Up-Regulation of MET but not Neural Cell Adhesion Molecule Expression by the PAX3-FKHR Fusion Protein in Alveolar Rhabdomyosarcoma¹

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Abstract

The 2;13 chromosomal translocation in alveolar rhabdomyosarcoma generates the chimeric protein PAX3-FKHR, which is a powerful transcriptional activator. We hypothesize that PAX3-FKHR regulates downstream effector genes involved in rhabdomyosarcoma tumorigenesis. We evaluated alterations in expression of MET and neural cell adhesion molecule that were proposed previously as downstream targets of wild-type PAX3. We used a myogenic tumor cell culture system and rhabdomyosarcoma tumor specimens to assess candidate gene expression in relationship to various PAX3-FKHR expression levels. We demonstrate that the expression of MET, but not neural cell adhesion molecule, correlates significantly with PAX3-FKHR expression. These findings indicate that MET, which encodes a receptor involved in growth and motility signaling, is a downstream target of PAX3-FKHR in alveolar rhabdomyosarcoma.

Introduction

RMS⁴ is a family of soft-tissue tumors related to the myogenic lineage that usually present in pediatric patients (1). Two major histopathological forms of pediatric RMS, ARMS and ERMS, are recognized. These two histopathological entities have differing clinical phenotypes and prognostic significance. Cytogenetic analysis has demonstrated nonrandom chromosomal translocations within ARMS tumors but not ERMS tumors; most ARMS cases have a translocation between chromosomes 2 and 13, t(2;13)(q35;q14) (2). The t(2;13) juxtaposes the 5′ portion of the PAX3 gene on chromosome 2 with the 3′ region of the FKHR gene on chromosome 13, resulting in a chimeric gene that encodes a fusion protein, PAX3-FKHR (3). This fusion juxtaposes the PAX3 DNA binding domain and FKHR transactivation domain to generate a powerful transcriptional activator that is more potent than wild-type PAX3 (4). The PAX3-FKHR fusion protein is hypothesized to lead to aberrant or inappropriate expression of a set of genes with binding sites that are normally regulated during development by the wild-type PAX3 protein. The inappropriate expression of these gene products is postulated then to contribute to oncogenic initiation or progression by stimulating cell behaviors, such as growth and motility, or inhibiting cell behaviors, such as apoptosis and terminal differentiation (5–7). Several candidate target genes of PAX3-FKHR can be proposed based on previous studies of wild-type PAX3 expression and transcriptional function. MET, which encodes the hepatocyte growth factor receptor, is a proto-oncogene that is necessary, like PAX3, for limb muscle development (8) and contains two potential PAX3 binding sites in its promoter (9). NCAM is expressed similar to PAX3 in the neural tube during embryogenesis (10, 11) and can be activated by microinjection of a PAX3 expression construct into Schwann cells (12). In this report, we tested whether expression of MET and NCAM is up-regulated by PAX3-FKHR using a transfected ERMS cell culture system and individual ARMS tumors to assess candidate gene expression in response to varying levels of PAX3-FKHR expression.

Materials and Methods

Tumor Specimens. Tumor specimens were collected and stored at −70°C. Total RNA was isolated using RNA STAT 60 extraction reagent (TEL TEST “B”). Tumor specimens were screened by RT-PCR for expression of the PAX3-FKHR and PAX1-FKHR fusion transcripts as described (13).

Stable Transfections. All plasmids were prepared by alkaline lysis and resin purification. The RD cell line (obtained from American Type Culture Collection) was transfected with pCD3M8, which contains the complete human PAX3-FKHR open reading frame in a 3710-bp insert cloned into the BamHI and XhoI sites of pCDNA3 (Invitrogen; Ref. 4). As a control, RD was also transfected with the pCDNA3 plasmid without PAX3-FKHR insert. For both transfections, cells were transfected with 10 μg of target construct by the calcium phosphate coprecipitation method (4). After 48 h, the cells were replated in media with G418 (500 μg/ml). Individual G418-resistant colonies were isolated and expanded. These clones as well as the parental RD cell line were maintained by weekly passage in DMEM with high glucose (Life Technologies, Inc.) containing 10% fetal bovine serum (HyClone).

Transfections and CAT Assays. Transient transfections were performed by the calcium phosphate coprecipitation method as described (4). To measure PAX3-FKHR transcriptional activity, 5 μg of plasmid 6xPRS-9/E1b CAT, containing six DNA binding sites for PAX3/PAX7, or 5 μg of E1bCAT (without PAX binding sites) were cotransfected with 5 μg of control plasmid pSV2APAP. After 48 h, transfected cells were lysed, and cell extracts were assayed for CAT activity. The amount of each lysate tested was normalized for transfection efficiency, determined by measuring expression of placent al alkaline phosphatase from the cotransfected control plasmid.

Riboprobe Plasmids. To detect MET, NCAM, and PAX3-FKHR expression, we assembled riboprobe plasmids by cloning portions of each cDNA into pSP72 (Promega). The PAX3-FKHR riboprobe plasmid contains a blunt-ended PvuII-NcoI fragment (396 bp) corresponding to exons 6–7 fused to FKHR exon 2 that was subcloned into the PvuII site of pSP72. The MET riboprobe plasmid was obtained by cloning a 404-bp PvuII-PstI fragment corresponding to exons 1–2 into PstI-Sma-digested pSP72. The NCAM riboprobe plasmid was obtained by cloning a 427-bp SalI-HincII fragment (containing amino acid residues 155–298) into SalI-PvuII-digested pSP72 (14). A GAPDH riboprobe plasmid (pTIRGAPDH; Ambion) served as an internal control for the RNase protection experiments. The MET cDNA was obtained from Dr. G. F. Vande Woude (NCI, Frederick, MD), and the NCAM cDNA was obtained from American Type Culture Collection.

RNase Protection Analysis. [32P]UTP-labeled antisense test and GAPDH riboprobes were synthesized from linearized riboprobe plasmids using SP6 RNA polymerase (MaxiScript; Ambion) and were used for RNase protection analysis (RPAII; Ambion). Total RNA (5–10 μg) was incubated with test and control riboprobes for 16 h at 37°C, and 5% formamide was added to the samples before electrophoresis on a 10% denaturing polyacrylamide gel.
GAPDH riboprobe. The mixtures were heat denatured, hybridized at 42°C for at least 18 h, and digested with RNases A and T1; the resultant protected fragments were electrophoresed in a denaturing 6% polyacrylamide gel. Band intensities were quantified using a PhosphorImager (Molecular Dynamics). A test RNA:GAPDH RNA ratio was calculated. The MET:GAPDH, NCAM:GAPDH, and PAX3-FKHR:GAPDH ratios were then normalized to the MET expression level in SJRH28 ARMS cells, NCAM expression level in SJRH28 ARMS cells, or PAX3 expression level in RD ERMS cells, respectively.

**Results**

**Development of a Cell Culture Model System.** Our first goal was to develop a cell culture system that would enable the assessment of potential downstream target genes. Specifically, we wanted to express the PAX3-FKHR fusion in a cell line that was derived from the myogenic lineage and isolate multiple subclones with variable expression levels of PAX3-FKHR. Furthermore, we hoped to achieve PAX3-FKHR expression levels that were comparable to the expression of PAX3-FKHR found in ARMS tumors. To accomplish this, we selected RD, an ERMS cell line, that does not have endogenous PAX3-FKHR expression and is derived from the myogenic lineage. The RD cell line was transfected with PAX3-FKHR in the mammalian expression vector pcDNA3. Initially, RT-PCR was performed on the resulting transfectants to assay expression of the PAX3-FKHR fusion transcript. All subclones derived from transfection of the PAX3-FKHR construct expressed detectable fusion transcript (11 of 11 subclones, designated PCD series; Fig. 1). As a negative control, the RD cell line was transfected with pcDNA3 without PAX3-FKHR insert. Five subclones that did not express PAX3-FKHR detectable by RT-PCR were chosen as negative controls and designated the FMD series.

RNase protection assays were then performed using total RNA isolated from these cells to quantify the level of PAX3-FKHR expression in these subclones. The PCD series had measurable and variable levels of PAX3-FKHR expression, whereas the FMD series had no detectable PAX3-FKHR expression (Fig. 1). Furthermore, the PCD series of subclones expressed PAX3-FKHR at levels comparable with that observed in the ARMS cell lines and tumors (15).

To determine whether the PAX3-FKHR transfectants had transcriptional activity that correlated with the expression levels of PAX3-FKHR, a reporter gene-transfection assay was performed on a subset of these clones. The reporter construct consists of the CAT gene cloned downstream of a multimer of PAX3 DNA binding sites. The activity of this construct was compared with that of a control containing the CAT gene without PAX3 binding sites. Transcriptional activity was documented in three of three PAX3-FKHR transfectants from the PCD series and not in the one negative control from the FMD series (Fig. 1). Moreover, PAX3-FKHR expression levels in the PCD series were positively correlated with the measured level of transcriptional activity (Fig. 1).

To assess any changes in the oncogenic properties of the RD cells expressing PAX3-FKHR, several cell culture growth properties were evaluated. Examination of several PAX3-FKHR-expressing clones and a control clone did not demonstrate any enhancement of transforming properties as assayed by focus formation and serum independence assays (data not shown). Therefore, PAX3-FKHR expression does not increase the oncogenic properties of these already transformed RD tumor cells.

**MET Expression.** The series of RD transfectants was then used to examine changes in MET expression induced by PAX3-FKHR. An RNase protection assay was designed to measure MET expression and was used to quantify MET transcript levels in the PCD and FMD clones (Fig. 2A). In the five negative control clones, we detected a mean MET expression of 5.9 with a SE of 0.9 unit. In the PCD series, higher levels of MET expression were present. A significant positive correlation between increasing levels of PAX3-FKHR and MET was observed, with a correlation coefficient of 0.873 and P < 0.0001 (Fig. 2B). The value obtained for the y-intercept of this graph comparing PAX3-FKHR expression to MET expression was consistent with the average MET expression in the negative control clones, and thus the y-intercept of this graph effectively represents MET expression in the absence of PAX3-FKHR. Therefore, PAX3-FKHR induces increased MET expression above baseline levels in RD cells.

To corroborate these findings in RMS cases, MET expression was assessed in ARMS specimens by RNase protection assays. Evaluation of MET expression in 15 ARMS tumor specimens (11 with PAX3-FKHR fusion and 4 with variant PAX7-FKHR fusion) demonstrated that MET was expressed at variable levels (Fig. 2D). Assessment of the 11 ARMS tumors with the t(2;13) translocation demonstrated a significant positive linear correlation between PAX3-FKHR expression and MET expression with a correlation coefficient of 0.686 and P < 0.02. (Fig. 2C). The PAX3-FKHR expression and MET expression patterns were consistent between our RD cell culture system and our ARMS tumor specimens. In fact, the slope and the y-intercept of the linear correlation in the RD transfectants (0.189 and 6.292, respectively) were comparable with the linear parameters from this analysis of t(2;13) ARMS tumors (slope = 0.129 and y-intercept = 7.075; Fig. 2B and C). The combined cell culture and tumor data indicate that when PAX3-FKHR is present, MET expression is enhanced in a dose-dependent manner. Therefore, it appears that the PAX3-FKHR fusion protein likely acts in part through a MET-dependent pathway and may influence oncogenicity through this pathway.

Evaluation of MET expression in nine ERMS tumor specimens revealed that MET was also expressed at variable levels (Fig. 2E). Moreover, the MET expression levels in some ERMS tumors were comparable with the levels in the ARMS tumors. PAX3-FKHR, therefore, is not necessary for regulating MET expression, and MET is a target for other transcription factors in the ERMS cell environment.

**NCAM Expression.** Similar to the MET experiments, the RNA samples from the PCD and FMD series were used in the RNase protection assay to measure expression levels of NCAM, and these NCAM levels were compared with the previously measured PAX3-
Fig. 2. RNase protection analysis of PAX3-FKHR and MET expression in RD subclones, ARMS tumors, and ERMS tumors. A, total RNA (5–10 µg) from the indicated RD subclones was hybridized with the indicated [32P]UTP-labeled test and control riboprobes. Shown are equivalent exposures of the PAX3-FKHR (upper panel, left) and MET-protected fragments (upper panel, right) and the corresponding GAPDH-protected fragments (lower panel) in PAX3-FKHR transfectant clones PCD1, PCD7, PCD8, PCD10, and PCD13 and negative control clones FMD12 and FMD25. The corresponding expression levels are noted below. B, scatter plot diagram with linear regression analysis of PAX3-FKHR and MET expression in the PAX3-FKHR transfectants (PCD series). RNase protection analysis was performed on these RD subclones, the protected bands were quantified by phosphorimaging, and a test RNA:GAPDH RNA ratio was calculated. MET results are shown as expression units relative to MET levels in the ARMS cell line SJRH28 (arbitrarily set to 10 units), and PAX3-FKHR results are shown as expression units relative to PAX3 levels in the ERMS cell line RD (arbitrarily set to 10 units). A significant positive correlation (P < 0.0001) between MET expression and PAX3-FKHR expression is observed. C, scatter plot diagram with linear regression analysis of PAX3-FKHR and MET expression in 11 t(2;13)-containing tumors. Similar to B, a significant positive correlation (P < 0.02) between MET expression and PAX3-FKHR expression is observed. D, relative MET expression levels in ARMS specimens. Results are calculated as described in B. E, relative MET expression levels in ERMS specimens. Results are calculated as described in B.

FKHR expression in the PCD series. The mean NCAM expression level in the FMD series (negative controls) was 0.66 with a SE of 0.31 unit, whereas the mean NCAM expression level in the PCD series (PAX3-FKHR transfectants) was 1.1 with a SE of 0.14 unit. There was no significant difference in NCAM expression between these two series of clones. In addition, there was no significant relationship between PAX3-FKHR and NCAM expression levels in the 11 PCD subclones, as indicated by the correlation coefficient of 0.527 and P of 0.096 (Fig. 3A).

NCAM expression was then assayed in the same tumors as in the
Fig. 3. RNase protection analysis of PAX3-FKHR and NCAM expression in RD subclones, ARMS tumors, and ERMS tumors. A. scatter plot diagram with linear regression analysis of PAX3-FKHR and NCAM expression in 11 PAX3-FKHR transfectants (PCD series). RNase protection analysis for PAX3-FKHR and NCAM expression was performed on these RD subclones, the protected bands were quantified by phosphorimaging, and a test RNA:GAPDH RNA ratio was calculated. NCAM results are shown as expression units relative to NCAM levels in the ARMS cell line SJRH28 (arbitrarily set to 10 units), and PAX3-FKHR results are shown as expression units relative to PAX3 levels in the embryonal rhabdomyosarcoma cell line RD (arbitrarily set to 10 units). No significant relationship between NCAM expression and PAX3-FKHR expression was observed in the PCD series. B. scatter plot diagram with linear regression analysis of PAX3-FKHR and NCAM expression in 11 t(2;13)-containing tumors. Similar to A, no significant relationship between NCAM expression and PAX3-FKHR expression is observed. C. relative NCAM expression levels in ARMS specimens. Results are calculated as described in A. E, t(2;13) tumors; D, t(1;13) tumors. D. relative NCAM expression levels in ERMS specimens. Results are calculated as described in A.

Discussion

In this study using a novel cell culture system and a large panel of RMS tumors, our data support the hypothesis that MET is a downstream target of the PAX3-FKHR fusion protein. Our findings that PAX3-FKHR enhances MET expression suggest that the oncogenic activity of the fusion protein acts at least in part through a MET-dependent pathway. MET is a proto-oncogene that encodes a tyrosine kinase receptor for hepatocyte growth factor/scatter factor (16). As indicated by the two names for this protein, hepatocyte growth factor/scatter factor has both growth- and motility-inducing activities. By binding this ligand, MET potentiates growth and appears to provide cells with the ability to migrate into and invade surrounding connective tissues. These activities may contribute to the invasive/metastatic phenotype and poor prognosis of ARMS. The induction of MET by PAX3-FKHR thus provides a potential link between a tumor specific chromosomal translocation and the aggressive phenotype of ARMS tumors.

Previous studies have indicated a significant interaction between
MET and PAX3 during limb muscle development. Splotch mice that have spontaneous Pax-3 mutations and MET knockout mice do not develop limb musculature (8, 17). In addition, MET expression is down-regulated in the Splotch mutant mouse, thus suggesting that MET functions downstream of Pax-3 in myogenic developmental pathways (9). Placement of MET downstream of PAX3 in a transcriptional pathway is further supported by the finding of two potential PAX3 binding sites in the MET promoter (9).

In addition to MET, NCAM was evaluated as a potential target gene of PAX3-FKHR. NCAM and PAX3 have overlapping expression patterns in the neural tube during mouse embryogenesis (10, 11). Differences have been detected in the level of NCAM expression and types of NCAM polypeptides in the neuroepithelium of Splotch homozygous mouse embryos as compared with wild-type embryos (18). Although there is some in vitro evidence for NCAM induction by Pax-3 in Schwann cells (12), we did not observe a significant correlation between PAX3-FKHR expression and NCAM expression in stably transfected RD ERMS subclones and ARMS tumors. It is likely that Schwann cells contain certain cofactors that are cell type specific and that the transcriptional environment in ARMS differs from that of the Schwann cell.

A small subset of ARMS tumors demonstrates a translocation involving chromosomes 1 and 13. The t(1;13) translocation results in a chimeric gene that encodes a PAX7-FKHR fusion protein (2). Studies indicate that there are significant clinical differences between patients with t(2;13) ARMS tumors and patients with t(1;13) ARMS tumors (19). Our expression studies indicated comparable MET levels between these two types of ARMS tumors, suggesting that MET may be a target for PAX7-FKHR and that MET expression does not explain the clinical differences between these two ARMS subtypes. More t(1;13) tumors need to be evaluated for definitive statistical assessment.

The control of MET expression may also be affected by the presence of wild-type PAX3 in these cells. We have determined that the level of PAX3 expression in wild-type RD cells is 10 units on the PAX3-FKHR expression scale shown in Fig. 2, B and C. This level of PAX3 may contribute to the basal level of MET expression found in our negative subclones, the FMD series. Within our PAX3-FKHR-expressing PCD transfectants, there is overexpression of PAX3-FKHR relative to PAX3 in two subclones and comparable PAX3 and PAX3-FKHR expression levels in nine subclones. The two former PCD subclones most closely mimic actual ARMS tumors, where it has previously been demonstrated that PAX3-FKHR is overexpressed relative to PAX3 (15). The finding of MET overexpression in these two subclones further emphasizes that MET is up-regulated in the setting of PAX3-FKHR overexpression.

To date few genuine targets of chimeric transcription factors have been identified in human malignancies. Some potential candidates have been postulated based on reporter gene-transient transfection assays and stable transfection of heterologous cell types, but few downstream target genes have subsequently been verified by expression studies in tumors. Several issues must be considered in the evaluation of the published target gene studies: (a) transfected genes in transient transfections are not present at their normal chromosomal location or in their normal chromatin configuration, and they are also present at an increased copy number; and (b) stably expressing a protein in a heterologous environment does not account for tissue-specific gene expression or appropriate cell specificity. The notable features of our RD cell culture model system are the use of a cell line of myogenic lineage and the stable expression of PAX3-FKHR in the transfectants at variable levels that were physiological. The variability of the levels allowed direct testing of our target genes, because we were able to measure expression of our target genes in the presence of increasing expression of PAX3-FKHR. The utility of this cell culture system was demonstrated by the similar MET expression characteristics in actual ARMS tumors. Our RD cell culture model system will facilitate the evaluation of further candidate target genes in combination with our panel of ARMS and ERMS tumor specimens.

References

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