Regulation of Target Genes of PAX3–FOXO1 in Alveolar Rhabdomyosarcoma

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Abstract. Background: The majority of alveolar rhabdomyosarcoma (ARMS) are distinguished through the paired box 3–forkhead box protein O1 (PAX3–FOXO1) fusion oncprotein, being generated by a 2;13 chromosomal translocation. This fusion-positive ARMS is the most clinically difficult type of rhabdomyosarcoma. The present study characterized four genes (gremlin 1 (GREM1), death-associated protein kinase-1 (DAPK1), myogenic differentiation-1 (MYOD1), and hairy/enhancer-of-split related with YRPW motif-1 (HEY1)) as targets of PAX3–FOXO1. Materials and Methods: The expression of the four genes, PAX3–FOXO1, and v-myc myelocytomatosis viral-related oncogene, neuroblastoma-derived (avian) (MYCN) was determined in various ARMS cell models and primary tumors. The roles of PAX3–FOXO1 and MYCN expression were examined. Results: Pulse-chase and cycloheximide experiments suggest that GREM1, DAPK1, and MYOD1 are directly regulated by PAX3–FOXO1. PAX3–FOXO1 appears to indirectly down-regulate HEY1 by up-regulating MYCN. Data reveal that the growth-suppressive activity of high PAX3–FOXO1 expression is closely-associated with up-regulation of the GREM1 and DAPK1 tumor-suppressor genes. Conclusion: This study characterized four downstream targets of PAX3–FOXO1 that contribute to the biological activities of growth suppression and myogenic differentiation.

Rhabdomyosarcoma is the most prominent type of pediatric soft tissue sarcoma, being associated with the skeletal muscle lineage. Among pediatric rhabdomyosarcoma, there are two major subtypes based on their histological appearance: embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) (1). ARMS has a higher frequency of metastases at the initial diagnosis than ERMS, commonly conferring a poorer prognosis than ERMS (2, 3). A common characteristic of ERMS is a loss of heterozygosity at 11p15, however ERMS has not been reported to exhibit a diagnostic genetic alteration. In contrast, chromosomal translocation is frequently observed for ARMS (4, 5). The translocation t(2;13)(q35;q14) generating the paired box 3–forkhead box protein O1 (PAX3–FOXO1) gene fusion was found to occur in 55% of ARMS cases, while the translocation t(1;13)(q36;q14) generating the paired box 7–forkhead box protein O1 (PAX7–FOXO1) gene fusion occurred in 22% of cases, and 23% of ARMS were fusion-negative (2). The 2;13 translocation present in ARMS is characterized by an overexpression of PAX3–FOXO1 relative to wild-type PAX3 (6).

Although PAX3–FOXO1-positive ARMS is the most clinically intractable fusion subtype of pediatric rhabdomyosarcoma, it can be difficult to histologically classify rhabdomyosarcoma into ARMS and ERMS in some cases (7), and no specific drugs are available for treating a specific fusion or histological subtype (1, 2, 7, 8). Therefore, there is a strong incentive to elucidate target genes of PAX3–FOXO1, which has the utility of being a therapeutic target and marker for the purpose of diagnosis and treatment of rhabdomyosarcoma. Several recent studies have utilized the gene expression profiles to classify rhabdomyosarcoma or identify target genes of only PAX3–FOXO1, or both PAX3–FOXO1 and PAX7–FOXO1, however, more research is needed to validate the biological function of the genes in ARMS development (9-15).

Recently Ahn and co-authors identified 34 potential target genes and validated the four genes, gremlin-1 (GREM1), death-associated protein kinase-1 (DAPK1), myogenic differentiation (MYOD1), and hairy/enhancer-of-split related with YRPW motif-1 (HEY1), as target genes of PAX3–FOXO1 in ARMS by analyzing gene.
expression profiles from two independent systems: primary tumors (PAX3–FOXO1-positive ARMS and fusion-negative ERMS) and a cell culture system expressing the inducible PAX3–FOXO1-estrogen receptor (ER) ligand binding domain construct (40). The present study focused on these four genes in order to investigate whether these target genes are directly or indirectly regulated by PAX3–FOXO1, the nature of interactions between target genes of PAX3–FOXO1, and whether specific targets of PAX3–FOXO1 are associated with growth suppressive activity of high PAX3–FOXO1 expression.

Materials and Methods

Tumor samples. Tumor specimens used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (13 PAX3–FOXO1-positive ARMS) were described previously (14). The presence of PAX3–FOXO1 in ARMS tumor specimens was confirmed by qRT-PCR (16).

Cell culture and cell growth assay. RD ERMS cell line and RD-derived PAX3–FOXO1 and PAX3–FOXO1-ER-positive cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (HyClone Thermo Scientific, Logan, UT, USA), 1% penicillin:streptomycin (P/S) (Invitrogen) and 1% antibiotic-antimycotic (AM) (Invitrogen). For cell growth experiments, 2×10⁵ cells constitutively expressing PAX3–FOXO1 in pK1 (pK-P3F) and 2×10⁶ cells carrying the pK1 vector alone (pK) were plated per well in six-well plates, trypsinized at the indicated time points, and viable cells were then counted.

DNA constructs. Retroviral constructs with relevant DNA inserts [inducible PAX3–FOXO1 in pK1 with puromycin resistance marker (pK-P3F-ER), constitutive PAX3–FOXO1 in pK1 with puromycin resistance marker (pK-P3F) and v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) (MYCN) in pK1 with hygromycin resistance marker (pKH-MYCN)] were previously generated by Dr. Frederic G. Barr’s laboratory (17-20). A modified ER ligand-binding domain was provided by Dr. G. Evan (21).

Establishment of ERMS cell culture systems inducibly or constitutively expressing PAX3–FOXO1 and ERMS cells overexpressing MYCN. Retroviral transduction was performed as described previously with modifications (19, 20). Cells that were transduced with retroviral DNA constructs were selected with puromycin (BD Biosciences, San Jose, CA, USA) at: 1 μg/ml for RD-derived cells with PAX3–FOXO1-ER in pK1 and 0.8 μg/ml for RD-derived cells with PAX3–FOXO1 in pK1. Cells carrying inducible PAX3–FOXO1-ER were treated with the ligand 4-hydroxytamoxifen (TMF) (Sigma-Aldrich, St. Louis, MO, USA), which activates and induces transcriptional activity of PAX3–FOXO1-ER by translocating PAX3–FOXO1-ER to the nucleus (18). RD cells were transduced with MYCN in pK1 with the hygromycin resistance marker (pKH-MYCN) or with pH vector only (pKH) and then were selected by incubation with hygromycin B (Roche Diagnostics, Indianapolis, IN, USA) at 250 μg/ml.

Results

PAX3–FOXO1 directly regulates GREM1, DAPK1, and MYOD1, while HEY1 may be indirectly regulated. The two approaches were followed to determine whether GREM1, DAPK1, MYOD1, and HEY1 are direct transcriptional targets regulated by PAX3–FOXO1. Firstly, pulse-chase time course experiments were conducted to determine how quickly the maximum expression change was achieved. RD ERMS-derived cells expressing an inducible PAX3–FOXO1-ER in pK1 (pK-P3F-ER) or pK1 vector-alone (pK) were treated without or with 100 nM TMF for one hour. Then TMF was removed and cells were cultured with medium for 3, 6, 12, and 24 h. The expression of GREM1, DAPK1, MYOD1, and HEY1 was determined by qRT-PCR at each time point (Figure 1). In RD cells expressing pK-P3F-ER, the maximum up-regulation of GREM1 (Figure 1A), DAPK1 (Figure 1B), and MYOD1 (Figure 1C) was achieved at 6 h. In contrast, the maximum down-regulation of HEY1 was observed at 12 hours (Figure 1D).

In the second approach, RD ERMS cells expressing an inducible PAX3–FOXO1-ER construct in pK1 (pK-P3F-ER) or pK1 vector-alone (pK) were treated with both

RNA extraction and qRT-PCR analysis. Total RNA was extracted using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA). The qRT-PCR assay was performed as described previously (14). The expression of test genes was normalized to the expression of 18S ribosomal RNA (18S rRNA). Taqman gene expression assays used (Applied Biosystems, Foster City, CA, USA) were: DAPK1 (assay ID# Hs00344891_m1), GREM1 (assay ID# Hs00171951_m1), and MYOD1 (assay ID# Hs00159528_m1) and sequences of these gene expression assays are not disclosed by Applied Biosystems. The sequences of forward and reverse primers and probes are for PAX3–FOXO1 [5’ CCT CAA CCM CAT GAA CCC3’ (M: either A or C); 5’ CCT TCA TTC TGC ACA CGA ATG A 3’; 5’ VIC TGG CAA TGG CCT TCT ACC TCA GAA TT TAMRA 3’], for HEY1 [5’ TGA CCG TGG ATC ACC TGA AA 3’; 5’ GCG TGC GCG TCA AAG TAA 3’; 5’ FAM TGC TGC ATA CGG CAG GAG GGA AAG TAMRA 3’], and for MYCN [5’ ACC ACA AGG CCC TCA GTA CCT 3’; 5’ AGC TGG ATT TCT TCT TCA TCT 3’; 5’ FAM CTC TTC ATC ATC TTC ATC ATC TGA ATC GTA ATC CA TAMRA 3’].

Extraction of cellular proteins and immunoblot analysis. The RD cells transduced with pKH or pKH-MYCN were seeded at 10⁵ cells per 100 mm dish in DMEM containing 10% FBS, 1% P/S, and 1% AM for 24 hours prior to hygromycin B treatment. Cellular proteins were extracted and immunoblot analysis was performed as described previously with modifications (22). MYCN (cat# sc-791) and actin (sc-1616) used as primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis. The correlation between MYCN and HEY1 expression levels determined by qRT-PCR in 13 PAX3–FOXO1-positive ARMS tumors was analyzed by Pearson’s correlation coefficient after log-transformation of gene expression values.
cycloheximide (a de novo protein synthesis inhibitor) and TMF for 24 h following one hour pre-treatment with cycloheximide (Figure 2). If the gene is a direct target, synthesis of intermediate transcription factors or other proteins will not be required to regulate the gene’s expression. Therefore, even in the presence of cycloheximide, the down-regulation or up-regulation of specific PAX3−FOXO1 target genes will still occur. Data indicated that even in the presence of cycloheximide, PAX3−FOXO1 induced by TMF resulted in the up-regulation of GREM1 (Figure 2A), DAPK1 (Figure 2B), and MYOD1 (Figure 2C).

Although HEY1 was down-regulated in the presence of cycloheximide and TMF (Figure 2D), a puzzling result was found for the effect of cycloheximide alone on HEY1 expression (Figure 2E). While cells transduced with the PAX3−FOXO1-ER in the pK1 vector showed no change in HEY1 expression in the presence of cycloheximide alone (Figure 2D), there was a significant decrease in HEY1 expression in cycloheximide-treated cells transduced with the pK1 vector only (Figure 2E). These findings suggest that cycloheximide inhibits the expression of a factor needed for HEY1 expression in these ERMS cells transduced with pK1, and that unstimulated PAX3−FOXO1-ER (control: untreated with TMF) is associated with an activity which reverses this inhibitory effect of cycloheximide on HEY1 expression. Based on these findings, it is unclear whether the effect seen in PAX3−FOXO1-ER-transduced cells treated with cycloheximide and TMF represents maintenance of the TMF-induced down-regulation of HEY1. Therefore, the results of this cycloheximide experiment for HEY1 cannot be used to evaluate whether HEY1 is a direct target of PAX3-FOXO1.

Taken together, both pulse-chase and cycloheximide experiments suggest that GREM1, DAPK1, and MYOD1 are directly regulated by PAX3−FOXO1. In contrast, after
excluding the cycloheximide result for HEY1, the longer time requirement for HEY1 down-regulation (Figure 2D) suggests the possible existence of intermediate transcription factor(s) that regulate HEY1 and must first be induced by PAX3−FOXO1.

PAX3−FOXO1 indirectly down-regulates HEY1 by up-regulating MYCN. Since results from the pulse-chase time course experiment suggests that HEY1 may be indirectly regulated by PAX3−FOXO1, further experiments were conducted to identify which gene(s) induced by PAX3−FOXO1 are also able to down-regulate HEY1 expression. We hypothesized that MYCN up-regulation induced by PAX3−FOXO1 may result in HEY1 downregulation, based on published reports. First, PAX3−FOXO1 up-regulated MYCN (15) and MYCN synergistically cooperated with PAX3−FOXO1 to enhance oncogenic activity (15, 23, 24). Second, expression profiles and RT-PCR data demonstrated that tumors from transgenic mice expressing MYCN were associated with decreased HEY1 levels.
overexpressing c-myc, that was targeted to the mammary gland, showed down-regulation of hey1 (25).

This hypothesis of HEY1 regulation by MYCN was tested by two experimental approaches: First, it was examined whether an inverse correlation exists between MYCN and HEY1 expression in a series of thirteen PAX3–FOXO1-positive ARMS primary tumors determined using qRT-PCR (Figure 3A). The Pearson’s correlation coefficient for HEY1 versus MYCN expressions was -0.7032, indicating that the MYCN expression was inversely correlated with HEY1 expression (Figure 3A). Second, a population of RD ERMS cells overexpressing MYCN (pKH-MYCN) was established (Figure 3B), and the expression of HEY1 in these cells by qRT-PCR was determined. These RD cells overexpressing MYCN (pKH-MYCN) showed about two-times lower HEY1 expression as compared with that of the RD cells carrying the vector pK1-Hyg-alone (pKH) (Figure 3C). These results are consistent with the hypothesis that PAX3–FOXO1 down-regulates HEY1 by up-regulating MYCN, and further support that HEY1 is an indirect downstream target of PAX3–FOXO1.

Growth suppression caused by high PAX3–FOXO1 expression is associated with up-regulation of GREM1 and DAPK1. Xia et al. reported that PAX3–FOXO1, when constitutively expressed at a level comparable to the expression levels found in ARMS tumors, is growth suppressive in immortalized murine NIH3T3 fibroblasts (19, 20). I hypothesized that the GREM1 and DAPK1 tumor-suppressor genes may contribute to this growth-suppressive activity of high PAX3–FOXO1 expression.

To test this hypothesis, RD cells were transduced with a construct in which PAX3–FOXO1 is constitutively expressed from the pK1 vector. As a control, RD cells were transduced with pK1 vector-alone and demonstrated continued growth over time (Figure 4A). In contrast, although RD cells transduced with PAX3–FOXO1 (Figure 4B) were seeded at a 10-times higher number than the control RD cells (Figure 4A), the growth of PAX3–FOXO1-expressing cells declined for the first 5 to 7 days (Figure 4B). At the later periods of 10, 14, and 21 days, these PAX3–FOXO1-expressing control cells (Figure 4B-Inset) recovered and grew at a similar proliferation rate as the pK1-transduced cells (Figure 4A-Inset). This initial period of growth decline (Figure 4B) coincided with the period of high PAX3–FOXO1 expression (Figure 4C). The PAX3–FOXO1 expression was higher during the early period, peaking at 5 and 7 days. It then went down and plateaued at approximately half the peak level (Figure 4C). It is noted that this growth suppressive period (3, 5, 7 days) with high PAX3–FOXO1 expression coincided with the period for higher expression of GREM1 (Figure 4D) and DAPK1 (Figure 4E). These findings are consistent with the hypothesis that GREM1 and DAPK1 are downstream targets responsible for growth suppressive effects of high PAX3–FOXO1 expression.

In contrast, the induced high expression of MYOD1 (Figure 4F) was similar during all periods. The expression of HEY1 (Figure 4G) stayed consistently low throughout all the periods and was the least affected by changes in PAX3–FOXO1 levels. These expression findings imply that different target genes of PAX3–FOXO1 have different dose-response effects. After the initial PAX3–FOXO1 down- and up-regulation event, HEY1 and MYOD1 are not sensitive to further changes in PAX3–FOXO1 expression ranges tested in this model, whereas GREM1 and DAPK1 are very sensitive to these PAX3–FOXO1 fluctuations.

Discussion

The current study demonstrates that PAX3–FOXO1 directly up-regulates GREM1, DAPK1, and MYOD1. This result is supported, in part, by the study of Cao et al., which showed that DAPK1 and MYOD1 genes are associated with proximal PAX3–FOXO1 binding sites, implying these genes as direct targets of PAX3–FOXO1 binding sites, implying these genes as direct targets of PAX3–FOXO1 (26).

To my knowledge, the present study is the first to demonstrate that PAX3–FOXO1 down-regulates HEY1 by up-regulating MYCN. Previous studies revealed a role of HEY1 in muscle differentiation. For example, overexpression of HEY1 inhibits MYOD1, an early myogenic differentiation marker, possibly through formation of inactive HEY1/MYOD1 heterodimers (27). Notch activation robustly induces the transcriptional repressors HEY1 and hairy/enhancer-of-split related with YRPW motif-like (HEYL) in myoblasts, but only the constitutive expression of HEY1 blocks myogenesis (28).

As demonstrated in some studies, PAX3–FOXO1 may facilitate early steps in myogenesis and thus repression of HEY1 expression is consistent with the PAX3–FOXO1-induced myogenic developmental program such as up-regulation of MYOD1.

Pulse-chase time course and cycloheximide experiments indicate that PAX3–FOXO1 directly up-regulates MYOD1. These findings are consistent with results reported in previous studies of MYOD1 expression in human ARMS (29, 30), PAX3/PAX7–FOXO1-positive cells (12), mesenchymal stem cells transduced with PAX3–FOXO1 (31), and NIH3T3 fibroblasts transduced with PAX3–FOXO1 (9, 32). The finding of PAX3–FOXO1 binding to the MYOD core enhancer in transduced NIH3T3 fibroblasts (32) and rhabdomyosarcoma cells (26) signified a direct transactivation of MYOD1 by PAX3–FOXO1. It is hypothesized that PAX3–FOXO1 up-regulates MYOD1 which causes a reinforcement of myogenic determination, while simultaneously suppressing terminal myogenic differentiation. Thus, ARMS cells remain in undifferentiated malignant states (33, 34). Evidence supporting this
hypothesis includes the finding of PAX3−FOXO1 inhibition of terminal differentiation in C2C12 myoblasts and MYOD-expressing 10T1/2 fibroblasts (33). Use of a siRNA to decrease for PAX3−FOXO1 expression in an ARMS cell line resulted in up-regulation of genes related to terminal myogenic differentiation (34).

The oncogenic activity of PAX3−FOXO1 is demonstrated, in part, by its stimulatory effects on cell proliferation (35, 36, 37), cell survival/anti-apoptosis (38, 39), as well as its inhibitory role on terminal myogenic differentiation (1, 33, 34). However, in addition to these effects, PAX3−FOXO1 can also trigger growth suppression and cell death in other settings (19, 20, 24). This paradoxical feature of PAX−FOXO1 being both growth-suppressive and oncogenic was shown in previous studies with immortalized murine fibroblasts and human myoblasts (19, 20, 24). These findings led me to postulate that chromosomal translocation and PAX3−FOXO1 fusion formation may first result in low fusion expression. This early step would be followed by activation of other transcriptional regulators or genetic alternations that block growth suppression pathways and permit later high PAX3−FOXO1 expression (20), which exerts additional oncogenic and tumorigenic activities (24).

The current study presents novel evidence that the up-regulation of GREM1 and DAPK1 is associated with the growth suppressive activity of high PAX3−FOXO1 expression. The growth inhibition caused by high PAX3−FOXO1 expression concurrently occurred with the high expression of GREM1 and DAPK1. When PAX3−FOXO1 expression was diminished, the expression of GREM1 and DAPK1 was also reduced. These findings are consistent with the hypothesis that high PAX3−FOXO1 expression inhibits cellular growth by up-regulating GREM1 and DAPK1.

In conclusion, the present study has established the basis to propose the four genes (GREM1, DAPK1, MYOD1, and HEY1) as targets that function in growth suppression or myogenic differentiation downstream of PAX3−FOXO1 in ARMS. These studies to define the biological function of PAX3−FOXO1 and its target genes and the associated phenotypes of ARMS will be useful in developing therapeutic targets as well as diagnostic and/or prognostic molecular markers and characterizing biological properties of PAX3−FOXO1-positive ARMS.

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References

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