple testing-corrected *P*-values were used for functional enrichment analyses.

### Quantitative Real-Time PCR

miRNAs and mRNAs were analyzed using miScript PCR system (Qiagen, Valencia, CA, USA) on Light cycler 480 (Roche, Indianapolis, IN, USA), following the manufacturer's recommendations. miRNAs were quantified with U6 small RNA serving as normalization control, and mRNAs with GAPDH as reference control. The fold expression and statistical significance were calculated using  $2^{-\Delta\Delta Ct}$  method.<sup>26,27</sup>

### Cell Culture

ARMS cell lines (Rh30 and Rh18), ERMS cell lines (JR1 and RD) and HEK293 were grown in Dulbecco's modified Eagle's Medium (DMEM, with 4 g/l glucose, 4 mM L-glutamine and 110 mg/l sodium pyruvate) (Hyclone, Logan, UT, USA) supplemented with 10% Fetal bovine serum (FBS, GIBCO) at 37 °C and 5% CO<sub>2</sub>. These RMS cell lines were authenticated by the presence of PAX3 or fusion PAX3- FOXO1 fusion proteins.

### Reporter Constructs

Two sets of luciferase reporter systems, psiCHECK-2-based vectors (Promega, Madison, WI, USA) and 3'UTR-sGG vectors (SwitchGear, Menlo Park, CA, USA) were used. 3'UTR-sGG vectors with mutations in specific miRNA-binding region were constructed using QuickChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA) following manufacturer's instructions. pGL4.73 (Promega) with a Renilla luciferase gene was co-transfected with all 3'UTR reporter vectors, working as an internal control to normalize transfection efficiency. Details of oligos used and mutation sites are given in Supplemental Method section.

### Transfection and Luciferase Assay

Three kinds of transfection reagents were used; 1) DNA only transfection: Lipofectamine 2000 (Invitrogen) was used to transfect DNA (reporter vectors) into cells; 2) DNA and miRNA precursor co-transfection: Attractene Transfection Reagent was used to transfect DNA (200 ng) and miRNA precursor (10 nM) together into cells; 3) miRNA (Ambion,

Austin, TX, USA) transfection: HiPerFect Transfection Reagent (Qiagen) was used to transfect microRNA precursors (10 nM), scrambled miRNA precursor, or pre-miR negative control #1 (10 nM). Dual-Luciferase Reporter Assay System (Promega) was used to perform dual-reporter assays using Synergy 2 (BioTek, Winooski, VT, USA).

### Total RNA and Protein Isolation from Cultured Cells

Culture media were changed after 24 hours of transfection. Total RNAs and proteins were extracted using mirVana PARIS kit (Ambion), 48 h after transfection.

### Western Blotting

Standard western blot analysis was carried out using protein extracts from pre- or post-treated cells. The following antibodies were used; PAX3 (ab53571, Abcam), CCND2 (ab3085, Abcam), E2F7 (sc-66870, Santa Cruz), GAPDH (39-8600, Invitrogen) and goat anti-mouse IgG-AP (sc-2008) or goat anti-rabbit IgG-AP (sc-2034) was used as secondary antibody.

### Apoptosis, Cell Cycle and Proliferation Assays

Vybrant apoptosis assay kit #8 (Molecular Probes, Eugene, OR, USA) was used to detect apoptosis. Cell cycle assay was conducted on cells resuspended with 2 µg/ml propidium iodide (PI) (MP Biomedicals, Solon, OH, USA) and 200 µg/ml RNase A (Qiagen) in PBS on FACSCalibur (BD Biosciences) and analyzed with Flowjo 7.5.5. Cell proliferation assay was done using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instruction.

## RESULTS

### miRNA Expression Patterns in ARMS and ERMS

Genome-wide miRNA expression profiling was performed on 41 RMS cases (21 ARMS and 20 ERMS) and compared with NSM ( $n = 7$ ). These 41 RMS cases represented various tumor stages and grades as indicated in Supplementary Information 1. Median age for both ARMS and ERMS patients was 6 years. Male gender representation for ARMS and ERMS was at 50% and 80%, respectively.

PCA of ARMS, ERMS and NSM miRNA profiling data revealed significant difference in miRNA profiles between normal and tumor tissues (Figure 1a). We noticed a unique and overlapping miRNA expression pattern among and between ARMS and ERMS cases (Figures 1b and c). First, we investigated the miRNAs that are commonly dysregulated in both ARMS and ERMS. The analysis revealed the presence of 235 miRNAs with  $P$ -value less than 0.001 and fold change  $>2$  between NSM and RMS samples (Figure 1b). The overexpressed miRNAs in RMS includes miR-183, -9 and -154. We also noticed a significant increase in expression of clusters of miRNAs from 14q32 locus (miR-136, -377, -154, -1185, -376a, -382) in both RMS types. On the other hand, the downregulated miRNAs in both types of RMS includes

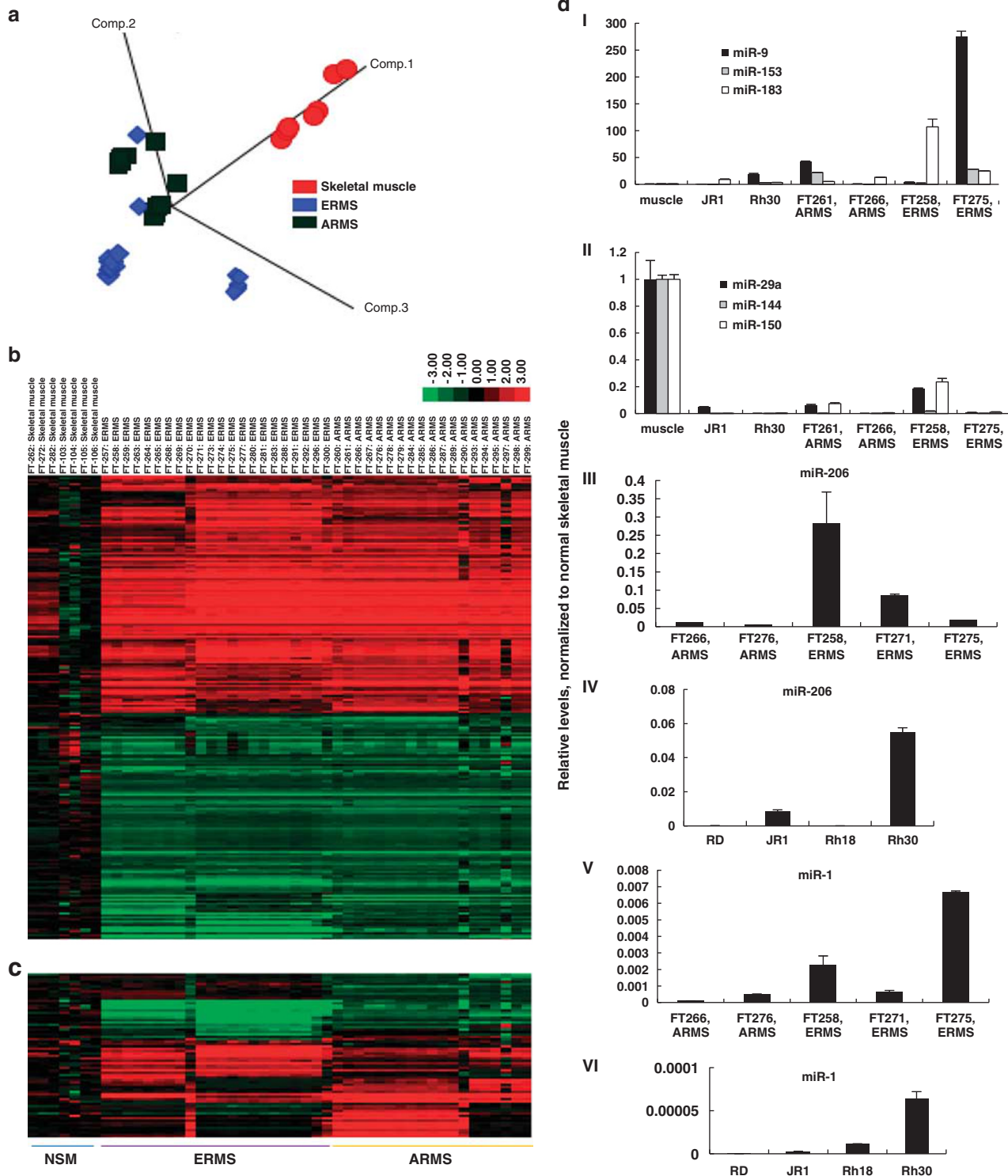
miR-1, -133, -29a-c, -144 and -150. The complete list of all the differentially expressed miRNAs in NSM and RMS are shown in Supplementary Information 2 and 2a. Next, we determined the miRNAs that are uniquely expressed in both RMS types. We identified 69 miRNAs with  $P$ -value  $<0.001$  and fold change  $>2$  between ERMS and ARMS samples (Figure 1c). These differentially expressed miRNAs between ARMS and ERMS includes miR-509-3p, -598, -135a, -7, -513, -506, -514. The list of differentially expressed miRNAs between ARMS and ERMS is given in Supplementary Information 3 and 3a.

miRNAs that are differentially expressed in RMS vs NSM were validated in a representative set of RMS patient samples via qRT-PCR (Figure 1d). We also determined that RMS cell lines (JR1 and Rh30) maintained miRNA expression patterns observed in tumors (Figure 1d) indicating that they may be suitable for functional analyses of dysregulated miRNAs (see below). When validating the microarray finding with qRT-PCR, we noticed that miR-1 and -206 were significantly downregulated compared with NSM (Figure 1d), which were not detected via microarray. Signal intensity analyses for microarray heatmap reveal that signals from miR-1 and -206 are highly saturated in NSM and RMS tissue samples (as shown in supplementary Information 3, page1), which may explain why the downregulation of miR-1/-206 in RMS is missed by microarray. Similar saturation of miR-1/-206 was noticed with spotted miRNA microarrays in our earlier studies.<sup>13</sup>

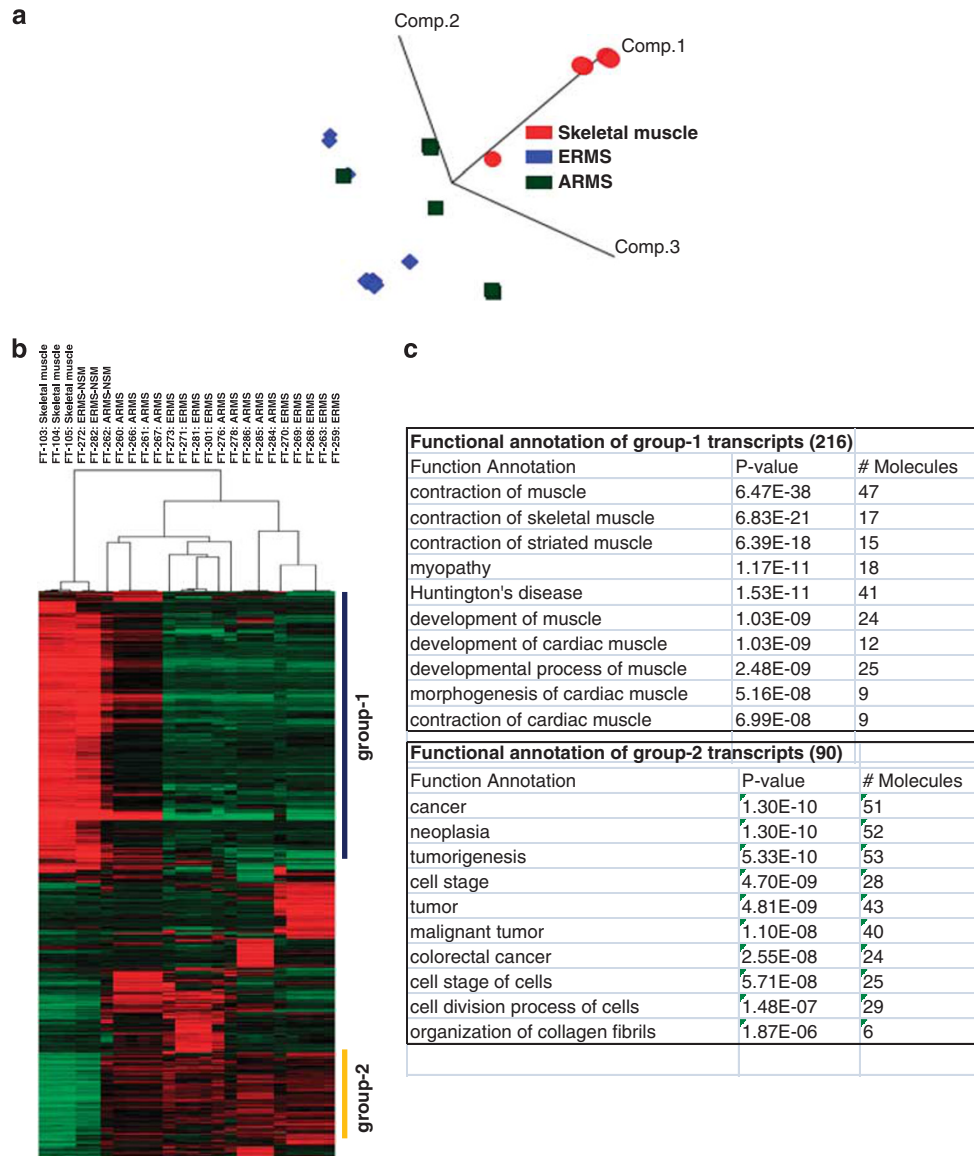
### Dysregulation of Muscle Differentiation Genes in RMS

To determine mRNAs that are deregulated in RMS, we carried out gene expression analysis in a subset of RMS cases. PCA of mRNA expression data showed clear segregation of tumors and normal muscle samples (Figure 2a). Further, we performed unsupervised hierarchical clustering of the mRNA profiles. The majority of NSM clustered separately from tumor samples (Figure 2b). However, one tumor-matched normal tissue sample (FT-262) collected from an ARMS patient (tumor sample FT-261 collected from the same patient) clustered in a group that contained ARMS tumor tissues, suggesting that tissue may bear tumorous genetic characters even with normal morphology and histology phenotype.

Next, we determined the functional significance of these differentially expressed genes between normal and RMS tissues using Ingenuity Pathway analysis (IPA). Notably, genes that are associated with muscle development and contraction were significantly downregulated in both RMS types compared with NSM. Conversely, genes implicated in neoplasia, cell division and tumorigenesis were overexpressed in both RMS compared with NSM (Figure 2c). We noticed upregulation of many cell cycle genes, including *CCND2* and *E2F7*, in both ARMS and ERMS. *CCND2* was the top-ranking gene in this analysis. The complete list of differentially expressed genes is given as Supplementary Information 4.



**Figure 1** miRNA profiling of rhabdomyosarcoma (RMS) and normal skeletal muscle tissues. **(a)** Principal component analyses of alveolar (ARMS), embryonal (ERMS) and skeletal muscle miRNA profiling data. **(b)** Unsupervised hierarchical clustering of miRNA profiles, showing 235 miRNAs with  $P$ -value  $< 0.001$  and fold change  $> 2$  in RMS samples compared with skeletal muscle. **(c)** 69 differentially expressed miRNAs between ERMS and ARMS samples, with  $P$ -value  $< 0.001$  and fold change  $> 2$ , data is shown relative to normal skeletal muscle. All heat maps are log base 2 transformed and the color scale is set to maximum of log base 2 = 3. **(d)** qRT-PCR of differentially expressed representative miRNAs in ARMS and ERMS patient samples as well as corresponding cell lines JR1 and Rh30 (I-II). Expression levels of miR-1 and -206 in ARMS and ERMS tumors tissues and cell lines (RD, JR1, Rh18 and Rh30) were quantified (III-VI). All expression levels were normalized to normal skeletal muscle shown as unity.



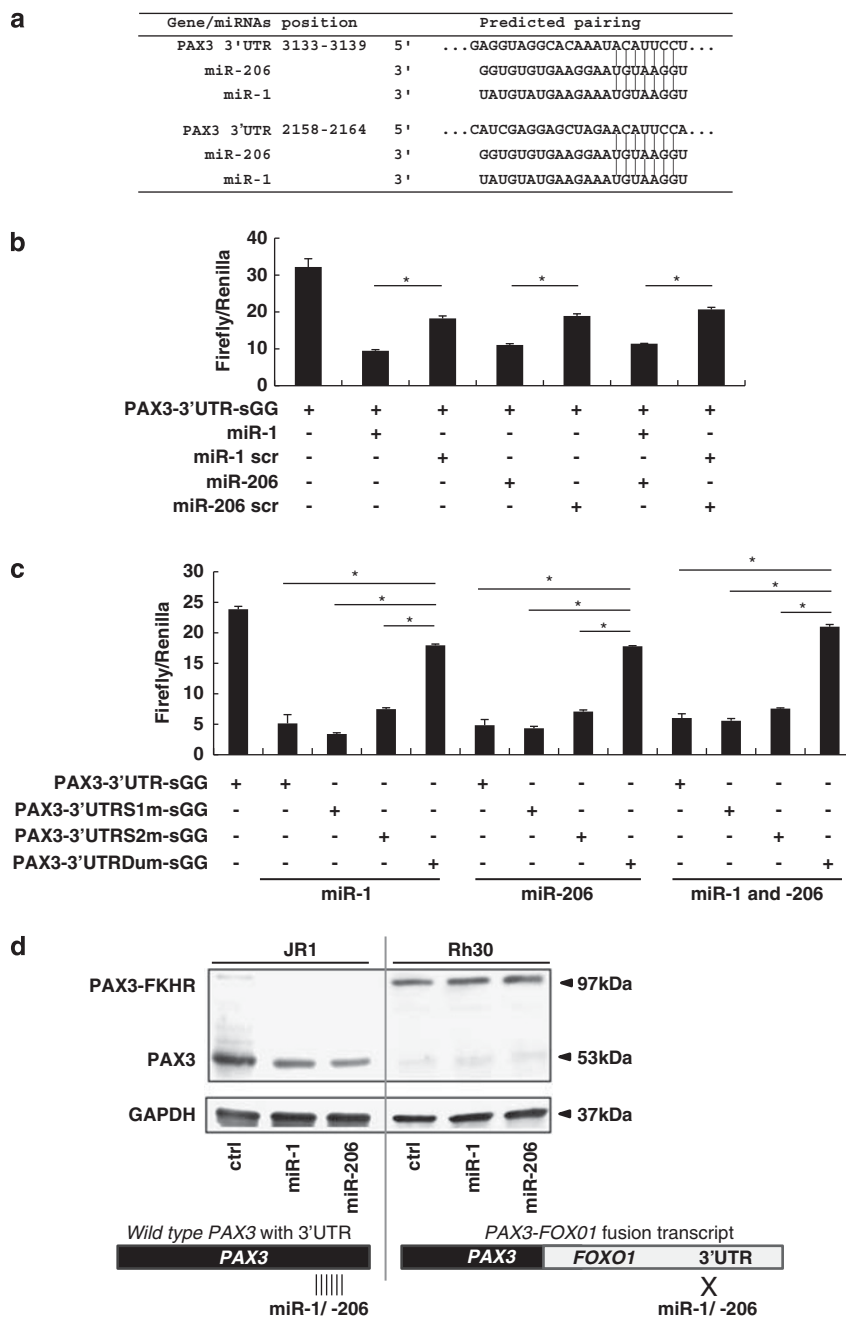
**Figure 2** mRNA profiling of rhabdomyosarcoma (RMS) tissues. **(a)** Principal component analyses of mRNA expression data. **(b)** Unsupervised hierarchical clustering of mRNA profiles and heatmap showing the differential expression of genes implicated in muscle differentiation (group-1) and tumorigenesis (group-2) in RMS and normal skeletal muscle. **(c)** Ingenuity Pathway functional analysis of the differentially expressed mRNAs from groups-1 and -2.

### miR-1 and -206 Regulates PAX3 Expression

As miR-1 and -206 are muscle-specific miRNAs and our qRT-PCR showed significant downregulation in RMS compared with NSM, we did a comprehensive target prediction analysis for both these miRNAs. Interestingly, PAX3, the critical gene in activation of myogenic program as well as RMS tumorigenesis, was predicted as one of the potential target for both miR-1 and -206. There are two potential miR-1 and -206 binding sites in the 3'UTR of PAX3 (accession number: NM\_001127366.2), site-1 (3133–3139 bp) and site-2 (2158–2164 bp) (Figure 3a). It is noteworthy that both miR-1 and -206 share identical seed sequences that can potentially target the same region. To verify the direct interaction between

miR-1 or -206 with PAX3 transcript, PAX3-3'UTR (PAX3-3'UTR-sGG) reporter construct was co-transfected with miR-1 and/or miR-206 precursors in HEK293 cells. Precursors with scrambled sequences of miR-1 or -206 served as controls. Significant repression of luciferase activity was observed after co-transfection of PAX3-3'UTR with miR-1 and/or -206 (Figure 3b) precursors compared with scrambled controls, which suggests that miR-1 and -206 can target PAX3.

As there are two predicted miR-1 or -206 binding sites within PAX3 3'UTR, reporter constructs were generated with mutations either in the first binding site (PAX3-3'UTRS1m-sGG), or the second binding site (PAX3-3'UTRS2m-sGG). Further, a construct with mutations in both sites



**Figure 3** miR-1 and -206 regulates *PAX3* expression through sequence-specific binding to its 3'UTR. **(a)** Predicted pairing of *PAX3*-3'UTR with miR-1 and -206. **(b)** *PAX3*-3'UTR-sGG co-transfected with miR-1 and/or -206 precursor into HEK293 cells result in decreased luciferase activity when compared with vector co-transfected with scrambled controls for miR-1 and/or -206, indicating a sequence-specific regulation of miR-1 or -206 through *PAX3*-3'UTR. **(c)** Luciferase reporter assay with *PAX3*-3'UTR-sGG wild type as well as constructs with mutation at either or both miR-1/-206 binding sites. Mutations in both binding site are required to rescue *PAX3* from miR-1/-206 repression. **(d)** *PAX3* protein levels quantified using western blot in JR1 and Rh30 cells. Exogenously expressing miR-1 or -206 led to decreased expression of *PAX3* in JR1 cells, whereas expression of chimeric protein *PAX3*-FOXO1 in Rh30 cells was not affected. Schematic shows the loss of 3'UTR of *PAX3* in *PAX3*-FOXO1 transcript and the potential escape mechanism from miR-1- and or -206-mediated regulation of *PAX3*. \* $P < 0.05$ .

(*PAX3*-3'UTRDum-sGG) was also generated. HEK293 cells were co-transfected with one of the above *PAX3*-3'UTR luciferase reporters along with miR-1 and/or -206 precursor. Wild-type *PAX3*-3'UTR showed decreased luciferase activity

when co-transfected with miR-1 and /or -206. Constructs with mutations at site-1 or site-2 showed decreased luciferase activity similar to that of wild-type construct. Importantly, a construct with mutation at both site-1 and site-2 rescued the

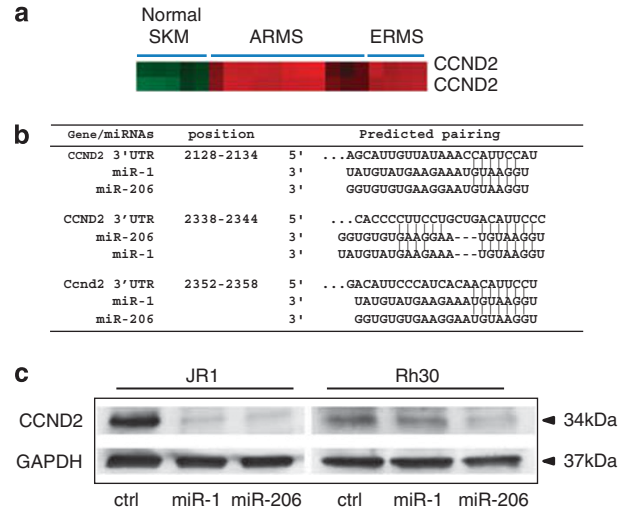
repressed luciferase expression caused by co-transfected miR-1 and/or -206 precursor (Figure 3c). These results indicate that both binding sites of miR-1 and -206 in *PAX3* are functional and there is minimal preference between these binding sites. These data also suggests that there was no preferential selection between miR-1 and -206 to regulate *PAX3* at least under the current experimental conditions.

### miR-1 and -206 Repress *PAX3* Protein Expression in ERMS but not in ARMS

We hypothesized that decrease in endogenous miR-1 and -206 in RMS may contribute to increased *PAX3* expression. *PAX3* is highly expressed in both ERMS and ARMS. However, in most ARMS, *PAX3* forms a fusion transcript with *FOXO1*. It has been reported that *PAX3* protein or *PAX3-FOXO1* fusion protein contributes to tumorigenesis and outcome in RMS.<sup>28</sup> To explore miR-1 and -206 regulation of *PAX3* in RMS, we transfected miR-1 or -206 precursor in JR1 (ERMS) and Rh30 (ARMS) cell lines and evaluated *PAX3* protein expression levels. The western blot analysis showed that JR1 cells transfected with miR-1 or -206 had significantly lower levels of *PAX3* expression (70% or 62% after miR-1 or -206 transfection, respectively) compared with untransfected cells. In contrast, in Rh30 cells, transfection of miR-1 or -206 did not have any effect on *PAX3* protein expression. In Rh30 cells, instead of a 53 kDa band as seen in JR1, the chimeric protein *PAX3-FOXO1* of ~97 kDa was observed (Figure 3d). The loss of *PAX3* 3'UTR in the fusion transcript eliminates binding sites for miR-1 and -206, thus resulting in escape from miR-1- or -206-mediated regulation (Figure 3d). In addition to the JR1 and Rh30, we also tested the expression of *PAX3* in RMS cells lines, such as RD (ERMS) and Rh18 (ARMS, without *PAX3-FOXO1* translocation), transfected with miR-1 or -206. Western blot analysis of these transfected cells revealed that miR-206 significantly downregulated *PAX3* expression in RD. However, in case of Rh18, we did not notice significant downregulation of *PAX3* expression. Scrambled miR-1 and -206 were used as controls (Supplementary Information 5, Supplementary Figure S1).

### miR-1 and -206 Represses *CCND2* in RMS

In our mRNA expression analysis, we noticed increased expression of several cell cycle genes including *CCND2* (> 8-fold) in RMS (Figure 4a). Interestingly, miR-1 and -206 have potential binding sites in *CCND2*-3'UTR (Figure 4b). Therefore, we hypothesized that as miR-1 and -206 are significantly downregulated in RMS, the expression of *CCND2* expression, similar to *PAX3*, may also be unrestricted in both RMS types. After transfecting miR-1 or -206 in JR1 and Rh30 cells, we tested *CCND2* protein level using western blotting (Figure 4c). In JR1, both miR-1 and -206 were very effective in decreasing *CCND2* protein levels. However, in Rh30 cells, miR-206 was found to be more effective in repressing *CCND2* levels than miR-1. Transfection efficiency and q expression of mature miR-1 and -206 in RMS cells were

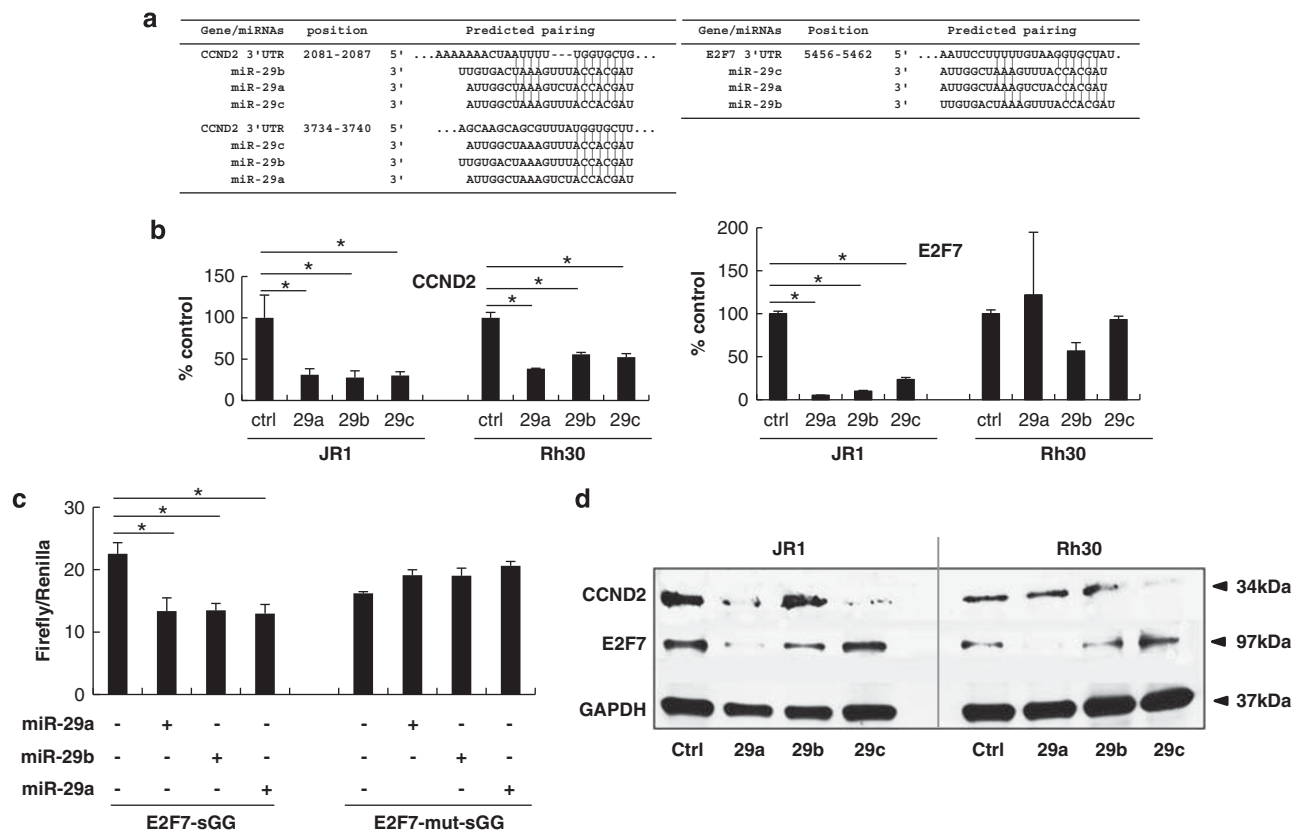


**Figure 4** miR-1 and -206 regulates *CCND2*. (a) mRNA microarray data represented as heatmap showing significant upregulation of *CCND2* transcript in both alveolar (ARMS) and embryonal (ERMS) tumor tissue compared with normal skeletal muscle. (b) Sequence predictions shows potential binding sites for miR-1 and -206 in the 3'UTR of *CCND2*. (c) Quantification of *CCND2* protein levels by western blot in JR1 and Rh30 cells transfected with either miR-1 or -206 precursor. Negative miRNA precursor served as control.

evaluated and confirmed using qRT-PCR (Supplemental Information 5, Supplementary Figure S2). It is noteworthy that compared with untransfected RMS cells, transfection with miRNA miR-1/ -206 precursors brought their levels up to ~800-folds higher. miR-206 levels were comparable to physiological levels noticed in NSM, however levels of miR-1 in the transfected RMS cells were still significantly lower than NSM.

### Cell Cycle Genes *CCND2* and *E2F7* are also Under the Regulation of miR-29

In addition to miR-1 and -206, our microarray and qRT-PCR data also showed significant downregulation of miR-29a, -29b and -29c (miR-29) in both ARMS and ERMS. Interestingly, miR-29 was predicted to target the cell cycle genes Cyclin D2 (*CCND2*) and E2F transcription factor 7 (*E2F7*) (Figure 5a). In order to validate the regulation of these cell cycle genes by miR-29, we performed qRT-PCR in either JR1 or Rh30 cells exogenously expressing miR-29a, b or c. We observed significant downregulation of *CCND2* transcript levels in both cell lines tested (Figure 5b, top panel). We further confirmed this regulation at protein level by western blotting using antibodies against *CCND2* in JR1 and Rh30 cells. Unlike *CCND2* transcripts, which were significantly repressed by all miR-29 members in both cell lines, the regulation in *CCND2* protein expression was specific to miR-29 family members (Figure 5d). Whereas miR-29c significantly downregulated *CCND2* expression in both JR1 and Rh30



**Figure 5** miR-29 targets cell cycle genes *CCND2* and *E2F7*. (a) Predicted pairing of *CCND2* and *E2F7* 3'UTR with miR-29. (b) qRT-PCR shows that ectopic expression of miR-29a, b, or c downregulate *CCND2* transcript levels in both JR1 and Rh30 cells. However, exogenously expressed miR-29 significantly downregulated *E2F7* transcript level in JR1 cells but not in Rh30 cells. \* $P < 0.05$ , vs cells transfected with control precursors. (c) Luciferase reporter assays using HEK293 cells; *E2F7*-3'UTR-sGG wild type co-transfected with miR-29 a, b or c precursors lead to repression of luciferase expression. However, no decrease in luciferase activity was observed when *E2F7*-mut-sGG with mutation at the miR-29-binding sites were co-transfected with miR-29. A full-length *E2F7*-3'UTR (NM\_203394.2) was used in the reporter assay. \* $P < 0.05$ , vs sGG vector (without cotransfection with miRNA precursor). (d) Western blotting show that exogenous expression of miR-29c result to downregulated *CCND2* protein in both JR1 and Rh30 cell lines, whereas ectopic miR-29a causes downregulation of *CCND2* protein in only JR1 but not Rh30 cells. In both RMS cell lines, miR-29a leads to significantly decreased expression of *E2F7* protein.

cells, miR-29a repressed *CCND2* was specific to JR1 cells. We also tested the expression of *CCND2* and *E2F7* in RD and Rh18 cell lines by transfecting miR-29 a or b or c individually by western blot. Expression of *CCND2* was downregulated significantly by all three miR-29 family members (miR-29a, -29b and -29c) in RD cells. However, in Rh18, we noticed that miR-29c had significant effect on *CCND2* expression compared with the other two miR-29 family members (Supplementary Information 5, Supplementary Figure S3).

Although *CCND2* was previously shown to be a target for miR-29,<sup>29</sup> the interaction between miR-29 and *E2F7* has not been experimentally validated. Hence, we carried out luciferase reporter assay to validate the direct interaction of miR-29 with *E2F7*. *E2F7*-3'UTR-sGG was co-transfected along with miR-29 a, b or c precursors in HEK293 cells. Construct with mutation in miR-29-binding site (*E2F7*-mut-sGG) served as control. Luciferase activities of wild-type *E2F7*-3'UTR vector were repressed when co-transfected with any of the miR-29 family miRNAs (Figure 5c), whereas construct with mutation did not show any significant repression of

luciferase activity. This suggests that miR-29a, -29b or -29c directly target *E2F7* by binding to the 3'UTR in a sequence-specific manner (Figure 5c).

Further, we validated the inverse correlation of miR-29 and *E2F7* expression levels in JR1 and Rh30 with ectopic expression of miR-29. Our data show decreased *E2F7* transcript levels only in JR1 cells (Figure 5b). However, overexpression of miR-29a, b or c in Rh30 did not result in significant decrease in *E2F7* transcripts. It may be possible that miR-29 regulation of *E2F7* mediated at the protein translational level.

To investigate this further, we examined *E2F7* protein expression in JR1, RD, Rh18 and Rh30 cells before and after transfection with either miR-29a, -29b or -29c. *E2F7* protein was significantly repressed in both JR1 and Rh30 cells by miR-29a (Figure 5d). We noticed similar effects on miR-29a/c in Rh18, however, there was no effect on *E2F7* protein levels with these miRNAs in RD cells (Supplementary Information 5, Supplementary Figure S4). Together, these experiments suggest that miR-29 family can exert both transcriptional and/or translational regulation of *CCND2* and *E2F7*. Further,



miR-29 family members can impart cell line-specific activity in regulating these cell cycle genes.

### Ectopic Expression of miR-29 Regulates Cell Cycle and Proliferation in RMS Cells

As miR-29 family members are significantly downregulated in RMS and our data shows that miR-29 can regulate genes implicated in cell cycle, we functionally assessed the role of miR-29 in RMS cells transfected with individual and combined miR-29 a, b and c miRNA precursors. With individual transfection of miR-29 members, we noticed that JR1 cells showed G1 arrest (Figure 6a). Consistently, combination of miR-29 a, b and c show significant G1 arrest in JR1 cells (Figure 6b). miR-29 transfection also significantly inhibited proliferation of JR1 cells either individually or in combinations (Figure 6c). We also noticed pro-apoptotic effect for miR-29 members transfected JR1 cells either individually (Figure 6d) or in combinations (Figure 6e). We also observed significant pro-apoptotic effect of individual miR-29 family members in Rh30 (Supplementary Information 5, Supplementary Figure S6a). However, in Rh30 cells, miR-29 transfections showed relatively mild effects in proliferation and cell cycle functions compared with JR1 (Supplementary Information 5, Supplementary Figure S6a and b).

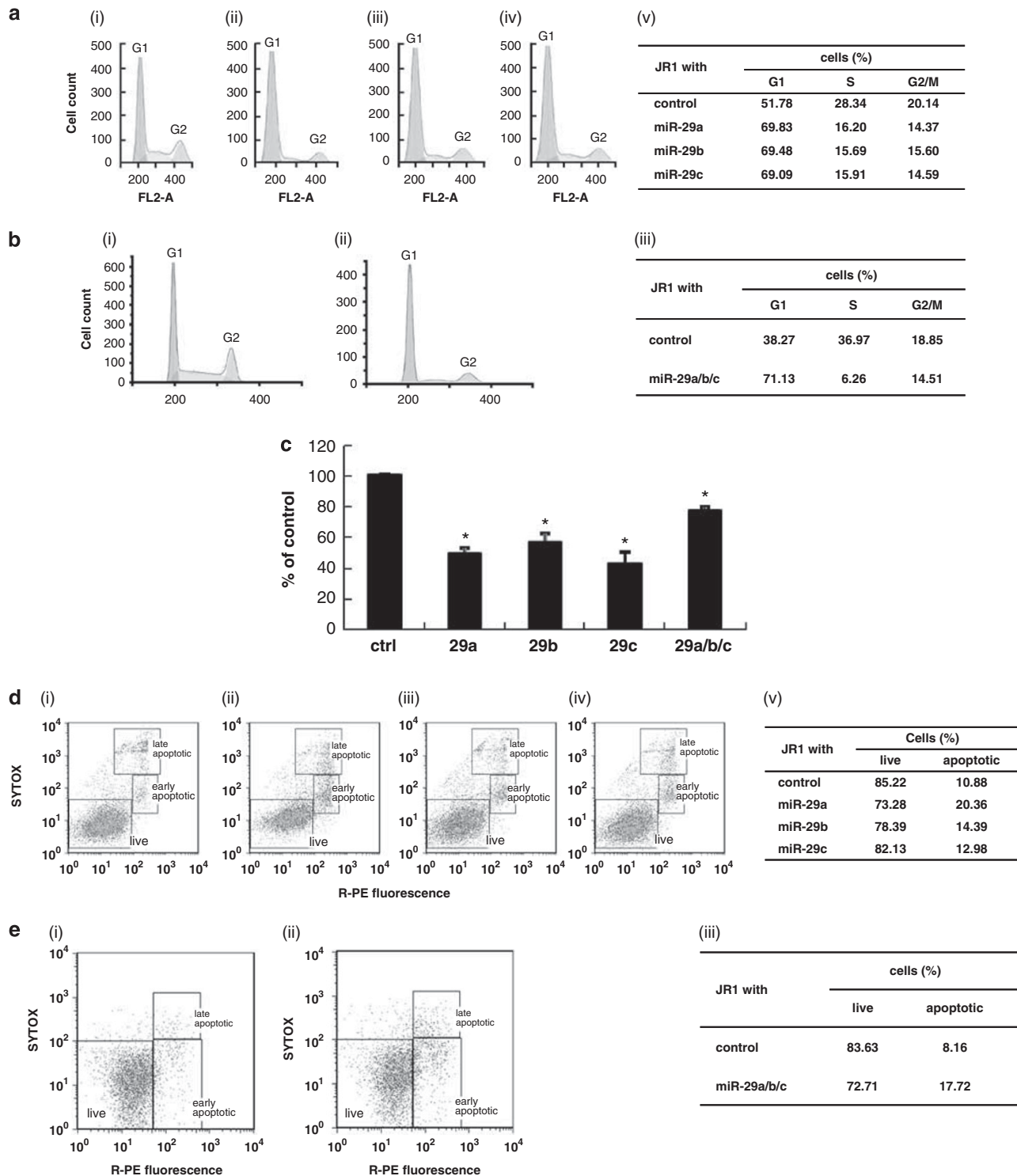
### DISCUSSION

In this study, we have outlined a unique miRNA signature in RMS compared with NSM that potentially contributes to pathogenesis. Further, our microarray analyses showed that ARMS and ERMS are characterized by the presence of shared sets of miRNAs. For example, miR-183 was upregulated and miR-1, -206 and -29 were consistently downregulated in both ARMS and ERMS. These observations are in agreement with previous reports from other groups and our laboratory.<sup>13–15</sup> Recently, we showed that miR-183, which is upregulated in RMS, functions as an oncogene by targeting the transcription factor *EGR1* and potentially have a role in tumor cell migration. This miR-183 network was also seen in other sarcoma such as synovial sarcoma, reiterating the presence of common deregulated miRNA networks in sarcomas.<sup>23</sup>

Dysregulation of genes implicated in muscle differentiation is an important component of RMS pathogenesis. Our gene expression analysis in RMS patient samples showed downregulation of muscle differentiation genes and upregulation of genes implicated in various tumorigenesis functions. miRNAs including miR-1, miR-206 and miR-29 that are known to be associated with muscle development and differentiation<sup>20,30</sup> are also significantly downregulated in RMS compared with NSM tissue. However, when compared with other sarcoma types, miR-1 and -206 are highly expressed in RMS tumors and cell lines, supporting the myogenic origin of RMS. In our miRNA microarray analysis, we also noticed that the probes for miR-1 and 206 were saturated due to high expression in the tumor tissues.<sup>18</sup> A recent study has shown that both miR-1 and -206 can be used as potential serum

markers for RMS.<sup>31</sup> Here in this study, we show that miR-1 and -206 are potential regulators of *PAX3* expression by binding to its 3'UTR. *PAX3* have a critical role in myogenesis<sup>32,33</sup> and increased expression of *PAX3* is implicated in the pathogenesis of RMS.<sup>34</sup> Specifically, in ARMS, fusion transcript *PAX3-FOXO1* produces a protein product, within which the structural integrity of both *PAX3* DNA-binding regions, the paired box and homeodomain, are retained. However, the 3'UTR of *PAX3* is lost during the formation of this functional fusion transcript<sup>35</sup> (Supplementary Information 5). In this context, the loss of 3'UTR region of *PAX3* due to formation of fusion transcript may allow the fusion transcript *PAX3-FOXO1* to escape miR-1, -206 or other potential miRNA-mediated regulation. A recent study also reported miRNA mediated regulation of *PAX3*.<sup>36</sup> A subset of ARMS is characterized by *PAX7-FOXO1* translocation; a recent study has shown that both miR-1 and -206 can potentially regulate *PAX7* in skeletal muscle satellite cells.<sup>37</sup> Based on these evidences, we propose that loss of 3'UTR of *PAX3/PAX7* and /or the downregulation of miR-1 and -206 are oncogenic events in rhabdomyosarcomagenesis (Figure 7). In ERMS, although *PAX3* is not associated with chromosomal translocation, we uncovered various *PAX3* 3'UTR abnormalities, including shorter transcript variants, deletions and mutations in sequences present between *PAX3* 3'UTR 1830–2127 bp (Supplementary Information 5, Figures 7a and b) in cell lines and representative RMS samples, which suggests alternative potential mechanisms for *PAX3* to escape from miRNAs repression. To check the effect of these deletions or mutations in miRNA-mediated regulation of *PAX3*, we generated two deletion (region 1830–2002 nt and region 1948–2119 nt) mutant constructs for *PAX3* 3'UTR and carried out luciferase reporter assays. *PAX3*- 3'UTR with deletion of 1948–2119 nt showed partial rescue of luciferase activity compared with the wild-type *PAX3* 3'UTR (Supplementary Information 5, Figure 7c). This suggests that the sequences adjacent to miRNA-binding sites in *PAX3* may also have critical roles in miR-1/206 binding and regulation.

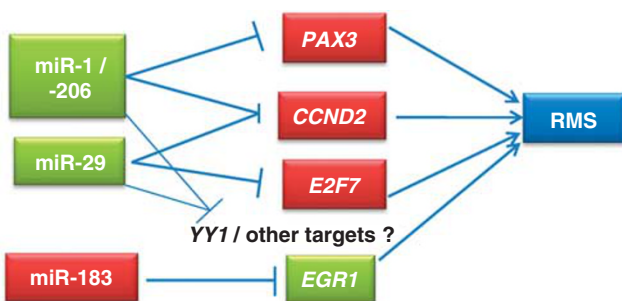
In our gene expression analysis of RMS patient samples, we also observed elevated expression of cell cycle genes such as *CCND2*. Elevated *CCND2* levels are observed in various cancers and are implicated in cell proliferation.<sup>38,39</sup> It was interesting to notice that miR-1 and -206 can also regulate *CCND2* transcript and protein levels. Overexpression of miR-1 showed strong promyogenic effect in RMS cells and downregulated expression of *CCND2* transcript levels.<sup>40</sup> As miRNAs can target and regulate several genes simultaneously, it is possible that downregulation of miR-1 and -206 allows the expression of *CCND2* and other potential oncogenic targets in RMS leading to increased cell proliferation. Recently, it was shown that TGF- $\beta$  inhibits differentiation of myogenic cells by downregulating miR-206 and -29 both of which targets *HDAC4*.<sup>41</sup> These findings reiterate that multiple miRNAs can cooperatively regulate genes involved in one or more cellular function/process.



**Figure 6** Cell function assays in JR1 cells ectopically expressing miR-29 a, b and c. **(a)** Cell cycle assay in JR1 cells transfected with 10 nM each of miR-29a or b or c. Compared with cells transfected with control precursor (I), miR-29a (II), -29b (III) or -29c (IV) transfected cells shows significant G1 arrest. Percentages of cells in each cell cycle phase are presented in (IV). **(b)** Cell cycle assays in JR1 cells transfected with miR-29 family members in combination (10 nM for each) shows significant G1 arrest (II) compared with control precursor transfected cells (I), and data are shown in (III). **(c)** Cell proliferation assays was conducted in JR1 cells 48 h after transfection with 10 nM miR-29 a or b or c, or all of the miR-29 family member together using CellTiter 96 Aqueous One Solution. Compared with control precursor transfected cells, JR1 cells transfected with miR-29 family members individually or in combinations shows significant inhibited cell proliferation. \* $P < 0.05$ . **(d)** Vybrant apoptosis assays in serum-starved JR1 cells transfected with 10 nM miR-29 a or b or c. The assay was conducted 48 hours after transfection and miR-29a (II), -29b (III), or -29c (IV) transfected cells showed increased apoptosis compared with control cells (I), and data are presented in (V). **(e)** Apoptosis assays in serum-starved JR1 cells transfected with miR-29 family member in combination (10 nM for each) show pro-apoptosis (II) compared with control cells (I), data are shown in (III).

Generation of both miRNA and mRNA expression profiles from the same set of RMS samples allowed us to investigate the correlation between miRNA and mRNA expressions. We investigated the negative correlation observed between miR-29 and cell cycle genes, including *CCND2* and *E2F7*. One of the significantly downregulated miRNAs in both ARMS and ERMS is miR-29 family. miR-29 is implicated in cell functions such as proliferation, apoptosis and regulation of DNA methylation.<sup>42,43</sup> A previous study has shown that miR-29 regulates myogenesis through feedback inhibition of YY1, an oncogene thus regulating cell proliferation.<sup>15</sup> In this study, we demonstrated that miR-29 targets both *CCND2* and *E2F7*, genes involved in cell cycle control, and activates muscle differentiation genes such as alpha actin and myogenin (Supplementary Information 5, Supplementary Figure S8). *CCND2* and *E2F7* were upregulated in both RMS types and the inverse correlation between expression of miR-29 and these cell cycle genes suggest that miR-29 may function as a potential tumor suppressor in RMS by targeting these genes in addition to YY1. Thus, downregulation of miR-29 in both ARMS and ERMS allows stable expression of genes that have oncogenic potential.

Consistent with the above observation, our cell cycle assay showed that ectopic expression of miR-29a, b or c caused cell cycle arrest at G1 phase in JR1 cells, which subsequently led to decreased proliferation in JR1 cells. A tumor suppressor role of miR-29 has been previously documented.<sup>15,43</sup> Interestingly, overexpression of miR-29 in Rh30 had less effect on G1 arrest and proliferation. Studies have shown that *E2F7* is essential for cell survival and embryonic development in mice and ectopic expression of *E2F7* block cell cycle transition resulting in G1 arrest.<sup>44,45</sup> As miR-29 regulates *E2F7* expression, it is possible that presence of *E2F7* in Rh30 allows cell proliferation even in the absence of *CCND2*. Taken together, these findings suggest that miR-29 family members may impart RMS cell type-specific function and /or require additional targets in mediating cellular functions.



**Figure 7** Schematic diagram showing the miRNA mediated gene regulations in rhabdomyosarcoma (RMS). *PAX3* and *CCND2* is regulated by both miR-1 and -206, and miR-29 regulates the expression of *CCND2* and *E2F7*, suggesting a presence of potential miRNA regulatory network that is deregulated in RMS. Previously, we have demonstrated miR-183-mediated regulation of *EGR1* in RMS. Downregulated and upregulated miRNAs and their target genes are color coded as green and red, respectively.

Others and we have shown that miR-1, -206 and -29 are significantly downregulated in RMS. It is possible that these miRNAs are influenced by transcriptional regulation such as epigenetic alterations and/or loss of transcription factors. For example, MYOD, a transcription factor activates the expression of miR-1 and -206. However in RMS, it is non-functional due to the formation of inhibitory dimers.<sup>8,46</sup> In addition, it has been shown that NF- $\kappa$ B and its target gene YY1 are highly expressed in RMS and epigenetically downregulate miR-29 b2/c.<sup>15</sup>

In this study, we noticed that RMS cell lines showed variable responses to the ectopic expression of miR-1, -206 or -29. For example, RD cells showed decreased expression of *PAX3* when transfected with miR-206, however Rh18 did not show any decreases in *PAX3* with either miR-1 or -206. Further, protein expression of *CCND2* and *E2F7* were also modulated at different levels in these RMS cell lines with exogenous expression of miR-29 family members. These differential modulations of target genes results in varying levels of apoptosis or proliferation noticed in these RMS cell lines. As miRNA-mediated regulation of target genes are influenced by various factors such as p53 mutational status<sup>43</sup> and the presence of competing endogenous RNAs,<sup>47</sup> it is possible that presence or absence of these factors may influence the levels of *PAX3* or *CCND2* in these RMS cells.

All the four RMS cell lines studied have different genetic background; Rh30 is an ARMS with a characterized translocation t(2;13)(q35;q14) to form a fusion protein *PAX3-FOXO1*. Rh18 is also ARMS but without translocation. Both JR1 and RD are ERMS cell lines. In addition, these RMS cells also vary in their p53 mutational status. Unlike Rh18 that carries wild-type p53, point mutations in p53 were observed in JR1, RD and Rh30 cells.<sup>48</sup> As p53 activates several miRNAs such as miR-34a, p53 inactivation in JR1, RD and Rh30 may lead to differential expression of p53-activated miRNAs, which in turn may be required to target *PAX3* or *CCND2*. For example, miR-29 could induce apoptosis in HCT116 (with wild-type p53) but not in p53-mutated SW480 colon cancer cell lines.<sup>43</sup> In addition, Muller Fabbri *et al*<sup>42</sup> observed significant reduction of DNMT3A levels with miR-29b, whereas no marked reduction was noticed with either miR-29a or -29c, even though miR-29 members share the identical seed region.

In addition, these RMS cells were established from different patients with or without treatment. For example, Rh30 and Rh18 are derived from tumors obtained at the time of diagnosis, whereas JR1 and RD were from treated patients or with relapsed tumors. Another possible reason for differential regulation of miRNA target genes is the surrounding sequence in 3'UTR of the target genes. We have noticed deletions/mutations in *PAX3* 3'UTR in RMS tumor tissues from patient as well as cell lines, and our luciferase reporter assay shows that deletion of certain 3'UTR region causes more significant impairment for miR-1/206 binding to *PAX3* 3'UTR than the other regions examined. As multiple factors

influences miRNA regulation of target genes, it is difficult to pinpoint specific mechanism that cause differential regulation of PAX3, CCND2 or E2F7 expression in RMS cells treated with miRNAs.

In conclusion, ARMS and ERMS are characterized by miRNAs that are commonly deregulated in both types. miR-1 and -206 are downregulated in RMS tumors and cell lines that lead to stabilization of two potential oncogenes PAX3 and CCND2. Further, loss of 3'UTR of PAX3 during the formation of fusion transcript or even loss of sequence adjacent to miRNA-binding sites may be an oncogenic mechanism to evade miR-1- and -206-mediated repression. Also, miR-29, which is downregulated in both RMS types, targets cell cycle genes CCND2 and E2F7 and may potentially function as a tumor suppressor. Taken together, our study suggests the presence of multiple deregulated miRNA networks in RMS and the RMS state is stabilized by the deregulation of multiple miRNAs and their target genes, supporting a tumor suppressor role for these miRNA.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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