Human Cancer Biology

FGFR4 Blockade Exerts Distinct Antitumorigenic Effects in Human Embryonal versus Alveolar Rhabdomyosarcoma

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Abstract

Purpose: Rhabdomyosarcoma (RMS) is a malignancy with features of skeletal muscle, and the most common soft tissue sarcoma of childhood. Survival for high-risk groups is approximately 30% at 5 years and there are no durable therapies tailored to its genetic aberrations. During genetic modeling of the common RMS variants, embryonal RMS (eRMS) and alveolar RMS (aRMS), we noted that the receptor tyrosine kinase (RTK) fibroblast growth factor receptor 4 (FGFR4) was upregulated as an early event in aRMS. Herein, we evaluated the expression of FGFR4 in eRMS compared with aRMS, and whether FGFR4 had similar or distinct roles in their tumorigenesis.

Experimental Design: Human RMS cell lines and tumor tissue were analyzed for FGFR4 expression by immunoblot and immunohistochemistry. Genetic and pharmacologic loss-of-function of FGFR4 using virally transduced short hairpin RNA (shRNA) and the FGFR small-molecule inhibitor PD173074, respectively, were used to study the role of FGFR4 in RMS cell lines in vitro and xenografts in vivo. Expression of the antiapoptotic protein BCL2L1 was also examined.

Results: FGFR4 is expressed in both RMS subtypes, but protein expression is higher in aRMS. The signature aRMS gene fusion product, PAX3-FOXO1, induced FGFR4 expression in primary human myoblasts. In eRMS, FGFR4 loss-of-function reduced cell proliferation in vitro and xenograft formation in vivo. In aRMS, it diminished cell survival in vitro. In myoblasts and aRMS, FGFR4 was necessary and sufficient for expression of BCL2L1 whereas in eRMS, this induction was not observed, suggesting differential FGFR4 signaling.

Conclusion: These studies define dichotomous roles for FGFR4 in RMS subtypes, and support further study of FGFR4 as a therapeutic target.

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Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood and adolescence, accounting for 40% of soft tissue sarcomas in this age range (1). RMS tumors exhibit features of skeletal muscle and are thought to derive from mesenchymal cell precursors that have failed to differentiate or otherwise developed aberrantly along the skeletal muscle axis. RMS treatment includes a multimodal approach of surgery, radiation, and chemotherapy, but despite the evaluation of experimental agents through cooperative group clinical trials, 5-year survival for high-risk groups remains approximately 30% (2).

In addition to clinical group and stage, risk stratification for RMS includes tumor histology. Originally defined by morphology under light microscopy, RMS histologic variants are known to bear unique cytogenetic and molecular abnormalities (3). The embryonal variant (eRMS), often found in the head, neck, and trunk, generally portends a favorable outcome, and is associated with LOH and abnormal imprinting on chromosome 11. The alveolar variant (aRMS), often found in the extremities, portends a worse outcome (survival <50% at 5 years; ref. 1), and is associated with a chromosomal translocation resulting in the PAX3-FOXO1 (also known as PAX3-FKHR) mutant fusion transcription factor. Although PAX3-FOXO1 is found exclusively in aRMS (4, 5) and thus a desirable drug target, as a transcription factor it has remained chemically intractable. Microarray studies suggest that RMS variants have distinct transcriptional profiles (6, 7) and therefore are distinct biologic entities. We and others have been seeking to understand the differences between the RMS variants, to

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Rhabdomyosarcomas (RMS) are the most common soft tissue sarcomas of childhood and adolescence. As with other sarcomas, there are several histologic variants of RMS, but the 2 predominant subtypes are embryonal RMS (eRMS) and the more aggressive alveolar RMS (aRMS). Accumulating evidence suggests that eRMS and aRMS are molecularly distinct entities that may require specific therapies. Therefore, understanding their common and unique cell signaling pathways will be critical for novel therapeutics development. Activating mutations in fibroblast growth factor receptor 4 (FGFR4) have recently been implicated in RMS metastasis. However, how FGFR4 functions in the RMS variants is not known. Herein, we provide evidence that although FGFR4 is expressed in both eRMS and aRMS, this receptor tyrosine kinase contributes to tumorigenesis by preferentially stimulating cell proliferation in eRMS, but promoting cell survival in aRMS. These data support the exploration of FGFR4 inhibitors as therapeutics for RMS.

Translational Relevance

Rhabdomyosarcomas (RMS) are the most common soft tissue sarcomas of childhood and adolescence. As with other sarcomas, there are several histologic variants of RMS, but the 2 predominant subtypes are embryonal RMS (eRMS) and the more aggressive alveolar RMS (aRMS). Accumulating evidence suggests that eRMS and aRMS are molecularly distinct entities that may require specific therapies. Therefore, understanding their common and unique cell signaling pathways will be critical for novel therapeutics development. Activating mutations in fibroblast growth factor receptor 4 (FGFR4) have recently been implicated in RMS metastasis. However, how FGFR4 functions in the RMS variants is not known. Herein, we provide evidence that although FGFR4 is expressed in both eRMS and aRMS, this receptor tyrosine kinase contributes to tumorigenesis by preferentially stimulating cell proliferation in eRMS, but promoting cell survival in aRMS. These data support the exploration of FGFR4 inhibitors as therapeutics for RMS.

Materials and Methods

Generation of cell lines and constructs

Early passage normal human skeletal muscle myoblasts (HSMM, Lonza) grown in defined media (SkGM-2) were stably infected with amphotropic retroviruses to express PAX3-FOXO1-puro (a gift from Dr Fred Barr, NCI) or empty vector, then selected in 0.25 μg/mL puromycin (Sigma-Aldrich). Human RMS cell lines RD (21), SMS-CTR (22), Rh36 (23), Rh3 (24), Rh28 (25), and Rh30 (26) were gifts from Dr Tim Triche (Children’s Hospital of Los Angeles, CA, USA) in 2005 and Dr Brett Hall (Nationwide Childrens, Columbus, OH, USA) in 2006 and cultured as described (9). Cell line identity was investigated in 2011 using STR analysis (Promega PowerPlex 1.2) conducted by the Fragment Analysis Facility at the Johns Hopkins Genetic Resources Core Facility (Supplementary Table S1). FGFR4 shRNA sequences were designed de novo or obtained from the literature (27) and annealed shRNA oligos (Supplementary Table S2) ligated into the pSUPER-retro-GFP/neo or plKO.1puro (Addgene 8453) plasmids. Wild-type FGFR4 was cloned from a JR cDNA library using high-fidelity PCR and subcloned into the EcoRI-SalI multicloning site of pBabe-puro and validated by sequencing. Cell lines expressing cDNAs or shRNAs were polyclonal.

Immunoprecipitation and immunoblotting

Cells were lysed in Tris/RIPA or 20 mmol/L Tris pH 7.5, 1% NP-40, 137 mmol/L NaCl, 10% glycerol, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA (for immunoprecipitation) with standard protease and phosphatase inhibitors. Protein concentration was measured by the DC assay (Bio-Rad). FGFR4 immunoprecipitations were conducted with anti-FGFR4 (C-16) and protein G sepharose (Santa Cruz). For immunoblotting, the following antibodies...
were used: anti-FOXO1A (FKHR) F6928, FLAG F3165, tubulin T4026 (Sigma), actin 8462, FGFR4 C-16, ERK1 C-16 (Santa Cruz), phospho-ERK1/2 (Th202/Tyr204), phospho-Akt (Ser273), Akt, caspase-3 (8G10, Cell Signaling), phospho-tyrosine (4G10, Millipore), FGFR4 (RD Systems), HRP-labeled goat anti-mouse or anti-rabbit antibody (Invitrogen-Zymed). Several bands were detected when immunoblotting for FGFR4 using the sc-124 antibody; this was previously reported and is related to posttranslational phosphorylation or glycosylation (28, 29).

**MTT, BrdUrd, and apoptosis assays**

The MTT assay was used as a surrogate measure for cell number (and therefore growth over time) and BrdUrd incorporation was used as a surrogate measure of proliferation according to the procedures described in (30). The double staining propidium iodide/Annexin V apoptosis flow cytometric assay (eBioscience, #88-8007) was used per manufacturer’s protocol as a secondary measure of apoptosis.

**RT-PCR**

Reverse transcription PCR was conducted as previously described (9). Primer sets for this work are shown in Supplementary Table S2.

**Tumor xenografts**

Under institutional IACUC-approved protocols, and as conducted (9, 31), 9 to 10 million cells/cell line were injected subcutaneously into the flanks of SCID/beige mice in triplicate or quadruplicate for genetic knockdown studies, or in replicates of 5 for FGFR drug inhibitor studies. In drug studies, mice were treated for short durations with PD173074 at 25 mg/kg/d [or dimethyl sulfoxide (DMSO) vehicle] intraperitoneally for 2 or 5 d/wk as previously reported (32), or 20 mg/kg/d for long durations (14 days, accomplished using an induction period of daily injections for 5 days, then every other day dosing for 9 days following). Mice were monitored biweekly. Tumor volume was estimated by external caliper measurements and calculated as \[(\text{width})^2 \times \text{length} / 2\]. In some experiments, measurement by calipers was difficult because of the tumor’s flat, oblong shape; therefore, decreases in estimated volume were verified by necropsy. Mice were sacrificed when tumors met IACUC-defined tumor burden or ill-health thresholds, and underwent necropsy with portions of tumor fixed in formalin or snap-frozen in liquid N2.

**Immunohistochemistry**

Human RMS TMAs were obtained from the Cooperative Human Tissue Network, which is funded by the National Cancer Institute; other investigators may have received specimens from the same subjects. The TMAs contain eRMS and aRMS cores; 134 individual eRMS or aRMS cores representing 19 eRMS and 39 aRMS tumors were scored. Staining for FGFR4 (FGFR4 C-16, Santa Cruz, ref. 33) was conducted with the assistance of the University of Florida Department of Pathology, Immunology, and Lab Medicine Pathology Core. The TMA was scored by 2 independent observers who were blinded to the annotated histologic type. For each core, FGFR4 positivity was determined on the basis of plasma membrane and cytoplasmic staining, and a semiquantitative score of the percentage of positive cells assigned as an integer (0 = no staining; 1 = <25% staining; 2 = 25–50% staining; 3 = >50% staining). In the case where there were replicate cores from a single tumor, an integer score was assigned on the basis of the overall impression of the cores combined. A 2-sample Wilcoxon test was used to compare the eRMS versus aRMS scores. Tumor xenograft immunohistochemistry from murine studies was conducted as described previously (30).

**Results**

**FGFR4 is differentially expressed in human eRMS versus aRMS cell lines and tumor tissue**

Although transcriptional profiling has showed upregulation of FGFR4 mRNA in human RMS cell lines and tumors (17, 19), we sought to examine FGFR4 expression at the protein level in aRMS versus eRMS tissue. Using immunoblot for FGFR4 protein expression, we examined a panel of 6 widely used human RMS cell lines (3 eRMS and 3 aRMS). Because the biologic behavior of fusion-negative aRMS is not clear, we studied only aRMS cell lines known to express the PAX3-FOXO1 fusion. Compared with nontransformed primary HSMM cells, FGFR4 protein was increased in all RMS cell lines examined (Fig. 1A), with highest expression in those of aRMS histology. Depending upon the exposure, 2 or 3 discrete FGFR4 bands were evident, likely related to differential phosphorylation or glycosylation, which has been described for FGFR4 (28, 29). Because we later found by short tandem repeat (STR) analysis that Rh3 and Rh28 cell lines likely derive from the same tumor (Supplementary Table S1 and as noted in ref. 34), it was important to confirm FGFR4 expression patterns in a larger cohort of human clinical RMS tumor samples. Using tissue microarrays (TMA) bearing cores of human RMS tumors and immunohistochemical staining for FGFR4, we found that FGFR4 was more highly expressed in aRMS compared with eRMS tissue (Fig. 1B). Taken together, these data suggest that FGFR4 protein expression is overall increased in RMS tumor tissue, with differences in expression levels noted between the eRMS and aRMS subtypes.

**Loss-of-function of FGFR4 in eRMS cells inhibits cell proliferation in vitro and tumorogenesis in vivo**

Because eRMS and aRMS are biologically distinct (2), we examined the role of FGFR4 in each separately. Using 2 independently targeting short hairpin RNAs (shRNA; designated sh1 and sh2), we knocked down FGFR4 protein expression in RD eRMS cells via stable viral transduction (Fig. 2A, left). Knockdown by either shRNA resulted in a decrease in intensity of FGFR4 bands, and in RD cells expressing sh1, the appearance of a faint intermediate-sized band (also seen in Fig. 3D) and a shift
in the migration of the lower band. The significance of these intermediate bands is not known. After selection, polyclonal FGFR4-deficient RD cell populations emerged, but displayed an obvious decreased growth rate in culture. This decrease in growth was quantified by MTT assay (Fig. 2B), and reproduced with manual counting (Supplementary Fig. S1). Morphologically, there was no increase in dead cells as assessed by trypan blue positivity compared to vector control (data not shown), suggesting that while cell viability was not affected, cell proliferation was slowed. To this end, we conducted bromodeoxyuridine (BrdUrd) incorporation assays, which showed defective RD cell proliferation in the setting of FGFR4 suppression (Fig. 2C, left). A second eRMS cell line, SMS-CTR, was also rendered FGFR4-deficient (Fig. 2A, right), and similarly showed defects in BrdUrd incorporation (Fig. 2C, right).

To determine whether FGFR4 suppression would block tumorigenesis in vivo, FGFR4-knockdown eRMS cells were evaluated as subcutaneous xenografts in immunodeficient mice. Compared with vector control, mice inoculated with RD or SMS-CTR cells expressing FGFR4 shRNAs still developed tumors (Fig. 2D), but at a longer latency and with smaller tumors at necropsy (not shown). Xenograft sections were probed for expression of Ki67, TUNEL and CD31 to quantify changes in proliferation, apoptosis, and vessel density, respectively (data not shown). In RD xenografts, although TUNEL and CD31 staining were unchanged, expression of FGFR4 shRNA was associated with a significant decrease in expression of Ki67, from 56.9% to 36.6% (P < 0.0003). These data suggest that similar to the in vitro studies, in eRMS cells FGFR4 was stimulating cell proliferation, although we cannot rule out diminished clonogenicity or poor survival of cells injected into mice as additional reasons for delayed xenograft growth.

FGFR4 promotes cell survival in human aRMS cells in vitro

In our survey of FGFR4 protein expression in human RMS tissue, we observed higher expression in PAX3-FOXO1-positive aRMS and histologically-defined aRMS tumors compared with eRMS tissue (Fig. 1), suggesting a correlation between PAX3-FOXO1 and FGFR4 expression. To investigate the relationship between PAX3-FOXO1 and FGFR4, we stably expressed PAX3-FOXO1 cDNA in HSMM cells, and found that endogenous FGFR4 protein expression was induced (Fig. 3A), suggesting that FGFR4 is downstream from PAX3-FOXO1. To determine the functional significance of this increased expression, we stably knocked down FGFR4 in HSMM cells expressing PAX3-FOXO1 (Fig. 3B), and found that cell viability was inhibited as measured by MTT (Fig. 3C). Additional experiments verified a correlation between FGFR4 expression and cell viability (Supplementary Fig. S2).

We next investigated FGFR4 loss-of-function in human fusion-positive aRMS cell lines. As opposed to eRMS cells (described earlier), which survived selection into polyclonal, passageable populations after viral transduction with FGFR4 shRNAs, human aRMS cells (Rh28 and Rh30) selected for stable FGFR4 knockdown became nonadherent and trypan blue-positive (data not shown), suggesting that FGFR4 loss was not compatible with cell survival. Despite repeated attempts with the identical reagents used in the eRMS studies, passageable aRMS cells exhibiting stable FGFR4 knockdown could not be generated. This phenotype also precluded in vivo analysis in our murine xenograft system. Suspecting that FGFR4 was required for aRMS cell survival, we examined Rh30 and Rh28 cell populations (completing selection for stable shRNA expression but beginning to detach from tissue culture plate) for biochemical evidence of cell death. As expected, FGFR4 was appropriately suppressed by the shRNAs (Fig.

Figure 1. FGFR4 protein expression is higher in human cell lines and tumors of alveolar (aRMS) histology. A, immunoblot of endogenous FGFR4, FOXO1, and PAX3-FOXO1 protein in human RMS cell lines (eRMS cell lines RD, SMS-CTR, Rh66; aRMS cell lines Rh3, Rh28, and Rh30). Through STR cell line analysis (Materials and Methods and Supplementary Table S1), Rh3 was found to be identical to Rh28, indicating that it originated from the same tumor. FOXO1 and PAX3-FOXO1 were co-detected by immunoblot for FOXO1. Actin was used as a loading control. B, standard immunohistochemistry was used to measure expression of endogenous FGFR4 in a human RMS tumor microarray. In total, 19 independent eRMS and 39 independent aRMS tumor were analyzed. Staining intensity was scored as described in Materials and Methods. *, P = 0.0092.
3D, lanes 5–6), but caspase-3 cleavage was also evident (Fig. 3D, lanes 5–6 and Fig. 3E, lanes 2–3), suggesting induction of apoptosis. As a second measure of apoptosis, we subjected Rh28 aRMS cells expressing FGFR4 shRNAs to flow cytometric measurement of propidium iodide and Annexin V. We found that compared with untreated (control) or empty vector (pLKO.1)-expressing cells, which had a baseline of 10% to 13% of cells in early or late apoptosis, the percentage of FGFR4 shRNA-expressing cells in late apoptosis increased to 32% to 44% (Fig. 3H). There was not a significant increase in early apoptosis, suggesting we had sampled the aRMS cells late in the apoptotic process.

FGFR4-associated cell survival has previously been noted in human breast cancer cells, thought to be mediated by the antiapoptotic protein BCL2L1 (BCL-X(L); refs. 16 and 35). Because BCL2L1 has also been linked to PAX3-FOXO1 expression (36, 37), we predicted that in our system FGFR4 was promoting aRMS survival through BCL2L1. To this end, we examined expression of BCL2L1 mRNA in HSMM cells ectopically expressing FGFR4, and observed an increase in BCL2L1 (Fig. 3F). Ectopic PAX3-FOXO1 did not increase BCL2L1 levels (data not shown). Next, we suppressed FGFR4 in Rh30 aRMS cells, and found that correspondingly, BCL2L1 decreased (Fig. 3G). Interestingly, in RD eRMS cells, no effect on BCL2L1 was observed with FGFR4 loss. Finally, we generated aRMS cell lines stably expressing epitope-tagged-BCL2L1, followed by vector, FGFR4 shRNA1 or shRNA2. Although BCL2L1 was able to block caspase-3 cleavage in these cells, they could not re-adhere to the tissue culture plate and ultimately lost viability as determined by trypan blue staining (data not shown), suggesting that additional cell survival mechanisms were disrupted by FGFR4 suppression.

Given the loss of viability in FGFR4-deficient aRMS cells, we reevaluated the eRMS cell lines in vitro and in vivo to assess for biochemical evidence of apoptosis. In vitro, stable expression of FGFR4 shRNAs in RD cells did not cause caspase-3 cleavage (Fig. 3D, lanes 2–3). In vivo, early harvesting of RD eRMS xenografts (day 11 after inoculation) did not reveal a “missed” increase in apoptosis (Supplementary Fig. S3). That is, although the baseline level of TUNEL staining was mildly elevated relative to that seen in the long-term tumor assays (5%–7% in Supplementary Fig. S3 vs. 2%–4%), there was no difference in TUNEL staining between vector and FGFR4 shRNA-expressing cells. To test...
the hypothesis that high levels of FGFR4, such as seen in aRMS cells, are associated with dependence on FGFR4 for cell survival, we ectopically overexpressed wild-type FGFR4 in eRMS cells (data not shown). This overexpression did not render them sensitive to apoptosis under conditions of FGFR4 suppression, as assayed by caspase-3 cleavage, suggesting that level of expression alone is not a determinant of phenotypic response to FGFR4 suppression. In summary, although FGFR4 suppression diminished cell survival in aRMS cells, in eRMS cells FGFR4 suppression had no such effect, suggesting a persistent dichotomy in the function of FGFR4 in RMS subtypes.
Small-molecule inhibition of FGFR4 in RMS tumorigenesis in vitro and in vivo

Because knockdown of FGFR4 in eRMS and aRMS cells using a genetic approach revealed different cellular responses, we next evaluated the effect of pharmacologic blockade of FGFR4 using a commercially available small-molecule inhibitor of FGF receptors, PD173074. PD173074 was originally observed to inhibit FGFR1 (38), but also shown to antagonize FGFR4 autophosphorylation and activity (32). In MTT assays, PD173074 inhibited both RD eRMS and Rh28 aRMS cell growth in a dose-dependent fashion (Fig. 4A and B). Similar results were observed in the SMS-CTR eRMS and Rh30 aRMS cell lines (Supplementary Fig. S4). Doses as low as 5 and 3 μmol/L (not shown) inhibited FGFR4 autophosphorylation (Fig. 4C) and signaling through the canonical extracellular signal–regulated kinase (ERK) pathway, whereas there was no effect on AKT activation (Fig. 4D). To determine whether FGFR blockade by a pharmacologic inhibitor would still yield dichotomous phenotypes in RMS subtypes, we evaluated aRMS and eRMS cells for caspase-3 cleavage in response to PD173074. In both Rh28 and Rh30 aRMS cells, PD173074 caused caspase-3 cleavage, whereas in RD eRMS cells, there was no evidence of such (Fig. 5A). Apoptosis was validated by PI/Annexin V staining, showing that aRMS cells treated with PD173074 displayed both increased early and late apoptosis (Fig. 3H). Interestingly, treatment with PD173074 caused downregulation of BCL2L1 in Rh30, but not RD cells (Fig. 5B), again suggesting that FGFR4 is preferentially sustaining survival signals in aRMS cells.

Finally, we evaluated the effect of PD173074 on the growth of Rh28 aRMS subcutaneous tumor xenografts in vivo. At a dose of 25 mg/kg, PD173074 caused tumor regression of established xenografts after 5 to 7 days.
Pharmacologic inhibition of FGFRs exerts distinct biochemical effects in eRMS versus aRMS cells. In vivo, pharmacologic inhibition of FGFR4 has a narrow therapeutic window with relatively high toxicity, and that future studies on the impact of FGFR4 blockade in human eRMS versus aRMS are required.

Discussion

Despite advances in supportive care and the evaluation of new agents, survival for children with high-risk RMS remains approximately 30% at 5 years (2). Expression of the PAX3-FOXO1 fusion gene in patients with metastatic aRMS portends a particularly poor outcome (39). Although it would be ideal to therapeutically interfere with tumor-specific proteins such as PAX3-FOXO1, transcription factors are currently chemically intractable. Identification of alternate RMS-specific proteins will be critical, and understanding their differential expression and regulation in RMS variants may permit refining of therapy.

This work focuses on FGFR4, a member of the FGFR RTK family. In our primary cell-based modeling of aRMS, we noted that stable expression of PAX3-FOXO1 in human myoblasts induced elevated FGFR4 protein. Analysis of human aRMS cell lines and tumor tissue, described here and by others (19, 20, 40, 41), continues to support a relationship between acquisition of the PAX3-FOXO1 fusion and expression of FGFR4. One limitation of this study is our retrospective discovery that the Rh3 and Rh28 cell lines likely derive from the same tumor, so that we in fact evaluated FGFR4 expression in 2 rather than 3 independent aRMS cell lines. However, the TMA studies of human clinical tissue do validate the observed pattern of greater FGFR4 protein expression in aRMS compared with eRMS tumor tissue. Moreover, mouse development studies show that Pax3 modulates Fgfr4 expression via a 3′ cis regulatory element (42), so it is reasonable to suspect that PAX3-FOXO1 is similarly co-opting PAX3 targets (43, 44) such as FGFR4. Recent ChIP-Seq analysis confirmed the presence of a PAX3-FOXO1 binding site in a distal regulatory element of human FGFR4 (45).

On the other hand, the significance and mechanism of FGFR4 expression in eRMS is not clear. Although we observe “upregulation” of FGFR4 in eRMS compared with primary human myoblasts, it is unknown what the baseline level of FGFR4 expression should be, because the precise cell of origin for eRMS is not identified. However, because wild-type PAX3 is expressed in eRMS, and genetically upstream of FGFR4 (46–48), it could have some role in controlling FGFR4 expression in eRMS. Posttranscriptional and post-translational mechanisms (as suggested by the multiple bands seen on FGFR4 immunoblot and the shift in these bands in response to shRNA knockdown) likely further modulate FGFR4 levels and activity. Future studies are required to determine the significance and roles of these FGFR4 protein species.

Although the mechanisms of expression of FGFR4 in the RMS subtypes are intriguing, even more so are the consequences of FGFR4 axis blockade. In our work, FGFR4 blockade in eRMS inhibited proliferation in vitro and tumorigenesis in vivo. On the other hand, FGFR4 blockade in aRMS induced cell death. BCL2L1-mediated reversal of caspase-3 induction did not completely rescue FGFR4 loss, raising the likelihood that additional survival pathways and processes such as anokis might be involved. However, the phenotypic difference we note in response to FGFR4 suppression does suggest a dichotomy between the function of FGFR4 in eRMS compared with aRMS cells. On the basis of our aRMS modeling, we speculate that acquisition of the PAX3-FOXO1 fusion is an early event in tumorigenesis, providing survival signals such as increased FGFR4 and...
BCL2L1 expression, which permit accumulation of later mutations resulting in aRMS. BCL2L1 does not seem to be a direct transcriptional target of PAX3-FOXO1, which is consistent with recent microarray studies in which BCL2L1 expression is not changed in the presence of PAX3-FOXO1 (40). Because our results are based on a limited number of human cell lines, we cannot exclude the possibility of cell-line specific effects. For example, RD cells harbor an activating Ras mutation (49), which may render them resistant to apoptosis. The profound response to FGFR4 suppression in aRMS cells does suggest an “addiction” to FGFR4 (50), consistent with the recent recognition of FGFR4 as an oncogene (51).

The identification of FGFR4 kinase domain activating mutations in 7% of RMS tumors, which promote metastasis in xenotransplanted models, further supports FGFR4 as a novel and important mediator of RMS progression (20). Because none of the human RMS cell lines routinely used in cell culture harbors an FGFR4-activating mutation (20, 41), it is not possible to strictly correlate our observations with the phenotypes described for the FGFR4 mutations. However, in murine cell systems designed to assess the impact of the activating mutations, the FGFR4 activating mutations K535 and E550 do provide additional drive to proliferate (41) and resist apoptosis (20). Because the majority of RMS tumors do not harbor these mutations, it will be important to continue to understand how upregulated, wild-type FGFR4 contributes to RMS.

In a manner similar to that being elucidated for the RTK MET (52), development of FGFR4 inhibitors will be critical. Although PD173074 itself is too toxic for therapeutic use, either because of broad FGFR inhibition or off-target effects, other small-molecule inhibitors of FGFRs are under development (53), as are high-affinity or neutralizing monoclonal antibodies to FGFR4 (54) and its cognate ligand FGF19 (55). It is not clear how important it will be for FGFR4 specificity, because although complete FGFR inhibition will likely be toxic, it is possible that FGFRs in addition to FGFR4 will be important in tumorigenesis. For example, FGFR1 has recently been noted upregulated in RMS (56).

In summary, in this work we show that FGFR4 protein expression is upregulated in human RMS cell lines relative to nontransformed human myoblasts, with highest expression in PAX3-FOXO1-positive aRMS cell lines. Immunohistochemistry of RMS tumor microarrays supports this finding in a larger cohort of patient tumor samples. PAX3-FOXO1 expression mediates upregulation of FGFR4, leading to increased myoblast viability. Loss-of-function of FGFR4, whether through genetic or pharmacologic means, seems to cause distinct effects in aRMS versus eRMS cell lines, suggesting dichotomous roles in these histologic subtypes. These results provide insight into the unique molecular origins of eRMS and aRMS, may be useful in the design of RMS histology-specific treatment algorithms, and support further study of FGFR4 as a rational drug target in RMS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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27. Estes NR, Thothassery JV, Kern FG. siRNA mediated knockdown of fibroblast growth factor receptors 1 or 3 inhibits FGF-induced anchor-age-independent clonogenicity but does not affect MAPK activation. Oncol Rep 2006;15:1407–16.


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