Identification of PAX3-FKHR-Regulated Genes Differentially Expressed Between Alveolar and Embryonal Rhabdomyosarcoma: Focus on *MYCN* as a Biologically Relevant Target

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Rhabdomyosarcoma is a family of myogenic soft tissue tumors subdivided into two main subtypes: alveolar (ARMS) and embryonal (ERMS). ARMS is characterized by a frequent 2;13 chromosomal translocation that creates a PAX3-FKHR fusion transcription factor. To identify downstream targets of PAX3-FKHR, we introduced an inducible form of PAX3-FKHR into human RD ERMS cells. Microarray analysis identified 39 genes (29 upregulated and 10 downregulated) that are modulated by PAX3-FKHR in RD cells and differentially expressed between ERMS and PAX3-FKHR-positive ARMS tumors. Functional annotation demonstrated that genes involved in regulation of transcription and development, particularly neurogenesis, are represented in this group. *MYCN* was one notable neural-related transcription factor-encoding gene identified in this set, and its regulation by PAX3-FKHR was further confirmed at the RNA and protein levels. The findings of cycloheximide inhibition and time-course studies are consistent with the hypothesis that the PAX3-FKHR protein acts directly on the *MYCN* gene at the transcriptional level. Functional studies established that MYCN cooperates with PAX3-FKHR to enhance oncogenic activity. In conclusion, we identified a selected set of biologically relevant genes modulated by PAX3-FKHR, and demonstrated that PAX3-FKHR contributes to the expression of MYCN and in turn MYCN collaborates with PAX3-FKHR in tumorigenesis.

INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue tumor (Gurney et al., 1995). Alveolar (ARMS) and embryonal (ERMS) are the main histopathologic subtypes of RMS, and show different clinical behaviors and genetic features (Parham, 2001). All subtypes show evidence of skeletal muscle differentiation, and are proposed to originate due to deregulated myogenic differentiation and growth pathways.

Most ARMS tumors are characterized by recurrent chromosomal translocations. The frequent translocation t(2;13)(q35;q14) is present in 55% of cases while the variant translocation t(1;13)(p36;q14) is present in 22%, and 23% are fusion-negative (Sorensen et al., 2002). The 2;13 and 1;13 translocations generate gene fusions that encode chimeric transcription factors consisting of the DNA binding domain of PAX3 or PAX7 fused to the transactivation domain of FKHR (FOXO1) (Barr, 2001).

The formation of the chimeric transcription factor is a key step in ARMS tumorigenesis. Previous studies suggest a gain of function with deregulated expression of downstream targets, which participate in pathways related to proliferation, survival, differentiation, and migration (Barr, 2001). A few genes have been defined as possible PAX3-FKHR targets, including *MET*, *PDGFRA*, *BCL2L1*, *CXCR4*, and *TFAP2B* (Epstein et al., 1998;

Supported by: NIH; Grant numbers: CA64202 (FGB), CA87812 (GEM), CA104896 (FGB), CA106450 (ML); Alveolar Rhabdomyosarcoma Research Fund; University Research Foundation; Children's Oncology Group Chair's Development Fund; Joanna McAfee Childhood Cancer Foundation; Consejo Nacional de Ciencia y Tecnología (CONACYT); Universidad Nacional Autónoma de México (UNAM); National Institute of Genomic Medicine, Mexico (INMEGEN).

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Received 4 October 2007; Accepted 4 February 2008
DOI 10.1002/gcc.20554
Published online 11 March 2008 in Wiley InterScience (www.interscience.wiley.com).

Ginsberg et al., 1998; Margue et al., 2000; Tomescu et al., 2004; Ebauer et al., 2007). In the present study, we used gene-expression profiling of two complementary systems, an inducible cell culture system and a panel of ARMS and ERMS tumors, to identify a new set of PAX3-FKHR target genes pertinent to ARMS pathogenesis. Although downstream PAX3-FKHR target genes were investigated in other recent cell culture studies (Davicioni et al., 2006), this inducible system has the advantage of identifying early genes induced by PAX3-FKHR, and these early genes are likely to be direct targets of this chimeric transcription factor.

MATERIALS AND METHODS

Tumor Samples and Cell Lines

Thirty-one frozen primary tumor specimens (15 ERMS and 16 ARMS) were used for microarray profiling and were described previously (Lae et al., 2007). An independent set of 38 RMS samples (19 ARMS and 19 ERMS) were used for PCR validation. The samples were derived from residual pathology specimens removed for diagnostic and/or therapeutic purposes from the Children's Hospital of Philadelphia, Memorial Sloan Kettering Cancer Center or the Hospital of the University of Pennsylvania. Additional tumor material was provided by the Cooperative Human Tissue Network. Each tumor sample used for microarray analysis was confirmed histopathologically to have >70% tumor cells. PAX3-FKHR and PAX7-FKHR were detected by either reverse transcription-polymerase chain reaction (RT-PCR) and differential hybridization after slot blotting (Barr et al., 1995) or real-time RT-PCR with specific probes (Barr et al., 2006).

The RD and SMS-CTR ERMS cell lines were maintained in Dulbecco's modified eagle medium (DMEM; Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS; HyClone, Logan, UT), and the RH6 and RH36 ERMS cell lines were maintained in DMEM with 15% FBS. The media were supplemented with penicillin, streptomycin, and fungizone (Gibco).

Inducible System, Plasmid Transfection, and Retroviral Transduction

The construct for the inducible form of the PAX3-FKHR fusion protein (P3FK/ER) was described previously (Tomescu et al., 2004). This construct was prepared by fusing PAX3-FKHR with the modified estrogen receptor ligand binding domain (Littlewood et al., 1995). Retroviral transduction of P3FK/ER in pK1 or vector controls was performed as described previously (Xia and Barr, 2004) to infect the ERMS cell lines. Cells selected in puromycin (1 μg/ml) were expanded for a limited number of population doublings before use in individual experiments. This procedure was repeated to generate multiple, independent transduced populations for P3FK/ER and for the controls with the empty vector pK1. To activate P3FK/ER, the cells were treated with the ligand 4-hydroxytamoxifen (4-OHT), which results in the transfer of P3FK/ER to the nucleus and induction of transcriptional activity.

RNA Extraction, Labeling, and Hybridization

For microarray analysis, RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). For validation studies, the RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX). For microarray studies, labeling and hybridization were performed according to Affymetrix standard protocols. As was previously performed for the tumor samples (Lae et al., 2007), labeled cRNA from the inducible system was hybridized onto the Affymetrix GeneChip Human Genome U133A array (Santa Clara, CA), which contains 22,215 probes. Microarray slides were scanned using the Affymetrix GCS 2500 scanner to collect fluorescence signal.

Microarray Data Analysis

Affymetrix Microarray Suite 5.0 was used to measure expression levels, and default values were applied to all analysis parameters. Data files were uploaded into GeneSpring (Agilent Technology, Santa Clara, CA), and normalization of expression levels of all spot replicates was performed. Probe sets were initially filtered to select only those with a present or marginal call in at least one sample. This yielded 17,605 probe sets, which contain most of the variation in gene expression information across all samples. Identification of differentially expressed genes between ARMS and ERMS samples was performed using significance analysis of microarray (SAM) software with default settings (Tusher et al., 2001). The statistical test Mixed ANOVA (Partek Pro, St. Louis, MO) was used to analyze the data from the inducible cell culture system. Functional annotation was assigned through GENATLAS (www.genatlas.org) and previously published literature. Gene classification was obtained through the web-accessible functional annotation tool DAVID 2007 (http://david.abcc.ncifcrf.gov/; Dennis et al., 2003).

Quantitative RT-PCR Analysis

cDNA synthesis from total RNA samples was performed using MultiScribe reverse transcriptase with random hexamers (Applied Biosystems, Foster City, CA). The cDNA was quantified by quantitative RT-PCR (qRT-PCR) using the 7900 Sequence Detection System (Applied Biosystems) and TaqMan Universal PCR master mix (Applied Biosystems). The sequences for the primer and probes for KCNN3 and MYCN were published previously (Tomita et al., 2003; Williamson et al., 2005). Gene expression levels were normalized using the level of 18S RNA. Each sample was tested in triplicate, and data are expressed as mean ± SD. Statistical differences between groups were analyzed by the Mann–Whitney test. Statistical significance was accepted at the $P < 0.05$ level (two-tailed).

Reporter Assay

Transcriptional activity was measured with the Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Madison, WI), performed according to the manufacturer's instructions. MYCN promoter constructs were provided by Dr. W. Carroll.

Western Blot Analysis

Protein lysates were prepared and assayed by Western blot analysis as described previously (Xia and Barr, 2004). The proteins were detected using antibodies directed against PAX3 (Abcam, Cambridge, MA) and MYCN (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Microarray Analysis

To identify PAX3-FKHR downstream targets that are relevant in ARMS tumors, we compared the gene expression profiles of two independent sets of samples. To study the degree to which PAX3-FKHR accounts for differences between ARMS and ERMS, we expressed an inducible form of PAX3-FKHR (P3FK/ER) in an ERMS cell culture system. In particular, three populations expressing P3FK/ER were treated with or without 30 nM 4-OHT and three populations transduced with vector only (pK1) were similarly treated. Use of this ERMS cell line permits examination of the effect of PAX3-FKHR expression in this closely related myogenic tumor background. Furthermore, the relevance of this ERMS cell line to tumor phenotype is shown in a nude rat injection model in which there is increased dissemination to bone sites, similar to ARMS metastasis, when these cells express PAX3-FKHR (LeGallo et al., 2005).

The other set of samples consisted of RNA isolates from 15 fusion-negative ERMS tumors and 16 PAX3-FKHR-positive ARMS tumors. Expression profiling and initial analysis of this set of tumors has been previously performed (Lae et al., 2007). Our overall strategy was to use an oligonucleotide-based microarray to determine the downstream PAX3-FKHR targets in the first system and the differences in gene expression between the two RMS subtypes in the second system. Then these two sets of data could be compared in a final step to find genes induced by PAX3-FKHR that are differentially expressed between ERMS and fusion-positive ARMS.

To identify PAX3-FKHR regulated genes in the inducible cell culture system, we analyzed the gene expression data using the mixed ANOVA statistical test, which is a factorial design that includes both between and within subjects variables. This design consisted of one within subject variable ("treatment"), with two levels (0 and 30 nM), and one between subjects variable ("construct"), with two levels (vector control and P3FK/ER). Genes were selected for further analysis if the P value was less than 0.01, and there was at least a 1.5-fold expression difference between the two conditions, P3FK/ER-treated (30 nM) and P3FK/ER-untreated (0 nM). Based on these parameters, this analysis revealed a total of 361 genes (178 upregulated and 183 downregulated). To validate the specificity of the PAX3-FKHR targets, we also compared gene expression between 4-OHT treated and untreated control RD pK1 cells as well as between RD pK1 controls and RD P3FK/ER cells, and did not find any statistically significant genes from the previous analyses.

SAM was used to identify genes that are differentially expressed between the 16 PAX3-FKHR-positive ARMS and 15 fusion-negative ERMS tumors, using our previously published expression profiling dataset (Lae et al., 2007). SAM uses a permutation-based multiple testing algorithm and identifies significant genes with a variable false discovery rate (FDR; Tusher et al., 2001). Filtering was performed using a FDR less than 5%, and a fold change of at least of 1.5. Using these conditions, there were 1,085 probe sets differentially expressed between the two morphological subtypes, 779 genes overexpressed in ARMS and 306 overexpressed in ERMS.

On the basis of these findings, we identified the genes that were both differentially expressed in the inducible cell culture system and between

TABLE 1. PAX3-FKHR Target Genes Identified by Microarray Analysis

		Fold change	
Affy ID	Common	Inducible	Tumors
A. Upregulated genes			
218468_s_at	GREM1	6.61	2.58
203680_at	PRKAR2B	5.75	2.99
209757_s_at	MYCN	3.31	4.05
49111_at	DKFZp762M127	3.69	4.69
204851_s_at	DCX	3.26	8.23
205903_s_at	KCNN3	2.57	8.80
203510_at	MET	2.38	2.47
203962_s_at	NEBL	2.35	2.69
210794_s_at	MEG3	2.28	7.91
209656_s_at	TM4SF10	2.23	2.39
218145_at	TRIB3	2.10	2.14
206306_at	RYR3	2.09	4.64
222186_at	DKFZp686F02110	2.03	1.51
203725_at	GADD45A	2.02	2.33
202517_at	CRMP1	1.95	3.61
205935_at	FOXF1	1.85	4.86
207231_at	DZIP3	1.76	2.39
206657_s_at	MYOD1	1.75	3.78
212761_at	TCF7L2	1.74	2.04
209123_at	QDPR	1.72	3.68
203139_at	DAPK1	1.72	2.02
211042_x_at	MCAM	1.69	3.49
218007_s_at	RPS27L	1.66	1.99
218829_s_at	CHD7	1.63	2.20
35671_at	GTF3C1	1.65	1.52
209105_at	NCOA1	1.61	2.38
212585_at	OSBPL8	1.60	1.61
213256_at	MARCH3	1.54	3.00
204458_at	LYPLA3	1.50	1.53
B. Downregulated genes			
204014_at	DUSP4	4.30	2.41
210095_s_at	IGFBP3	2.46	2.63
218839_at	HEY1	2.10	2.70
213479_at	NPTX2	2.05	7.41
202670_at	MAP2K1	2.01	1.62
201368_at	ZFP36L2	1.81	1.90
210839_s_at	ENPP2	1.72	2.49
206114_at	EPHA4	1.61	4.03
205372_at	PLAG1	1.50	5.96
204301_at	KIAA0711	1.55	4.51

ARMS and ERMS. To assure that the expression values were consistent in most tumor samples, we filtered the genes based on the raw expression values. Upregulated genes were selected if the raw expression value was greater than 150 in at least 14 of 16 ARMS tumor samples, and for downregulated genes, the genes were selected if the raw expression value was greater than 150 in 13 of 15 ERMS. After removing probe sets that belonged to the same gene and showed similar gene expression profiles, a nonredundant set of 39 genes was obtained, of which 29 were upregulated and 10 were downregulated (Table 1).

TABLE 2. DAVID Functional Annotation

Biological process	Count	*P* value
Transcription regulator activity	10	8.9 E −4
Protein kinase activity	6	6.3 E −3
Nervous system development	5	1.6 E −2
Cell communication	14	1.8 E −2
Development	9	3.3 E −2
Signal transduction	12	5.6 E −2

To validate the results of the microarray analysis, seven genes (*KCNN3*, *MET*, *RYR3*, *DCX*, *TRIB3*, *FOXF1*, and DKFZp762M127) were examined by qRT-PCR. First we verified that all genes were induced in an independent dose–response experiment with the inducible cell culture system (data not shown). We also evaluated the expression of these genes by qRT-PCR in an independent set of 38 RMS tumor samples (19 PAX3-FKHR-positive ARMS and 19 ERMS); three of these genes were examined in a previous study (Lae et al., 2007) and the remainder were examined in the current study (data not shown). Of the seven genes examined, all were confirmed to be differentially expressed between the two RMS subtypes except MET, which did not show a significant statistical difference between ARMS and ERMS. Of note, previous studies also showed MET overexpression in both ERMS and ARMS tumors (Taulli et al., 2006).

Functional Annotation

The set of genes that exhibited either increased or decreased expression levels was analyzed for significant enrichment with respect to various categories of gene function using the DAVID 2007 annotation software. Categories corresponding to biological processes or molecular function that achieved the highest degree of significance are shown in Table 2. The analysis pointed to three major groups of genes whose expression was altered by PAX3-FKHR: (a) genes participating in development, (b) genes encoding protein kinases, and (c) genes involved in transcriptional regulation. Analysis of the genes in the category of development specifically pointed to proteins that participate in nervous system development. These genes included *GREM1*, *KCNN3*, *HEY1*, *CRMP1*, and *DCX*. Examination of the PAX3-FKHR-induced genes that participate in regulation of transcription indicated that 4 of these 10 genes encode products classified as helix–loop–helix transcriptional factors (bHLH); the genes included in this group are *NCOA1*, *HEY1*, *MYOD1*, and *MYCN*.

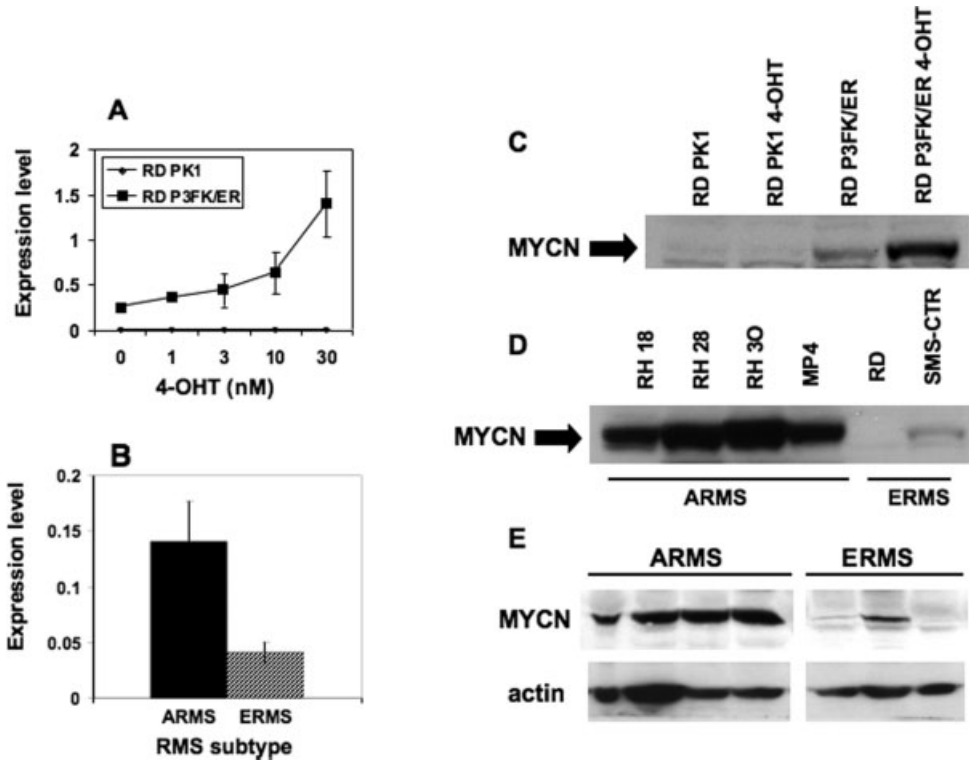

Figure 1. PAX3-FKHR induction of MYCN expression and relationship of MYCN expression to RMS subtype. A. RD ERMS cells were transduced with the inducible PAX3-FKHR construct (P3FK/ER) or vector control (pK1), and treated with the indicated concentration of 4-OHT. RNA was isolated 24 hr after induction, and MYCN transcript expression was measured by qRT-PCR. Values were normalized to corresponding 18S RNA expression levels and represent the ratio of the means (±SD) of triplicate measurements. B. MYCN mRNA was measured by qRT-PCR in an independent set of PAX3-FKHR-positive ARMS ($n = 19$) and fusion-negative ERMS ($n = 19$) tumor samples. The mean (±SD) of the mRNA levels in the two RMS subsets is shown. C. RD cells were transduced as in (A) and treated with 0 or 30 nM 4-OHT. Lysates were isolated 24 hr after induction, and MYCN protein expression was measured by Western blot analysis. D. MYCN protein expression was measured in lysates of the indicated ARMS and ERMS cell lines by Western blot analysis. E. MYCN protein expression was measured in lysates of four PAX3-FKHR-positive ARMS and three ERMS tumor samples by Western blot analysis.

MYCN Is Upregulated by PAX3-FKHR and Has a Higher Expression in ARMS

For further analysis of one target gene, we selected *MYCN*, which encodes a bHLH transcription factor normally functioning in neural development and also implicated in tumorigenesis (Thomas et al., 2004). As shown in Figure 1A, MYCN transcripts are regulated by PAX3-FKHR in a dose–response manner in the inducible RD cell culture system. To further confirm the induction of MYCN expression in a myogenic tumor environment, we transduced additional ERMS cell lines (SMS-CTR, RH6, and RH36) with the P3FK/ER construct. Treatment of these additional P3FK/ER-transduced ERMS cell lines with 4-OHT also showed an increase in MYCN expression (data not shown). Therefore, MYCN is upregulated by PAX3-FKHR in multiple, independent ERMS cell environments.

MYCN expression was determined by qRT-PCR in an independent set of 38 diagnostic primary RMS samples, 19 PAX3-FKHR-positive ARMS, and 19 ERMS. As shown in Figure 1B, the PAX3-FKHR-positive ARMS tumors expressed higher levels of MYCN mRNA than the fusion-negative ERMS cases. This difference in MYCN expression between the two subtypes was statistically significant ($P = 0.0004$).

We next used Western blot analysis to examine MYCN expression at the protein level in the inducible RD cell culture system, and in RMS cell lines and tumor samples. Protein lysates were harvested from RD cells incubated in the absence and presence of 30 nM 4-OHT for 24 hr. RD controls showed no MYCN protein expression, while untreated cells transduced with the P3FK/ER construct demonstrated a low basal level of MYCN protein expression, which was increased substantially by 4-OHT treatment (Fig. 1C). Analysis of MYCN protein expression in four ARMS and two ERMS cell lines revealed that MYCN protein expression is higher in the ARMS cell lines compared to the ERMS cell lines (Fig. 1D). Finally, examination of MYCN protein expression in seven

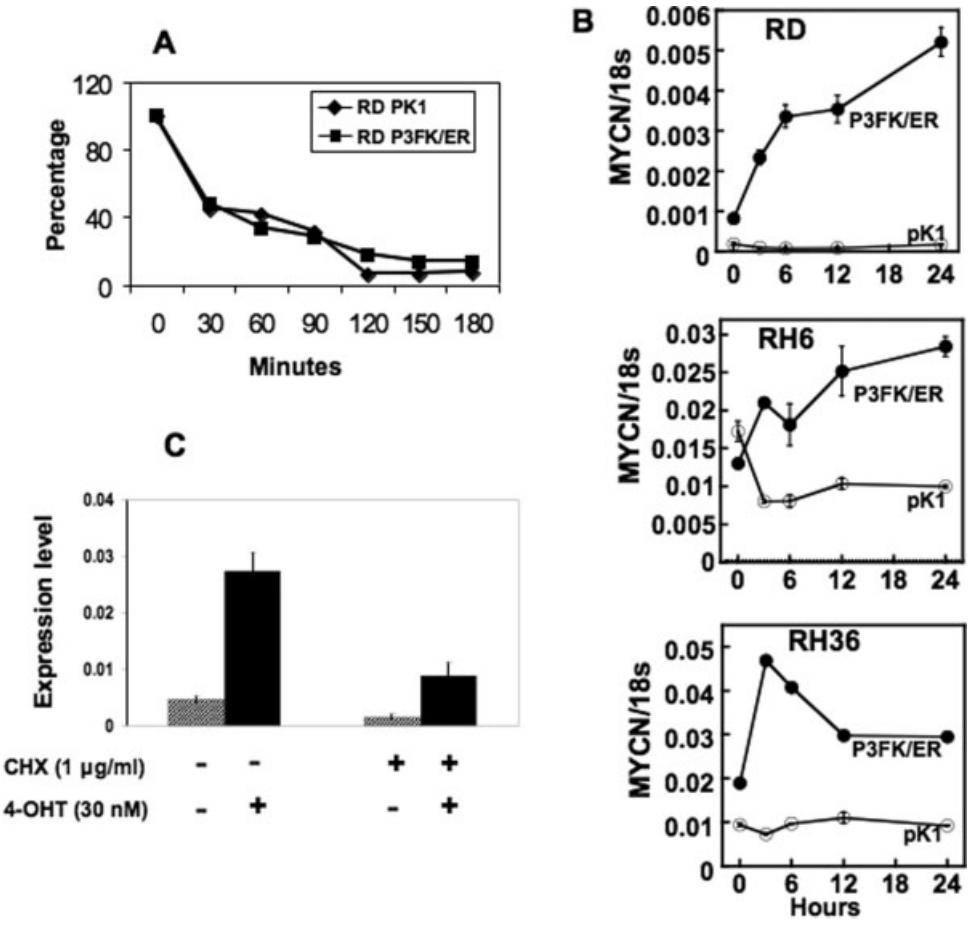

Figure 2. Studies of mechanism of PAX3-FKHR-induced MYCN expression. A. To measure the degradation rates of MYCN mRNA, 10^6 P3FK/ER- or pK1-transduced RD cells were seeded in 10-cm dishes. After 24 hr, 30 nM 4-OHT was added to the medium. After 24 hr of treatment, RNA synthesis was blocked by adding actinomycin D (5 µg/ml). Cells were harvested at the indicated time points following actinomycin D treatment and RNA was isolated. MYCN mRNA expression was measured by qRT-PCR as in Figure 1, and was shown as the percentage of MYCN expression at the beginning of actinomycin D treatment. B. To monitor the appearance of MYCN mRNA, ERMS cells (RD, RH6, and RH36) transduced with P3FK/ER or vector alone (pK1) were treated with 100 nM 4-OHT for 1 hr, washed with PBS, and then incubated with fresh medium. After isolating RNA at the indicated time points, MYCN mRNA expression was measured by qRT-PCR as in Figure 1. C. To evaluate the relationship of the action of PAX3-FKHR to the *MYCN* gene, P3FK/ER-transduced RD cells were incubated with or without CHX (1 µg/ml). After 1-hr incubation, cells were treated with or without 4-OHT for 24 hr. Total mRNA was isolated, and MYCN expression was measured by qRT-PCR as in Figure 1.

RMS tumor samples, consisting of four ARMS and three ERMS tumors, again demonstrated higher MYCN protein expression in the ARMS tumors than in the ERMS tumors (Fig. 1E).

MYCN mRNA Stability Is Not Modified by PAX3-FKHR

We next studied the mechanism by which PAX3-FKHR contributes to MYCN expression in ARMS, and investigated whether PAX3-FKHR augments MYCN mRNA at the level of mRNA degradation or production. First, we focused on degradation of MYCN mRNA and compared RD cells transduced with P3FK/ER or vector only, and treated with the transcriptional inhibitor actinomycin D, after incubation with 4-OHT (Fig. 2A). The amount of MYCN mRNA was measured by qRT-PCR in each cell population at time points after actinomycin D treatment. In two independent experiments, the MYCN expression pattern following actinomycin D treatment was nearly the same between P3FK/ER and vector-transduced cells. MYCN mRNA turnover was relatively rapid, with an average half-life of 30 min for both cell lines. These results suggest that the effect of PAX3-FKHR on MYCN expression is not posttranscriptional and thus direct attention to the possibility that this effect instead involves a transcriptional mechanism.

Analysis of *MYCN* as a Direct Target of PAX3-FKHR

To determine whether *MYCN* is a direct transcriptional target for PAX3-FKHR, we first

performed a pulse-chase time course experiment. RD, RH6, and RH36 cells expressing P3FK/ER were exposed to 4-OHT (100 nM) for 1 hr, washed with PBS to remove the inducing agent, and then incubated with medium. MYCN mRNA expression was determined by qRT-PCR at time points after this induction, and revealed that expression was increased as early as 3 hr in the three different systems. Of note, there are differences among the three cell lines in the ensuing expression pattern. In RD and RH6 cells, MYCN expression continued to increase up to at least 24 hr, while in RH36 cells, expression peaked at 3 hr and then decreased and plateaued to a lower level at 12 hr (Fig. 2B). This overall pattern of early increased expression is similar to other PAX3-FKHR targets, like MET and CXCR4 (data not shown). These findings are therefore consistent with the premise that PAX3-FKHR increases MYCN expression at the transcriptional level.

To distinguish between a direct and indirect effect of PAX3-FKHR on *MYCN* transcription, we performed cycloheximide (CHX) inhibition experiments. RD P3FK/ER cells were pretreated with the protein synthesis inhibitor CHX prior to treatment with (or without) the inducing agent 4-OHT. Measurement of the level of MYCN mRNA by qRT-PCR in the cells treated with and without CHX and/or 4-OHT thereby shows whether PAX3-FKHR induction of MYCN expression can occur without any additional protein synthesis. As shown in Figure 2C, CHX did not affect the overall PAX3-FKHR induction of MYCN. CHX pretreatment resulted in a decrease in basal MYCN expression in cells without 4-OHT treatment, suggesting the presence of a labile activator of MYCN expression. Although the basal level was lower, treatment of the cells with 4-OHT in the presence of CHX resulted in a sixfold induction of MYCN mRNA, equal to induction of MYCN mRNA by 4-OHT in the absence of CHX. These findings are consistent with the premise that the PAX3-FKHR protein acts directly on the *MYCN* gene.

To test whether PAX3-FKHR can bind to and transactivate regulatory elements in the *MYCN* gene, we used luciferase reporters coupled to different fragments of the 5′ region of the human *MYCN* gene (Hiller et al., 1991; Wada et al., 1992; Sivak et al., 1997, 1999). These reporters extended from −1871 to +1058 relative to the transcription start site, and included exon 1 and a portion of intron 1. In cells expressing the P3FK/ER construct and treated with increasing doses of 4-OHT, there was at most a small level of activation of 5′-*MYCN* reporter activity (16–27% increase over untreated; Fig. 3A), which did not account for the observed increase in RNA expression. Testing of reporter constructs with sequential 5′ deletions of the 5′ *MYCN* region still did not detect any difference in reporter activity (data not shown). These results indicate that this *MYCN* region could not recapitulate the PAX3-FKHR-mediated increase in MYCN expression.

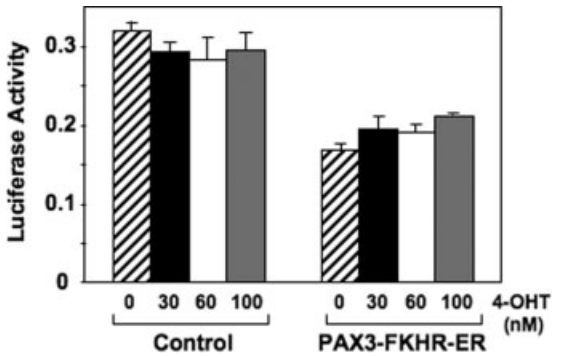

Figure 3. Reporter transfection analysis of the *MYCN* promoter. Reporter transfection analysis was performed with a firefly luciferase construct containing 2.0 kb 5′ *MYCN* region and the pRL-TK control construct constitutively expressing renilla luciferase (Promega). After seeding 5×10^4 P3FK/ER-expressing RD cells in 24-well dishes and incubating the cells for 24 hr, the cells were cotransfected with 0.4 μg 5′ *MYCN* reporter construct and 0.015 μg of pRL-TK construct. After 24 hr, the cells were then treated with the indicated 4-OHT concentrations. For all transfections, cell lysates were harvested after 24 hr, and the activities of firefly and renilla luciferases were measured by luminometry. The results are expressed as the ratio (±SD) of the means of triplicate measurements of firefly to renilla luciferase activity.

MYCN Cooperates with PAX3-FKHR to Enhance Transforming Activity

As *MYCN* has been identified as an oncogene with effects on cellular activities such as proliferation and differentiation, we hypothesize that MYCN upregulation by PAX3-FKHR in ARMS cells contributes to the oncogenic activity of PAX3-FKHR in this environment. We and others have shown that NIH3T3 cells transduced with PAX3-FKHR have low but detectable transformation efficiency (Xia and Barr, 2004). Of note, MYCN is not significantly upregulated in PAX3-FKHR-expressing NIH3T3 cells (data not shown). We speculate that part of the reason for the low transformation efficiency of PAX3-FKHR in NIH3T3 cells is that MYCN is not upregulated by PAX3-FKHR in this cellular background. However, the unresponsiveness of MYCN expression to PAX3-FKHR in these cells provides a useful tool, because it permits us to determine the individual contributions of PAX3-FKHR and MYCN to transformation in this model cell culture system. We postulate that enforced

upregulation of MYCN in the NIH3T3 environment will enhance PAX3-FKHR oncogenic function, and will simulate what would happen if MYCN was upregulated by PAX3-FKHR (along with other cellular influences).

To examine the latter hypothesis concerning the effect of enforced MYCN expression on PAX3-FKHR activity, we established a series of NIH3T3 populations, which express P3FK/ER, MYCN, or both. In this experiment, the retroviral vector was pBabe, which expresses lower levels of P3FK/ER and, unlike the retroviral vector pK1, does not result in growth suppression (Xia et al., 2007). Transforming activity was assessed in two independent assays: soft agar colony formation and focus formation (Fig. 4). To make the soft agar assay more stringent and to concentrate on detection of cooperativity between PAX3-FKHR and MYCN, we plated a small number of cells (2×10^3 cells compared to the usual 2×10^4 cells per assay). Under these conditions, PAX3-FKHR alone and vector controls do not result in detectable transformation, whereas MYCN alone demonstrates a low level of transformation. However, the combination of MYCN and PAX3-FKHR results in a substantial increase in transforming activity relative to controls. In the focus formation assay, cells expressing PAX3-FKHR alone or MYCN alone demonstrate a low level of focus formation that is near the background level. However, in NIH3T3 cells expressing both PAX3-FKHR and MYCN, there was again a large increase in transforming activity compared to MYCN or PAX3-FKHR alone, with a 4-OHT-dose response increase in focus formation. These observations indicate a clear role for MYCN in stimulating PAX3-FKHR-mediated oncogenic activity in this system.

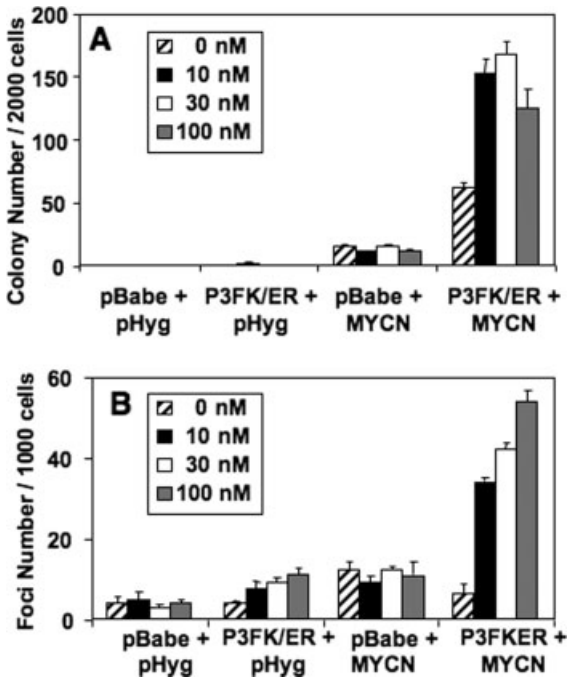

Figure 4. Phenotypic assays of MYCN and PAX3-FKHR oncogenic activity. A. For the soft agar colony formation assay, 2×10^3 cells were transduced with the indicated retroviral constructs (Xia et al., 2007), incubated in Iscove's medium (Gibco) containing 10% FBS with 0.35% agar (Agar Noble, Difco Laboratories, Detroit, MI), and seeded on top of 0.7% agar in the same medium. Cells were then incubated with the indicated 4-OHT concentration, and colonies were scored after 3 weeks. Three plates were counted for each condition, and results were expressed as the mean (±SD). B. For the focus formation assay, 10^3 cells were transduced with the indicated retroviral constructs (Xia et al., 2007), mixed with 10^6 nontransduced NIH3T3 cells in 100-mm dishes, and treated with the indicated concentrations of 4-OHT. After 2 weeks, three plates were stained and counted for each condition, and results were expressed as the mean (±SD).

DISCUSSION

In the present study, we identified target genes regulated by the PAX3-FKHR oncoprotein that are biologically relevant in ARMS tumors. The gene expression profiles of an inducible cell culture system were compared with the genes that showed differential expression patterns between ERMS and ARMS tumors. A set of 39 genes was identified, and the expression pattern of representative genes was verified. Functional annotation showed an enrichment of transcriptional regulators, many of which are involved in development. *MYCN*, which encodes a transcription factor and oncoprotein, was identified as a prime example of this category in this gene set. Our subsequent studies verified that MYCN expression is modulated by PAX3-FKHR and, importantly, demonstrated that MYCN collaborates with PAX3-FKHR in transformation.

Functional annotation of the PAX3-FKHR target genes in this study showed that a significant subset of genes is involved in development, and remarkably many of them are associated with nervous system development. This finding can be explained by the fact that wild-type PAX3 is expressed during specific stages of development and participates in myogenesis and neurogenesis (Robson et al., 2006). A previous study introduced PAX3-FKHR into NIH3T3 cells, and showed activation of a myogenic transcription program, including upregulation of MyoD1 and myogenin (Khan et al., 1999). In our system, although MYOD1 is still activated, prominent activation of a myogenic program is not detected because the starting cell already has a myogenic background. Instead, we detected dramatic changes in neural development genes. This pattern may indicate that expression of

PAX3-FKHR in myogenic cells imposes mixed expression of myogenic and neurogenic genes. We postulate that PAX3-FKHR may require fewer cofactors than wild-type PAX3 to activate target genes, and thus can activate genes in the myogenic environment whose expression is normally restricted to the neural environment by the need for cofactors that collaborate with PAX3.

MYCN has an essential role during neurogenesis, and amplification of MYCN occurs in several neurogenic tumors as well as RMS (Schwab, 1999). During neural development, MYCN promotes pathways controlling survival and cell cycle progression in early progenitors. This premise is supported by studies in which overexpression of MYCN in postmitotic sympathetic neurons stimulates reentry into S-phase and survival (Wartiovaara et al., 2002). Furthermore, the loss of its function specifically disrupts the ability of neuronal progenitor cells to expand, differentiate, and populate the brain (Knoepfler et al., 2002). In human neuroblastoma, *MYCN* amplification is associated with tumor progression and poor clinical outcome. Higher copy number and expression levels have also been identified in ARMS subsets, and some studies have proposed that high *MYCN* copy number and/or expression defines a high-risk group in ARMS (Hachitanda et al., 1998; Williamson et al., 2005).

Previous studies have shown that MYCN expression is generally higher in ARMS than ERMS tumors (Williamson et al., 2005). One mechanism that explains a subset of ARMS cases with high MYCN expression is *MYCN* gene amplification. However, our results indicate a more common mechanism for MYCN expression in ARMS tumors. We propose that PAX3-FKHR or PAX7-FKHR is involved in the activation of intermediate MYCN expression levels in most fusion-positive ARMS tumors. Our cell culture studies suggest that the mechanism for this effect of PAX3-FKHR on *MYCN* is at the direct transcriptional level. However, our reporter transfection studies in the RD cell line with the inducible P3FK/ER construct could not recapitulate the transcriptional regulation of *MYCN* by PAX3-FKHR with an exogenous reporter construct. Although other cell culture-based reporter assays have localized positive regulatory elements to the near 5′ region of the *Mycn* gene (Sivak et al., 1997), transgenic mouse studies have indicated that additional sequences are needed to recapitulate developmental expression patterns (Charron et al., 2002). Thus, there may be other regulatory regions in the *MYCN* locus, and one or more of these regions may be responsible for transcriptional activation of *MYCN* by PAX3-FKHR.

Normal cells have a mechanism of growth inhibition by cell–cell contact and a growth requirement for anchorage. When introduced alone into NIH3T3 cells, PAX3-FKHR can provide a low level of anchorage independence but cannot attenuate contact inhibition as measured in soft agar and focus formation assays, respectively (Xia and Barr, 2004). The introduction of MYCN into this NIH3T3 system substantially enhances the transformation efficiency of PAX3-FKHR in both focus formation and soft agar colony assays. These results suggest that MYCN may be important for PAX3-FKHR function in ARMS cells, and thus MYCN has a postulated role in the maintenance of the malignant phenotype. These phenotypic studies have been performed in murine fibroblasts to take advantage of the special expression features of these cells. However, since ARMS is a human tumor of the myogenic lineage, these studies do not fully address the phenotypic role of PAX3-FKHR and MYCN in the actual tumor lineage. Comparable studies have been performed in human myoblasts and resulted in similar oncogenic effects, and thus in this case, NIH3T3 cells seem to provide a reasonable model of the corresponding human tumor lineage (Xia and Barr, manuscript in preparation).

Davicioni and colleagues published a study in which the microarray component was somewhat similar to the present one. These investigators cross-referenced the results of an expression profiling study comparing fusion-positive and -negative RMS tumors to data from RD ERMS cell lines expressing PAX3-FKHR or PAX7-FKHR to identify a PAX-FKHR "expression signature" consisting of 81 genes (Davicioni et al., 2006). Comparison of that study with the current study showed that 17 of the 39 genes identified in our study are also present in the 81-gene list. A major difference between our study and that conducted by Davicioni and colleagues was that we assayed genes regulated by an inducible form of PAX3-FKHR after 24 hr, whereas in the system used by Davicioni and colleagues, the fusion gene was constitutively expressed in RD cells and was assayed 48 or 72 hr after retroviral transduction. As a consequence of this difference, our system at 24 hr was better suited to detect earlier regulated genes, whereas after 48–72 hr, the identified genes included later events.

Recently, a study was published in which a series of mRNA expression changes was identified

after treating an ARMS cell line with a pair of small interfering RNAs directed against the PAX3 region 5′ of the PAX3-FKHR fusion point (Ebauer et al., 2007). In particular, this study identified 51 activated genes that were also differentially expressed between fusion-positive ARMS and ERMS tumors. Comparison of these 51 genes identified four genes in common with our group of 39 differentially expressed PAX3-FKHR target genes. One of the genes in common between our study and the Ebauer study is *MYCN*. Therefore, this alternative strategy also indicates that *MYCN* is a biologically relevant target of PAX3-FKHR in ARMS cells.

As demonstrated by our studies with *MYCN*, the gene list identified in our study will help to understand the biology and clinical behavior of ARMS tumors. As the function and interactions of these genes are further elucidated, these genes will provide insights into the regulatory networks and signaling pathways that point to key differences between the ARMS and ERMS subsets. Therefore, this list provides the starting point for multiple studies of critical pathways in ARMS biology. Furthermore, as demonstrated in other recent profiling studies (Davicioni et al., 2006), specific genes within this signature may have clinical utility as markers for prognosis and for development of new specific therapeutic targets.

ACKNOWLEDGMENTS

We thank Dara D. Holder for technical contributions to this paper and John Tobias for bioinformatic consultation.

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