Defining the Cooperative Genetic Changes That Temporally Drive Alveolar Rhabdomyosarcoma

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Abstract

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood and adolescence. Most cases can be classified as embryonal (eRMS) or alveolar (aRMS), depending upon appearance under light microscopy. Although ongoing clinical trials have led to improved survival for patients with eRMS, children with aRMS still face a 5-year survival rate of <30% (1). Even more discouraging is the outcome for children with aRMS whose tumors harbor the PAX3-FKHR fusion gene; when metastatic, their 5-year survival is <10% (2). Although it is accepted that PAX3-FKHR is a characteristic and most likely founding mutation of aRMS (3–5), functioning in part by illegitimately activating myogenic transcription programs (6), it is not clear how this protein cooperates with other changes to promote aRMS.

To elucidate the molecular changes giving rise to RMS, we first showed that human myoblasts could be driven to a tumorigenic state by expression of the SV40 DNA tumor virus large T and small t antigens, which disable the p53 and Rb pathways and activate Myc pathways, respectively; oncogenic H-Ras, which provides a proliferative signal; and hTERT, which reactivates telomerase and immortalizes cells (7–9). This work identified myoblasts as a putative cell of origin for RMS, and validated the roles of pathways commonly dysregulated in human cancer in the development of RMS (10). Based on these studies, we recently showed that such myoblasts can be induced to proliferate inappropriately by expression of PAX3-FKHR, and that this was accompanied by epigenetic silencing of p16INK4A via methylation of its promoter (11). Similarly, loss of p16INK4A and tissue-specific expression of PAX3-FKhr in murine muscle models led to aRMS-like tumors (12). Taken together, we speculate that PAX3-FKhr and the accompanying loss of p16INK4A, which disables the RB pathway, may be initiating events in aRMS. Because p16INK4A silencing is often accompanied by loss of p14ARF (13), thereby also disabling the p53 pathway, and both MYCN amplification (14) and telomere stabilization (summarized in ref. 9) are observed in clinical samples of RMS, we tested whether these additional changes cooperated with PAX3-FKhr loss to drive myoblasts to become aRMS tumors.

Materials and Methods

Generation of cell lines. Early passage normal human skeletal muscle myoblasts (HSMNs; Lonza), grown in defined medium (Clonetics SkGM-2 Bullet kit) were stably infected with amphotrophic retroviruses derived from PK1-PAX3-FKhr-puro, pBABE-hTERT-hygro (9), pWZL-FLAG-murine-MycN-blasticidin, or vector. Cells were selected in 0.25 μg/mL puromycin (Sigma) or 50 μg/mL hygromycin B for 7 d, or 250 μg/mL G418 (Life Technologies Invitrogen) for 10 d. HSMNs were characterized by the vendor as >90% desmin positive. Unless indicated, for presenescent expression, retroviral infections were initiated at population doubling (pd) 2 to 4. Senescence bypass in HSMNs generally starts at ~ pd 15, although it can be influenced by culture conditions. HSMNp53+/p53−, M1/L1/p53−, and M1/L1+H− cells contained all transgenes by pd 21, 31, and 13, respectively. Human RMS cell lines were grown in RPMI 1640 (Life Technologies) with 10% fetal bovine serum. HSMNs previously engineered to generate eRMS morphology, via expression of SV40 T/t-oncoproteins, hTERT, and oncogenic H-Ras (9), provided control cell lysates for transgene expression.

Immunoblotting. Cells were lysed in Tris/radioimmunoprecipitation assay buffer with standard protease inhibitors and passed through a 21g needle to shear DNA. Protein concentration was measured by the DC assay (Bio-Rad). Sixty to 100 μg of lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with primary monoclonal antibodies anti-FOXO1A (FKhr) F6928, FLAG F3165, tubulin T4026 (Sigma), p16INK4A 554079 (BDPharmingen), actin SC-8462 (Santa

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Cruz), or primary polyclonal antibody anti-PAX3 1607802 (Geneka). Membranes were reacted with a secondary horseradish peroxidase–labeled goat anti-mouse or anti-rabbit antibody (Invitrogen-Zymed), and developed using chemiluminescence (Amersham).

**Reverse transcription-PCR.** Total cellular RNA was isolated using the RNA-Beek kit (TEL-TEST). After spectrophotometric quantitation, 2 μg were subject to reverse transcription using the Omniscript RT kit (QIAGEN) with Oligo-RT primers (Life Technologies Invitrogen). Standard PCR using primer sets for MYOD1, MYOGENIN (15), PAX3-FKHR, FLAG-hTERT (16), murine-FLAG-MycN, p14ARF, and p16INK4A (11) was performed, with product separated on 2% agarose (Supplementary Table S1). Glyceraldehyde-3-phosphate dehydrogenase and water controls were included to verify equal RNA and specificity of cDNA input, respectively.

**Tumor xenografts assays to measure in vivo tumorigenic ability.** Although cell transformation may be measured in vitro as colony growth in soft agar, because neither the human RMS JR nor HSMMPF+H+M cell lines formed colonies, we proceeded directly to tumorigenesis assays in vivo. Under Institutional Animal Care and Use Committee–approved protocols, and as performed (9), cell lines were proven to be free of replicating retrovirus or Mycoplasma, expanded in culture, then 10 million cells per cell line were injected s.c. into the flanks of severe combined immunodeficient (SCID)/beige mice and monitored for tumor appearance and volume (Fig. 1C). The human aRMS cell line JR (naturally expressing PAX3-FKHR) and the HSMMPF cell line expressing only hTERT (HSMMPF+H) were included as positive and negative controls, respectively. As anticipated, JR cells rapidly formed tumors, whereas HSMMPF+H cells failed to generate tumors during the 90 days that mice were monitored (Fig. 1C). Importantly, HSMMPF cells additionally expressing hTERT and MycN, and lacking p14ARF/p16INK4A expression (HSMMPF+H+M), also formed tumors (Fig. 1C), albeit at a longer latency than the positive controls. This latency is consistent with the variation observed for other human RMS cell lines, such as the eRMS cell line RD, which requires over 3 weeks to induce s.c. tumor growth (Fig. 1C).

To assess the morphologic characteristics of the resulting xenograft tumors, we were subject to standard H&E staining (Fig. 1D). Whether derived from the human RMS cell lines or from HSMMPF+H+M cells, all tumors consisted of monomorphous small blue cells, a morphological characteristic of pediatric sarcomas. The tumors deriving from HSMMPF+H+M-M cells also contained interspersed myoblastic cells, reflecting their recent derivation from primary skeletal muscle (Fig. 1D). Tumors were next subject to a tripartite of immunohistochemical stains that are the clinical standard for identifying RMS: desmin, skeletal muscle–specific actin, and myoglobin (18). As expected, control RD eRMS and JR aRMS tumors stained positive for desmin and skeletal muscle-specific actin (Fig. 2A, a–b and d–e). Similarly, the HSMMPF+H+M-M derived tumors stained positively for these markers (Fig. 2A, c and f). Myoglobin staining was strongest in the HSMMPF+H+M xenograft (Fig. 2A, i). Thus, ectopic expression of hTERT and MycN in postnecescent HSMMPF myoblasts promotes tumor growth resembling RMS as judged by clinical immunohistochemical standards.

To further classify the HSMMPF+H+M-M tumors as either eRMS or aRMS, they were subject to a second tier of immunohistochemical stains (Myod1 and myogenin) that distinguishes these variants (18). Using nuclear expression of these transcription factors, the control eRMS RD xenografts were found to appropriately exhibit diffuse staining for both markers, whereas the control aRMS JR xenografts appropriately exhibited diffuse staining for MyoD1 but patchy staining for myogenin (Fig. 2B, a–b and d–e). Tumors derived from HSMMPF+H+M-M cells exhibited diffuse staining for MyoD1, but patchy staining for myogenin, consistent with an alveolar pattern (Fig. 2B, c and f). Thus, HSMMs engineered to stably express PAX3-FKHR (with concomitant silencing of the INK/ARF locus), hTERT, and MycN can recreate alveolar RMS.
Effect of altering order of acquisition of genetic changes.

Because PAX3-FKHR is found exclusively in aRMS, it is thought to be a genetic lesion acquired early in the stepwise process of tumorigenesis. Although this is not proven, other chromosomal translocation–driven malignancies such as chronic myelogenous leukemia are similarly thought to result from early translocation events, based on the paradigm of BCR-ABL expression in susceptible precursors (19). To determine whether the order of acquisition of genetic changes is important in aRMS, we generated a second set of genetically defined HSMM cell lines whereby, as before, PAX3-FKHR was introduced first, followed by bypass of senescence, then introduction of hTERT, then MycN (HSMMPF+H+M); or whereby MycN was introduced first, followed by hTERT, then PAX3-FKHR (HSMM+H+PF). hTERT was not used as an initial genetic change because although it prevents crisis associated with telomere shortening, it does not enable HSMMs to bypass the senescence checkpoint (9). Thus, here hTERT is used as a second or third genetic change. Cell lines were then assayed for transgene expression and p14ARF/p16INK4A expression (Fig. 3A), then tested as xenografts (Fig. 3B). As before, we found that only HSMMs expressing all three genetic changes—PAX3-FKHR, hTERT, and MycN, in that order—could form tumors (HSMMPF+H+M). However, HSMMs expressing MycN, hTERT, then PAX3-FKHR (HSMM+H+PF) were unable to do so. Because PAX3-FKHR–mediated bypass of senescence (11) is an early step in this model, we asked whether this event might be the pivotal difference between these matched...
cell lines. Therefore, we assessed the effect of early MycN expression by stably expressing it in presenescent HSMMs, and found that although it enabled bypass of senescence (Fig. 3C), likely through epigenetic down-regulation of p14ARF (20), MycN-mediated bypass was not accompanied by the low p16INK4A and high PAX3-FKHR levels seen in PAX3-FKHR–mediated bypass (11). To the contrary, postsenescent HS MMMycN cells exhibited high p16INK4A (Fig. 3C, inset M postsenescent; Fig. 3A, M+H, M+H+PF) and low PAX3-FKHR protein expression (Fig. 3A).

Although we suspected that the altered order of genetic changes in the HSMMM+H+PF underlay their lack of tumorigenesis, mechanistically we wondered whether this was due to failure of p16INK4A down-regulation (or PAX3-FKHR up-regulation) to a critical threshold. Because p14ARF was similarly down-regulated in both MycN and PAX3-FKHR–mediated bypass, it was not likely the cause. To this end, we attempted to knock down p16INK4A (using an shRNA; ref. 11) or increase PAX3-FKHR (using overexpression constructs) in these cells. However, p16INK4A levels paradoxically increased in response to the shRNA, and PAX3-FKHR levels remained constant (data not shown), suggesting a tolerated setpoint of PAX3-FKHR expression, a phenomenon noted previously (21). Thus, it seems that early expression of PAX3-FKHR results in a specific level of expression of both itself and the INK/ARF locus, which effect later tumorigenesis.

Last, to confirm the requirement for not only early PAX3-FKHR expression, but for PAX3-FKHR–mediated bypass of senescence accompanied by p16INK4A loss in this model of aRMS, we generated a cell line (HSMMPF+M) in which PAX3-FKHR was expressed first and MycN second, but importantly, both expressed before the senescence checkpoint. After bypass, p16INK4A and PAX3-FKHR levels were high and low, respectively (Fig. 3D, top) reminiscent of bypass mediated by MycN in the nontumorigenic HSMMM+H+PF cells. This predicted that HSMMPF+M cells, even after addition of hTERT, would not form tumors. However, when assayed as xenografts, all HSMMPF+M injected sites formed tumors, albeit after a delay (Fig. 3D, bottom). HSMMPF+M tumor lysates showed down-regulated p16INK4A compared with that in preinjection cultured cells (Fig. 3D, bottom), suggesting a selective advantage for p16INK4A loss in vivo, and supporting our hypothesis that PAX3-FKHR–driven tumors require p16 INK4A down-regulation. PAX3-FKHR transcripts from these tumors were not increased (Fig. 3D, bottom), suggesting that low expression can be adequate for tumorigenesis. However, this must be interpreted cautiously, as we cannot rule out other mutations acquired in vivo, and at least in HSMMPF+M+PF cells, PAX3-FKHR transcript level does not predict protein expression (Fig. 3D), suggesting regulation of PAX3-FKHR at the translational or posttranslational level. Taken together, these data suggest a specific order of acquisition of genetic lesions.

Figure 2. Immunohistochemical evaluation of tumor xenografts. A, to evaluate whether the tumor xenografts resulting from HSMM+PF cells transduced with hTERT and MycN resembled RMS, they were evaluated by immunohistochemical markers desmin (c), skeletal muscle-specific actin (f), and myoglobin (i), and MyoD1 (c) and myogenin (f). Tumor xenografts derived from eRMS and aRMS cell lines are included as controls for both sets of immunohistochemistry. Brown color, immunoreactivity. Magnification, ×400.
required to convert HSMMs to tumors mimicking aRMS, with early expression of PAX3-FKHR critical for tumorigenesis.

Summary. We have used a rational modeling approach to identify a set of genetic changes required to generate aRMS in the laboratory. Based on mutations identified in human aRMS tumor specimens, this includes gain-of-function of PAX3-FKHR with concomitant loss-of-function of p16INK4A/p14ARF (RB/p53 pathways), and gain-of-function of MycN and hTERT (Fig. 4). In addition, the order of acquisition of genetic lesions is critical, as only those HSMMs serially transduced to express PAX3-FKHR first, followed by hTERT/MycN, formed tumors in vivo. This supports the prediction that oncogenic translocations mediate important early events underlying later tumorigenesis. In RMS, early expression of PAX3-FKHR in a susceptible cell may provide the initiating step of senescence bypass; acquisition of subsequent critical oncogenes enables full conversion to the malignant phenotype. The biological value of this approach is in demonstrating that the same precursor cells, HSMMs, can be steered toward an eRMS or aRMS phenotype.

Figure 3. Evaluation of order of transgene expression in aRMS tumorigenesis. A, a second set of genetically defined cell lines was generated from HSMMs and, again, validation of ectopically expressed transgenes and assessment of p14ARF and p16INK4A levels were performed as described in Fig. 1B. PAX3-FKHR expression was also examined by immunoblotting with an anti-FKHR antibody, which detects endogenous FKHR and ectopic PAX3-FKHR, and an anti–NH2-terminal-PAX3 antibody, which detects endogenous PAX3 and ectopic PAX3-FKHR. B, cells lines from A and D were injected as s.c. xenografts in SCID/beige mice to determine their tumorigenic ability in vivo. C, to determine the effect of MycN expression in primary human myoblasts, HSMMs were stably transduced with a mammalian MycN [+] or vector control (−) and monitored over time for pd. Inset, immunoblot of p16INK4A expression in cell lysates prepared from presenescent or senescent HSMMs, compared with HSMMs that have bypassed senescence because of MycN or PAX3-FKHR expression. D, top, HSMMs were stably transduced with PAX3-FKHR and MycN before undergoing the senescence checkpoint. After senescence bypass hTERT was added, yielding the HSMMPF+M+H cell line. PAX3-FKHR and p16INK4A protein levels were assessed by immunoblotting as described in A. Presenescent native HSMMs and postsenescent HSMMs expressing PAX3-FKHR are included as controls. Bottom, level of expression of PAX3-FKHR and p16INK4A, as assessed by RT-PCR in xenograft tumors derived from the HSMMPF+M+H cell line, compared to that in the parental line and the tumorigenic HSMMPF+H+M cell line. Abbreviations and use of the T/t+/H+R cell line as described in Fig. 1.
depending upon the genetic lesions introduced. The therapeutic value is in defining a minimum number of genetic lesions (cellular pathways) required to convert normal human skeletal muscle precursors into cells that can form aRMS. This knowledge may be useful in the development of rational drug combinations to treat this cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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