Mouse Mesenchymal Stem Cells Expressing PAX-FKHR Form Alveolar Rhabdomyosarcomas by Cooperating with Secondary Mutations

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Abstract

Alveolar rhabdomyosarcomas (ARMS) are highly malignant soft-tissue sarcomas that arise in children, adolescents, and young adults. Although formation and expression of the PAX-FKHR fusion genes is thought to be the initiating event in this cancer, the role of PAX-FKHR in the neoplastic process remains largely unknown in a progenitor cell that is undefined. We hypothesize that PAX-FKHR determine the ARMS progenitor to the skeletal muscle lineage, which when coupled to the inactivation and/or activation of critical cell signaling pathways leads to the formation of ARMS. Because a number of studies have proposed that mesenchymal stem cells (MSC) are the progenitor for several of the sarcomas, we tested this hypothesis in MSCs. We show that PAX-FKHR induce skeletal myogenesis in MSCs by transactivating MyoD and myogenin. Despite exhibiting enhanced growth in vitro, the PAX-FKHR–expressing populations do not form colonies in soft agar or tumors in mice. Expression of dominant-negative p53, or the SV40 early region, elicits tumor formation in some of the PAX-FKHR–expressing populations. Additional activation of the Ras signaling pathway leads to highly malignant tumor formation for all of the populations. The PAX-FKHR–expressing tumors were shown to have histologic, immunohistochemical, and gene expression profiles similar to human ARMS. Our results show the critical role played by PAX-FKHR in determining the molecular, myogenic, and histologic phenotype of ARMS. More importantly, we identify MSCs as a progenitor that can give rise to ARMS. [Cancer Res 2008;68(16):6587–97]

Introduction

Rhabdomyosarcoma (RMS) is a heterogeneous family of soft-tissue sarcomas arising in children, adolescents, and young adults (1). Common among RMS tumors is their myogenic phenotype, ranging from well-differentiated cells resembling embryonic muscle to variably undifferentiated round cells (2). There are two main histopathologic RMS subtypes, alveolar (ARMS) and embryonal (ERMS), which differ in their clinical presentation, response to therapy, and prognosis (3). Among patients with RMS, those with ARMS have a worse prognosis because their tumors are very aggressive, have often metastasized at diagnosis, and respond poorly to treatment (4, 5). Two distinct chromosomal translocations are found exclusively in ARMS and lead to the formation of the PAX3-FKHR and PAX7-FKHR (PAX-FKHR) transcription factors (6). As a consequence of these translocations, the intact DNA-binding domains of the PAX3 and PAX7 proteins are fused to the potent transcriptional transactivation domain of FKHR. As a result, PAX-FKHR transcriptional activity is greater than for PAX3 and PAX7 (7–9). PAX-FKHR likely function in ARMS, in part at least, by deregulating PAX3- and PAX7-specific target genes, signaling pathways, and biological processes. Despite exhibiting the classic alveolar histology, nearly one quarter of ARMS lack a PAX-FKHR translocation (10). By microarray analysis, we have shown that translocation-positive ARMS have a gene expression signature that is distinct from translocation-negative ARMS and ERMS (11). PAX-FKHR expression above a threshold level was necessary for the induction of this expression signature. Therefore, the PAX-FKHR transcriptional program plays an important role in determining the molecular phenotype of ARMS.

Myogenic differentiation is a hallmark of RMS. The most widely used immunohistochemical markers for the diagnosis of RMS are MyoD, myogenin, and desmin (12). Despite expressing MyoD and myogenin, key regulators of skeletal muscle determination and differentiation (13), ARMS show limited structural evidence of muscle differentiation and seem to be in a primitive stage of skeletal myogenesis (2, 3). However, few clues exist as to the cellular genesis of ARMS because these tumors do not resemble known normal tissue and can arise at diverse anatomic sites not always associated with skeletal muscle. The progenitor for ARMS has not been established, and whether the muscle phenotype stems from the cell lineage or is a consequence of PAX-FKHR–induced myogenesis remains unclear. Khan and colleagues (14) first showed by cDNA microarray analysis that expression of PAX3-FKHR in mouse NIH3T3 fibroblasts activates a myogenic transcription program that includes MyoD and myogenin, as well as genes involved in muscle signaling processes. We have since shown that PAX-FKHR transactivate MyoD directly by physically binding to sequences within its 258-bp core enhancer (15). PAX3-FKHR has also been shown to directly transactivate myogenin, independent of MyoD (16). These studies have led us to hypothesize that PAX-FKHR determine an unknown progenitor to the myogenic lineage by transactivating MyoD and myogenin, which when coupled to the disruption of key cell signaling pathways leads to the formation of ARMS. Identification of the ARMS progenitor remains an important goal because more accurate model systems are needed to study the disease process.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Here, we show that mesenchymal stem cells (MSC) expressing either PAX3-FKHR or PAX7-FKHR, in combination with p53/RB/Ras pathway disruptions, form tumors with histologic, immunohistochemical, and gene expression profiles similar to human ARMS. Based on these results, we propose that MSCs are a potential progenitor for ARMS.

Materials and Methods

MSC isolation and characterization. MSCs were isolated from the bone marrow of C57BL/6 mice. Marrow cells were pooled, dissociated by passing through a 25-gauge needle, filtered through a 70-μm nylon mesh filter, and plated into complete culture medium [Iscove’s modified Dulbecco’s medium supplemented with 20% fetal bovine serum (FBS), 20% horse serum (HS), 100 μg/mL 2-mercaptoethanol, 1 μg/mL hydrocortisone, penicillin, and streptomycin]. Once attached, the cells were periodically washed to rid the culture of nonadherent cells. At the first passage, cells were gently trypsinized to leave behind macrophages and fibroblasts that tend to adhere tightly to the culture dish. To enrich for the MSC population, the culture was immunodepleted of CD45-positive hematopoietic cells by magnetic-activated cell sorting (FACS) using phycoerythrin-conjugated antibodies: CD45, CD90, and SSEA-1 (BD PharMingen); CD105 (Southern Biotech). The resulting population was tested for cell surface marker expression by fluorescence-activated cell sorting (FACS) using phycoerythrin-conjugated antibodies: CD45, CD90, and SSEA-1 (BD PharMingen); CD105 (Southern Biotech).

In vitro differentiation. For osteogenic and adipogenic differentiation, 1 × 10^6 cells were seeded into a six-well plate and grown until confluent. Osteogenesis was then induced in medium containing 10 mM dexamethasone, 20 mM β-glycerophosphate, and 50 μg/mL t-ascorbic acid 2-phosphate. Adipogenesis was induced in medium containing 0.5 μg/mL dexamethasone, 0.5 μg/mL isobutylmethylxanthine, and 50 μg/mL indomethacin. After 21 d in differentiation medium, cells were fixed in 10% buffered formalin and stained with either Alizarin Red S (osteogenesis) or Oil Red O (adipogenesis). Myogenic differentiation was carried out according to Wakiatani and colleagues (18). Cells (5 × 10^4) seeded into a six-well plate were treated with 10 μg/mL 5-azacytidine for 24 h. Thereafter, the cells were maintained in medium containing 10% FBS and 5% HS for up to 30 d. Myogenesis was scored for multinucleated myotube formation and myosin heavy chain expression.

Quantitative reverse transcription-PCR and Western blotting. Total RNA from cultured cells and tumors was extracted using TRIzol (Invitrogen). Quantitative PCR was carried out on an ABI 7900HT Fast Real-time PCR System (Applied Biosystems) using QuantiTect SYBR Green (Qiagen) as described previously (11, 15). Relative expression levels were normalized to Gapdh from no less than three independent determinations using the ΔΔct method (19). All primer sequences are shown in Supplementary Table S1. Protein isolation and Western blot analysis were done as described (15). The antibodies used were as follows: MyoD and myogenin (BD PharMingen); Ras (Calbiochem); DCX (Chemicon); HA (Covance); AP-2, ME2F, Myf5, p53, and SV40-LT (Santa Cruz Biotechnology); and β-actin and FKHR (Sigma).

Retroviral constructs, transduction, and FACS. MSCV-ires-gfp retroviral constructs containing hemagglutinin (HA) epitope-tagged PAX3-FKHR or PAX7-FKHR have been described (11, 15). Viral supernatants were made in 293T cells by cotransfecting expression vector with plasmids pCG and pHIT123 encoding gag-pol and env, respectively (15). MSCs were transduced in complete culture medium containing 8 μg/mL polyene, subjected to FACS 72 h later for green fluorescent protein (GFP) expression, and pooled as polyclonal populations. Subsequent transductions with pBABE-zeo-LTg (20), pBABE-puro-Hras12V (21), pBABE-hygro-p53D (20, 22), or their corresponding control vectors (ref. 23; Addgene) were done similarly, except that polyclonal populations were isolated following selection in 500 μg/mL zeocin, 2 μg/mL puromycin, or 100 μg/mL hygromycin, respectively.

Tumorigenicity assays. Subcutaneous tumor formation was tested by injecting 5 × 10^6 cells into the shoulder region of 6-week-old nu/nu (Harlan Sprague-Dawley) or NIH-III (Charles River) mice. Mice were checked regularly for palpable tumors. Tumor size was measured weekly using calipers. Measurements of the height (H), width (W), and depth (D) were taken and converted into relative tumor volume (H × W × D; reported as mm³). An injection was scored nontumorigenic when a palpable tumor did not form after 120 d. When a tumor reached sufficient size, the animal was euthanized and the tumor was harvested for histologic analysis, gene expression analysis, or cell line reconstitution.

Results

Activation of skeletal myogenesis in PAX-FKHR-expressing MSCs. Wild-type MSCs were isolated from the bone marrow of C57BL/6 mice following depletion of CD45-positive hematopoietic cells by magnetic-activated cell sorting. The resulting population had a heterogeneous morphology and was composed of spindle-shaped, large flat, and rapidly self-renewing cells (Fig. L4). Although cell surface marker expression in MSCs varies depending on the mouse strain and anatomic site in which they are isolated (24, 25), FACS analysis confirmed that the population was positive for SSEA-1, CD90, and CD105 (Fig. L8). The population also expressed Sca-1 and CD44 (data not shown), but not CD45 as expected. The population was capable of undergoing adipogenesis, osteogenesis, and myogenesis (Fig. 1C and D).

HA-tagged PAX3-FKHR and PAX7-FKHR were expressed from the MSCV-ires-gfp retroviral expression vector (Fig. 2A), allowing transduced cells to be FACS sorted based on GFP expression (26). The HA tags have been shown not to alter the transcriptional function or biological activity of these proteins (11, 15, 27, 28). Duplicate polyclonal populations infected with vector, PAX3-FKHR, or PAX7-FKHR virus were isolated. Similar amounts of PAX-FKHR were expressed in each of the PAX-FKHR populations (Fig. 2F). Reanalysis using a COOH-terminal FKHR antibody showed that PAX-FKHR expression was lower than in the ARMS cell lines RH30 and JR/C (Fig. 2C). All of the PAX-FKHR populations expressed MyoD and myogenin, whereas the parental and vector populations did not. Myf5 was not expressed in any of the populations. MEF2, which cooperate with MyoD and myogenin to regulate muscle-specific gene expression, were expressed in all of the populations. Despite expressing PAX-FKHR, these populations maintained the characteristic MSC morphologies and seemed similar to the parental and vector populations (Fig. 2D). Apoptosis and...
senescence were not detected, suggesting that PAX-FKHR expression was compatible with continued growth and survival of these cells.

PAX-FKHR expressing MSCs exhibit enhanced growth in vitro, but do not form tumors in mice. To compare in vitro growth properties between the populations, proliferation and soft agar assays were done (Supplementary Table S2). Compared with parental and vector populations, all of the PAX-FKHR populations had increased growth rates (i.e., reduced doubling times). These differences were most pronounced when cells were analyzed in 10% FBS, although they were also seen in complete culture medium (20% FBS plus 20% HS) and medium containing 2% FBS (data not shown). The PAX-FKHR populations were also less contact inhibited and consistently had an average 4-fold higher saturation density at confluency. The parental and vector populations were not markedly different from each other, suggesting that the transduction process had no obvious effects on growth in vitro.

Despite these growth advantages, none of the populations exhibited anchorage-independent growth in soft agar. More importantly, none of the populations formed tumors in mice (Supplementary Table S2).

MSCs expressing PAX3-FKHR and the SV40 early region form tumors resembling human ARMS. Because PAX-FKHR alone was not transforming, we set out to deregulate normal growth control in the PAX-FKHR populations to determine whether they would then form tumors that were similar to human ARMS. To do so, the SV40 early region containing the large T (SV40-LT) and small t oncoproteins was transduced into each of the populations. Among its many functions, SV40-LT inactivates the RB and p53 tumor suppressor proteins (29, 30). Single transductions with pBABE-zeo-LTg (or the control vector pBABE-zeo) were carried out on the two vector, PAX3-FKHR and PAX7-FKHR populations. Transduced cells were selected in zeocin, pooled, and expanded as polyclonal populations. Similar amounts of SV40-LT were expressed in each of the populations transduced with pBABE-zeo-LTg (Fig. 3A). PAX-FKHR expression remained similar to the levels expressed in the original populations. In vitro growth analyses confirmed that all of the SV40-LT populations grew faster in culture, reached higher saturation densities, and formed colonies in soft agar (Supplementary Table S2; Supplementary Fig. S1). In contrast, the pBABE-zeo–transduced populations exhibited in vitro growth properties similar to their corresponding original populations.

Next, s.c. tumor formation was tested. After an average latency of 48 days, one of the two PAX3-FKHR/SV40-LT populations (P3F2-LT) formed tumors at 8 of 8 injection sites (Fig. 3B; Supplementary Table S2). One of the two vector/SV40-LT populations (V1-LT) also formed tumors, but only at 50% (4 of 8) of the injection sites and with a longer average latency of 67 days (Fig. 3C; Supplementary...
Table S2). Despite expressing equivalent SV40-LT, the other populations were nontumorigenic and no evidence of tumor formation was found at necropsy. As expected, none of the pBABE-zeo–transduced populations formed tumors. All tumors resulting from P3F2-LT and V1-LT were harvested for histologic analysis (Fig. 3D). By H&E staining, P3F2-LT tumors appeared as small, round cells with large nuclei and little cytoplasm. The classic alveolar spaces were absent, although the cells were densely packed, forming a nodular morphology around fibrovascular septa, consistent with the solid variant of human ARMS. Rhabdomyoblasts were present within the tumors. Immunohistochemistry confirmed that P3F2-LT tumors exhibit evidence of skeletal muscle differentiation. The tumors showed strong cytoplasmic desmin staining, as well as diffuse nuclear MyoD and myogenin staining. Tumors originating from V1-LT did not resemble ARMS. They were composed of spindle-shaped cells with a lot of atypical fibrillar material, lacked fibrovascular septa, and exhibited no evidence of skeletal muscle differentiation. When cell lines reconstituted from P3F2-LT and V1-LT tumors were rejected, they formed tumors with faster growth kinetics (Fig. 3B and C) and similar histologic phenotypes compared with their corresponding original tumor (data not shown).

Constitutive H-ras activation leads to enhanced ARMS-like tumor formation in all PAX-FKHR/SV40-LT populations. The lack of tumor formation in most SV40-LT populations suggested that an additional pathway disruption was needed to elicit a fully malignant phenotype. We addressed this by expressing constitutively active H-rasG12V in each of the populations. Single transductions with pBABE-puro-HrasG12V (or the control vector pBABE-puro) were carried out on the two vector, PAX3-FKHR and PAX7-FKHR populations expressing SV40-LT. Transduced cells were selected in puromycin, pooled, and expanded as polyclonal populations. Duplicate populations are labeled as 1 and 2. PAX-FKHR expression was determined using a HA antibody, β-actin served as a loading control. C, ectopic PAX-FKHR expression in the P3F2 and P7F1 populations was compared with endogenous PAX-FKHR expression in the ARMS cell lines RH30 and JR/C using a cross-reactive FKHR antibody. D, representative phase contrast images show similar morphologies in each of the populations.

Figure 2. Creation of PAX-FKHR–expressing MSCs. A, diagram of the retroviral constructs used to express PAX-FKHR. Three tandem HA epitope tags are represented by the black boxes at the 3’ end of the PAX-FKHR cDNAs. B, Western blot analysis shows skeletal myogenesis in the PAX-FKHR (P3F, P7F) populations compared with the parental (MSC) and vector populations. Duplicate populations are labeled as 1 and 2. PAX-FKHR expression was determined using a HA antibody; β-actin served as a loading control. C, ectopic PAX-FKHR expression in the P3F2 and P7F1 populations was compared with endogenous PAX-FKHR expression in the ARMS cell lines RH30 and JR/C using a cross-reactive FKHR antibody. D, representative phase contrast images show similar morphologies in each of the populations.

Constitutive H-ras activation leads to enhanced ARMS-like tumor formation in all PAX-FKHR/SV40-LT populations. The lack of tumor formation in most SV40-LT populations suggested that an additional pathway disruption was needed to elicit a fully malignant phenotype. We addressed this by expressing constitutively active H-rasG12V in each of the SV40-LT populations. Single transductions with pBABE-puro-HrasG12V (or the control vector pBABE-puro) were carried out on the two vector, PAX3-FKHR and PAX7-FKHR populations expressing SV40-LT. Transduced cells were selected in puromycin, pooled, and expanded as polyclonal populations. H-rasG12V expression was confirmed in each of the pBABE-puro-HrasG12V–transduced populations, seen as an increase in expression compared with the level of endogenous Ras in the pBABE-puro–transduced populations (Fig. 4A). PAX-FKHR and SV40-LT expression remained similar to the levels expressed in the original populations.

Soft agar analysis showed that the SV40-LT/H-rasG12V (designated LTR) populations formed colonies with greater efficiency and size (Supplementary Table S2; Supplementary Fig. S1B). When injected into mice, all of the LTR populations formed tumors with 100% frequency and no latency (Fig. 4B; Supplementary Table S2). By H&E staining and immunohistochemistry, the PAX-FKHR/LTR populations formed tumors resembling ARMS with evidence of myogenic differentiation, whereas the vector/LTR populations formed undifferentiated spindle-shaped sarcomas (Fig. 4C). Notably, some of the PAX-FKHR/LTR tumors exhibited the classic alveolar histology with alveoli-like spaces.

P3F2-LT tumors have a gene expression profile similar to human ARMS. PAX-FKHR–expressing MSCs resemble human ARMS. At the expression level, this was based on positive staining for MyoD, myogenin, and desmin (Figs. 3D and 4C). PAX-FKHR–expressing human ARMS share an expression profile with translocation-negative ARMS and ERMS (2, 3, 31), which includes the three aforementioned proteins. However, using microarray analysis, we have identified an expression signature that distinguishes PAX-FKHR–expressing ARMS from these other tumors (11). Therefore, to determine whether our model accurately mimics human ARMS, we examined global gene expression using Affymetrix GeneChip Mouse Exon 1.0 ST arrays. This analysis was restricted to P3F2-LT and V1-LT tumors to examine tumors containing the fewest pathway disruptions. To identify genes differentially expressed between five P3F2-LT and three V1-LT tumors, empirical Bayes moderated t-statistics were computed using LIMMA (32). A statistical significance cutoff (P < 0.01) was applied, as well as a biological significance cutoff so as to only include genes exhibiting expression differences ≥1.5-fold. This
analysis identified 797 up-regulated and 1,871 down-regulated genes (Supplementary Tables S3 and S4). Gene ontology (GO) analysis of these two lists identified overrepresented biological themes. Within the up-regulated list, GO categories included myogenesis and proliferation (Fig. 5A, left; Supplementary Table S5). Five of the top 10 GO categories within the down-regulated list were related to cell adhesion (Fig. 5A, right; Supplementary Table S6).

To validate the microarray analysis, RNA expression for several of the genes was compared by quantitative reverse transcription-PCR (Fig. 5B). The genes to be validated were selected based on their reported association with RMS (MyoD, myogenin, Igf2, Pax3, and Pax7) or ARMS (Tcfap2b, Cnr1, Dcx, Mycn, and Fgfr4). Although Pax7 expression was not statistically different by microarray analysis, it was included in the validation because it is expressed in ARMS and ERMS (31). As expected, MyoD and myogenin were differentially expressed in P3F2-LT versus V1-LT tumors. In addition, Igf2, Fgfr4, Mycn, Tcfap2b, Cnr1, and Dcx were also differentially expressed. Although the Pax3 and Pax7 results were somewhat variable, the data show that both were expressed in the P3F2-LT tumors. A tight correlation existed between the microarray and quantitative RT-PCR analyses (Supplementary Fig. S2). Using reconstituted cell lines derived from P3F2-LT and V1-LT tumors, differential MyoD, myogenin, Tcfap2b, and Dcx
protein expression was confirmed by Western blot analysis (Fig. 5C, left). By quantitative RT-PCR, differential (Tcfaq2b, Cnr1, Dcx, Fgf4, Igf2, and Mycn) or positive (Pax3 and Pax7) expression of most of these genes was confirmed in representative PAX-FKHR/LTR tumors (Supplementary Fig. S3). Differential MyoD, myogenin, and Tcfaq2b protein expression was also confirmed in P3F2-LTR and P7F1-LTR tumor reconstituted cell lines (Fig. 5C, right).

We next compared the gene expression profile of P3F2-LT tumors (Supplementary Tables S3 and S4) to human ARMS. To first derive an ARMS expression profile, comparative microarray analysis was done between six PAX-FKHR–expressing ARMS tumors (11) and six human bone marrow–derived MSC populations (BM-MSC), which had been analyzed using Affymetrix HG-U133A arrays. Performing LIMMA analysis as before (P < 0.01; expression changes ≥1.5-fold), 907 up-regulated and 1,849 down-regulated probe sets were identified as being differentially expressed (Supplementary Tables S7 and S8). GO analysis of the up- and down-regulated probe sets identified myogenesis and cell adhesion categories (Supplementary Tables S9 and S10), respectively, similar to the P3F2-LTR versus L1-LTR analysis (Supplementary Tables S5 and S6). The two highest ranked GO categories overrepresented in both up-regulated lists were identical. Four of the top 10 GO categories overrepresented in both down-regulated lists were identical, and five of the categories involved cell adhesion. Next, mouse and human orthologue gene pairs were mapped across the two microarray systems (mouse versus human) and platforms (Exon 1.0 ST versus HG-U133A) to generate gene lists from both that could be compared. Of the 2,392 and 1,925 differentially expressed mouse and human orthologue pairs, respectively, 99 of the up-regulated and 409 of the down-regulated
genes were common (Fig. 5D; Supplementary Tables S11 and S12). Overall, 21.2% (508/2,392) and 26.4% (508/1,925) of the mouse and human orthologue pairs overlapped, respectively. Individually, the overlap was restricted to 13.8%/16.0% and 24.4%/31.3% of the up-regulated and down-regulated mouse/human orthologue pairs, respectively. To determine whether these overlaps were significant, Monte Carlo simulations were done (33). In each simulation, we randomly assigned observed intensities to a set of orthologous

![Figure 5](https://example.com/image5.png)

**Figure 5.** Microarray analysis confirms ARMS-specific gene expression in P3F2-LT tumors. A, overrepresentation analysis of GO annotations shows categories enriched in the up-regulated (left) and down-regulated (right) gene lists shown in Supplementary Tables S3 and S4, respectively. The GO categories for each are plotted against the negative log of the P. Note that only the top 10 down-regulated GO categories are shown. B, quantitative RT-PCR shows RMS-associated and ARMS-specific gene expression in three individual P3F2-LT tumors (compared with two individual V1-LT tumors). The expression of each gene was normalized to Gapdh and shown relative to the highest expressing tumor. Columns, mean from triplicate reactions; bars, SE. C, Western blot analysis shows transgene and ARMS-specific protein expression in cell lines reconstituted from representative LT (left) and LTR (right) tumors, respectively. D, Venn diagram comparison between mouse P3F2-LT tumors and human PAX3-FKHR–expressing ARMS tumors. As indicated, up-regulated genes/probe sets are shown on top and down-regulated gene/probe sets are shown on bottom. The gray box outlines the mouse and human orthologue gene pairs that could be mapped; those outside the box could not be mapped and were not included in the comparison. Of the 2,392 and 1,925 differentially expressed mouse (P3F2-LT versus V1-LT) and human (ARMS versus BM-MSC) mapped orthologue gene pairs, respectively, 99 up-regulated and 409 down-regulated genes overlapped.
genes for two conditions, and the overlap was determined. The probability of the observed overlap was highly significant ($P < 10^{-38}$) and therefore unlikely to have occurred by chance alone. GO analysis of the overlapping up- and down-regulated orthologue pairs identified similar categories as before (Supplementary Tables S13 and S14). In summary, all of the gene expression results highlight how closely our model mimics human ARMS.

**Inactivation of p53 in PAX3-FKHR–expressing MSCs leads to ARMS-like tumor formation.** For the described experiments, we relied upon the SV40 early region to elicit malignant transformation and to inactivate p53 so that H-RasG12V would not trigger senescence (21). However, SV40 is not involved in human ARMS and is consequently not physiologic. We therefore repeated our studies using a dominant-negative form of p53 (p53DD), alone and in combination with H-rasG12V, to mimic p53 loss by more physiologic means (22). Single transductions with pBABE-hydro-p53DD and pBABE-hydro were carried out on representative vector (MSC-V1) and PAX-FKHR (MSC-P3F2, MSC-P7F1) populations. Polyclonal populations were isolated following selection in hygromycin. Similar amounts of p53 were expressed in each of the populations transduced with pBABE-hydro-p53DD (Fig. 6A). Endogenous p53 levels increased in the p53DD populations, indicating that p53DD was functional. PAX-FKHR and MyoD expression remained unchanged in the PAX-FKHR populations. In soft agar, the p53DD populations formed colonies (Supplementary Table S2; Supplementary Fig. S1C). However, when injected s.c. into mice, only P3F2-DD formed tumors at two of four injection sites (Supplementary Table S2). When injected i.m., P3F2-DD formed tumors at every injection site with a shorter average latency. Despite also expressing p53DD, the other populations were nontumorigenic. By H&E and immunohistochemical staining, P3F2-DD tumors had a phenotype consistent with the solid variant of human ARMS (Fig. 6B). The tumors were composed of small, round cells with large nuclei and little cytoplasm. The cells were densely packed around fibrovascular septa forming a nodular morphology. The tumors exhibited strong cytoplasmic desmin staining, as well as diffuse nuclear MyoD and myogenin staining.

**Constitutive H-ras activation in the PAX-FKHR/p53DD populations leads to enhanced tumor formation.** Next, constitutively active H-rasG12V was expressed in each of the p53DD populations. Single polyclonal populations transduced with pBABE-puro-HrasG12V were isolated, and expression of the transgenes was confirmed by Western blot analysis (Fig. 6C). In soft agar, the p53DD/H-rasG12V (designated DDR) populations formed colonies with greater efficiency and size compared with the p53DD populations (Supplementary Table S2; Supplementary Fig. S1D). When injected into mice, all of the DDR populations formed tumors with 100% frequency and no latency (Fig. 6D; Supplementary Table S2). By H&E staining and immunohistochemistry, the PAX-FKHR/DDR populations formed tumors resembling ARMS with evidence of myogenic differentiation, although the vector/DDR populations formed undifferentiated spindle-shaped sarcomas (Fig. 6B). By quantitative RT-PCR, the P3F2-DD and PAX-FKHR/DDR tumors exhibited RMS-associated and ARMS-specific gene expression (Supplementary Fig. S4).

**Discussion**

Identification of the ARMS progenitor remains an important goal within this field, because more reliable and accurate models are needed to study ARMS pathogenesis. Because myogenesis is a hallmark of RMS, it has been suggested that these tumors are derived from differentiating progenitor cells that become blocked at primitive stages of skeletal myogenesis at some point during the disease process. For ERMS, numerous studies suggest that this occurs in satellite cells (31, 34). In studies analogous to ours, Linardic and colleagues (35) were able to model ERMS in human postnatal skeletal muscle myoblasts by expressing the SV40 large T and small t oncoproteins, H-rasG12V and hTERT. For ARMS, however, the issue of whether the myogenic phenotype stems from the cell lineage or arises as a consequence of PAX-FKHR–induced myogenesis is less straightforward. Until now, a study has not shown that PAX-FKHR could induce a primary, nonmyogenic progenitor cell to form tumors that resemble ARMS.

Our results show that PAX-FKHR can determine MSCs to the myogenic lineage by transactivating the skeletal muscle regulators MyoD and myogenin, but not Myf5. This expression pattern recapitulates a similar pattern found in human ARMS (2, 12). Although MyoD and myogenin are expressed in most ARMS, Myf5 is expressed at much lower levels or not at all. Perhaps more striking, our results show that PAX-FKHR can activate a myogenic transcription program in MSCs that mimics a similar program found in human ARMS. This was evident from the tumor histopathology and immunohistochemistry, but was further reinforced by the gene expression profiling and GO analyses. GO categories overrepresented in the list of genes up-regulated in P3F2-LT tumors included skeletal muscle and neuromuscular junction development, as well as muscle contraction. Activation of this transcription program was undoubtedly mediated through MyoD and myogenin. PAX-FKHR have been shown to induce a similar expression pattern in cultured NIH3T3 fibroblasts (14, 15, 36). Khan and colleagues (14) showed that PAX3-FKHR up-regulates MyoD, myogenin, and numerous genes involved in muscle signal transduction, energy metabolism, and synaptic transmission. We have since shown that PAX-FKHR regulates MyoD transcription by binding directly to paired domain and homeodomain sites within its 258-bp core enhancer (15). Zhang and colleagues (16) have similarly shown that PAX3-FKHR directly regulates myogenin. We suspect that PAX-FKHR used these same mechanisms in MSCs.

We began this study expecting PAX-FKHR to drive the molecular expression, and the myogenic and histologic phenotypes of the resulting tumors, which our results confirmed. This was based on our observation that PAX-FKHR can determine a significant portion of the ARMS expression profile (11). As shown by competitive microarray analysis, the gene expression profile of P3F2-LT tumors significantly overlapped with the expression profile of human ARMS. These results suggest that our model accurately mimics ARMS at the global gene expression level. Besides activating myogenesis, PAX-FKHR transactivated many of the diagnostic discriminator genes that distinguish PAX-FKHR–expressing ARMS from translocation-negative ARMS and ERMS (11). These genes included Tcfap2h, Dcx, Car1, Pipox, Fgfr4, Mycn, Eya2, and MEOX1. However, because the microarray analysis identified genes that were differentially expressed in P3F2-LT versus V1-LT tumors, it underestimated similarities in the gene expression profiles of P3F2-LT and ARMS tumors by excluding those genes that were already expressed in the parental MSCs and did not change in the P3F2-LT and V1-LT tumors. **Met** was one such example. Notably, the GO analysis identified loss of cell adhesion as a major biological theme in the P3F2-LT tumors.
Considering that loss of cellular cohesion is thought to result in the classic alveolar spaces seen in human ARMS tumor sections, it is perhaps not surprising that some of our PAX-FKHR–expressing MSC tumors showed this phenotype. Based on these results, this may be a suitable model to study PAX-FKHR–mediated loss of cell adhesion in ARMS.

PAX-FKHR alone was not transforming in our model, which could have stemmed from the low PAX-FKHR expression in the MSC populations. However, Xia and Barr (37) have shown that physiologic PAX-FKHR expression is not tolerated in transduced cells. Although low levels of PAX3-FKHR promote transformation in NIH3T3 fibroblasts, high levels are growth suppressive. If this same mechanism occurred in MSCs, then we should expect the noticeably lower PAX-FKHR expression in MSCs when compared with the PAX3-FKHR–expressing ARMS cell lines RH30 and JR/C. Alternatively, it is possible that our in vitro grown mouse MSCs lost some of their permissiveness for malignant transformation. There are always limitations or deficiencies with in vitro
modeling, which do not exist in the in vivo patient setting. In this case, it may have been the ability of PAX-FKHR alone to elicit malignant growth.

Human ARMS contain a number of mutations that likely secondarily to the formation and expression of PAX-FKHR. These secondary mutations are necessary for the ARMS progenitor to progress to a fully malignant tumor (38–40). For example, although the percentage of ARMS tumors containing p53 mutations is somewhat low, this pathway is disrupted collectively through p53 mutation and/or loss, c-Myc amplification and overexpression, and p14ARF deletion. In this study, p53, RB, and RAS pathway disruptions were tested for their synergistic effects in eliciting tumor formation. Although ras point mutations have not been found in human ARMS, H-RasG12V was used to elicit malignant transformation in all of the populations so that their tumor histopathology could be studied. This was necessary because not all of the DD and LT populations formed tumors, and clonal outgrowth occurred in only some of these populations after a latency period during which a further genetic mutation may have been sustained. As such, H-RasG12V does not represent a physiologic secondary mutation, although this pathway may still function in ARMS because alternative mechanisms of RAS activation that are independent of activating point mutations may be present. Despite their reported roles in skeletal myogenesis, disruptions to these pathways were unable to inhibit the induction of myogenesis by PAX-FKHR. H-RasG12V did, however, down-regulate desmin expression in the LTR and DDR tumors. H-RasG12V also down-regulated Pax3, Pax7, Igf2, Fgfr4, Mycn, and Dcx expression in the DDR tumors. Because we were able to create ARMS-like tumors by expressing p53G100 alone, and in combination with H-RasG12V, the role of RB inactivation was not fully resolved. We are currently testing the effects of other ARMS-associated secondary mutations in this model, namely MNCY, which is amplified and/or overexpressed in a significant number of ARMS tumors (39). The goal of these studies is to model ARMS pathogenesis in MSCs (mouse and human) using as few as two true physiologic genetic mutations.

It has been suggested that satellite cells are the ARMS progenitor. To test this, Keller and colleagues (41) used a conditional knock-in mouse model to express Pax3-Fkhr in the Pax7-expressing satellite cell subcompartment. Although this led to a significant reduction in the postnatal myoblast pool, no tumors formed. Conditional Pax3-Fkhr expression in postnatal, differentiating myf-6–expressing myofibers, however, gave rise to tumors with a histologic appearance similar to the solid variant of ARMS (42). Tumors arose at a very low frequency (<1%) with a latency period of at least 1 year. As with our study, their results question the oncogenicity of PAX-FKHR. Tumor incidence increased in Pax3-Fkhr homozygous mice containing either homozygous p53 or Ink4a/Arf knockout alleles. To date, the progenitor for these tumors has not been fully characterized. Moreover, no comprehensive whole-genome approach was used to show whether the Pax3-Fkhr knock-in model accurately mimics the human disease. Last, it is uncertain as to how this progenitor cell is related to MSCs.

There is emerging evidence to suggest that MSCs are the progenitor for several of the sarcomas. Studies have shown that EWS-FLI1 can transform mesenchymal progenitor cells, leading to the formation of tumors resembling Ewing’s sarcoma (43, 44). In a correlative study, silencing of EWS-FLI1 in Ewing’s sarcoma cell lines caused their gene expression profile to converge toward that of MSCs, resulting in cells that were able to differentiate along the adipogenic and osteogenic lineages (45). Riggi and colleagues (46) have shown that FUS-CHOP expression in mesenchymal progenitor cells leads to the formation of myxoid liposarcoma. More recently, MSCs were transformed into malignant fibrous histiocytoma by inhibiting the Wnt signaling pathway (47). Along with our study, there is compelling evidence for MSCs being the progenitor for these related tumors. As such, our study provides a new model from which to study ARMS pathogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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