Abstract
Rhabdomyosarcoma, a malignancy showing features of skeletal muscle differentiation, is the most common soft tissue sarcoma of childhood. The identification of distinct clinical presentation patterns, histologic tumor types, and risk groups suggests that rhabdomyosarcoma is a collection of highly related sarcomas rather than a single entity. In an effort to understand this seemingly heterogeneous malignancy, we constructed a genetically defined but malleable model of rhabdomyosarcoma by converting less differentiated human skeletal muscle cell precursors (SkMC) and committed human skeletal muscle myoblasts (HSMM) into their malignant counterparts by targeting pathways altered in rhabdomyosarcoma. Whereas the two cell types were both tumorigenic, SkMCs gave rise to highly heterogeneous tumors occasionally displaying features of rhabdomyosarcoma, whereas HSMMs formed rhabdomyosarcoma-like tumors with an embryonal morphology, capable of invasion and metastasis. Thus, despite introducing the same panel of genetic changes, altering the skeletal muscle cell of origin led to different tumor morphologies, suggesting that cell of origin may dictate rhabdomyosarcoma tumor histology. The ability to now genetically induce human rhabdomyosarcoma-like tumors provides a representative model to dissect the molecular mechanisms underlying this cancer. (Cancer Res 2005; 65(11): 4490-5)

Introduction
Malignant tumors resembling skeletal muscle, collectively known as rhabdomyosarcomas, are the most common soft tissue sarcoma of childhood, with a 3-year failure-free survival of high-risk patients of only 20% (1). Tumor histology plays a significant role in the prognosis of rhabdomyosarcoma (1), and combined with variations in clinical group and stage, suggest that the etiology of rhabdomyosarcoma is heterogeneous. There are only a few in vivo experimental systems to study the variable molecular events leading to rhabdomyosarcoma. A number of human rhabdomyosarcoma tumor cell lines have been established, but these lines represent the final stages of rhabdomyosarcoma development and hence are not amenable to dissecting early events. Rhabdomyosarcoma tumors appear at low or variable incidence in a variety of transgenic mouse backgrounds (2, 3), and recently, mouse models have been generated for both the embryonal and alveolar histologic variants of rhabdomyosarcoma (4, 5). However, tumorigenesis can be different between humans and rodents (6, 7); hence, there is value in studying cancer in human cells. To elucidate the cellular mechanisms underlying human rhabdomyosarcoma, we therefore sought to define the molecular events sufficient to drive normal human skeletal muscle cell precursors towards a cancerous fate.

Rhabdomyosarcoma shares a number of changes common to other human malignancies. Specifically, the p53 tumor suppressor pathway is impaired in up to 50% of rhabdomyosarcoma tumors and cell lines (2, 8, 9) and rhabdomyosarcoma can occur in children with germ line inactivation of p53 (10). The RB tumor suppressor pathway also seems dysregulated in rhabdomyosarcoma through amplification of cyclin-dependent kinase CDK4 and/or deletion of the tumor suppressor p16INK4A (2, 9, 11). The MYCN proto-oncogene is up-regulated in rhabdomyosarcoma (2, 12), targeting 40% of genes similarly activated by c-Myc (13). Telomere stabilization and correspondingly cell immortalization is illegitimately restored in rhabdomyosarcoma by reactivation of the hTERT catalytic subunit or through alternative telomere lengthening mechanisms (14–16). Lastly, activation of the Ras pathway (17) occurs in rhabdomyosarcoma through point mutations in Ras (2, 18, 19), activation of upstream tyrosine kinase receptors (2), or loss of the negative regulator neurofibromin (20). To recapitulate the genetic alterations of rhabdomyosarcoma in a human model system, we therefore disrupted these pathways (21) in human primary cells. The cell of origin of rhabdomyosarcoma is unknown but suggested to be from cells developing at any point along the skeletal muscle cell axis (2). Hence, we chose to introduce these alterations in human primitive fetal skeletal muscle cell precursors (SkMC) as well as human postnatal skeletal muscle myoblasts (HSMM) already committed to the skeletal muscle lineage. We used expression of the SV40 large T oncoprotein to inactivate the tumor suppressors p53 and RB, small t oncoprotein to inactivate PP2A, leading to stabilization of the c-Myc oncoprotein (22), hTERT to impart immortalization (23), and oncogenic (V12G) Ras to provide self-sufficiency in growth signals (17).

Materials and Methods

Cell lines. Low-passage normal human fetal SkMCs or normal HSMMs from a teenage donor (Clonetics Cell Systems, Cambrex Corp., East Rutherford, NJ) were sequentially infected with amphotropic retroviruses derived from pBABE-neo-T-Ag, pBABE-bleo-FLAG-H-RasV12S (7), pBABE-hygro-FLAG-hTERT, pBABE-puro-FLAG-H-RasV12G (21), or the corresponding empty vectors and sequentially selected for 7 to 10 days in medium supplemented with 0.25 μg/mL puromycin (Sigma Chemical Co., St. Louis, MO), 50 μg/mL hygromycin B, 250 μg/mL G418, or 800 μg/mL Zeocin (all from Life Technologies Invitrogen, Carlsbad, CA). Cells were verified to be of skeletal muscle lineage by expression of one or more skeletal muscle markers: SkMCs were desmin positive, skeletal muscle–specific variants of rhabdomyosarcoma (4, 5). However, tumorigenesis can be different between humans and rodents (6, 7); hence, there is
anti-FLAG-M2 (Sigma Chemical), anti-desmin M0760, anti-muscle-specific actin M0635, or anti-myoglobin A0324 (DakoCytomation, Carpinteria, CA), using established protocols (7).

For immunohistochemistry, 5-µm sections of paraffin-embedded tissue were subject to heat-induced antigen retrieval using the steam method and incubated with antibodies (DakoCytomation) against myoglobin (A0324, 1:3,000), muscle-specific actin (M0635, 1:200), desmin (M0760, 1:50), myogenin (M3539, 1:50), and MyoD1 (M3512, 1:50) at 37°C for 45 minutes. Biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was used, followed by a Peroxidase/DAB detection system (Vector Laboratories). Sections were counterstained with hematoxylin.

For tumor xenograft assays, 10^7 cells were suspended in 50 µL PBS and injected into the right gastrocnemius muscle. For orthotopic assays, 3 × 10^5 cells suspended in 50 µL PBS were injected into the right gastrocnemius muscle. For metastasis assays, 4 × 10^5 cells suspended in 200 µL medium were injected into the tail vein. Each cell line was tested in quadruplicate. All experiments were done under the Duke Institutional Animal Care and Use Committee–approved protocols.

**Results and Discussion**

**Genetic transformation of skeletal muscle cell precursors.** Unlike adult carcinomas, which derive from the malignant transformation of epithelial cells, the histogenesis of pediatric mesenchymal tumors such as rhabdomyosarcoma is less clear. It has been suggested that rhabdomyosarcoma tumors may derive from the transformation of cells developing at any point along the skeletal muscle cell axis (2), generically termed here as “skeletal muscle cell precursors.” Although historically these precursors were presumed to be satellite cell myoblasts located beneath the basement membrane of the skeletal myofiber, skeletal muscle cell precursors are also believed to include multipotent stem cells, which derive from the bone marrow but reside in skeletal muscle tissue, and possibly skeletal muscle myonuclei, which may be stimulated to reenter the cell cycle after specific cues (25). In an effort to model rhabdomyosarcoma, we postulated that transformation of an unselected primary skeletal muscle cell population might yield tumors akin to rhabdomyosarcoma. Therefore, a heterogeneous population of skeletal muscle cell precursors derived from human fetal muscle, termed SkMC cells, was serially infected and selected for antibiotic resistance with amphotrophic retroviruses encoding T/t-Ag, hTERT, and H-Ras^V12G, or a combination thereof, in which one or more transgenes was substituted with an empty vector. Stable polyclonal cell lines representing all possible combinations of T/t-Ag (T), and/or hTERT (H), and/or H-Ras^V12G (R), and/or vector (V), were used to determine the effect of each transgene on the malignant transformation of primary human fetal SkMCs. For xenograft assays, 10^7 cells were suspended in 50 µL Matrigel (BD Biosciences, San Jose, CA) and injected s.c. into the flank of a severe combined immunodeficient/Beige mouse as previously described (7). For orthotopic assays, 3 × 10^5 cells suspended in 50 µL PBS were injected into the right gastrocnemius muscle. For metastasis assays, 4 × 10^5 cells suspended in 200 µL medium were injected into the tail vein. Each cell line was tested in quadruplicate. All experiments were done under the Duke Institutional Animal Care and Use Committee–approved protocols.

**Figure 1.** Evaluation of transgene expression, cell growth, and in vitro tumorigenic capacity of primary human fetal SkMCs. (A) Transformation and tumorigenesis. Soft agar growth was assessed after 4 weeks as described previously (7). For xenograft assays, 10^7 cells were suspended in 50 µL Matrigel (BD Biosciences, San Jose, CA) and injected s.c. into the flank of a severe combined immunodeficient/Beige mouse as previously described (7). For orthotopic assays, 3 × 10^5 cells suspended in 50 µL PBS were injected into the right gastrocnemius muscle. For metastasis assays, 4 × 10^5 cells suspended in 200 µL medium were injected into the tail vein. Each cell line was tested in quadruplicate. All experiments were done under the Duke Institutional Animal Care and Use Committee–approved protocols.
were generated and confirmed to appropriately express the desired transgenes (Fig. 1A–B). A cell line expressing 5-fold lower levels of \( \text{H-Ras}^{V12G} \) was also generated (Fig. 1A; M-THR\textsuperscript{Lo}) to address the effect of oncogenic Ras expression on rhabdomyosarcoma tumorigenicity, as low oncogenic Ras expression has been found to limit tumor growth in human mammary tumors (26).

### Table 1. Xenograft tumor formation in immunocompromised mice

<table>
<thead>
<tr>
<th>Parental cell type</th>
<th>Cell line</th>
<th>Injection route</th>
<th>Mice developing tumors/mice injected</th>
<th>Earliest time to palpable tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkMC</td>
<td>M-TVV</td>
<td>SQ</td>
<td>0/4</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>M-THV</td>
<td>SQ</td>
<td>0/8</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>M-TVVR</td>
<td>SQ</td>
<td>0/4</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>M-THR\textsuperscript{Lo}</td>
<td>SQ</td>
<td>6/8</td>
<td>11 wks</td>
</tr>
<tr>
<td></td>
<td>M-THR</td>
<td>SQ</td>
<td>4/4</td>
<td>4.5 wks</td>
</tr>
<tr>
<td>HSMM</td>
<td>MY-THR</td>
<td>SQ</td>
<td>5/5</td>
<td>2 wks</td>
</tr>
<tr>
<td></td>
<td>MY-THR</td>
<td>IV</td>
<td>4/5</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>MY-THR</td>
<td>IM</td>
<td>4/4</td>
<td>8 wks</td>
</tr>
</tbody>
</table>

NOTE: Polyclonal cell lines derived from normal human SkMCs or normal human myoblasts (HSMM) stably expressing SV40 T/t-Ag (\( T \)), and/or hTERT (\( H \)), and/or \( \text{H-Ras}^{V12G} \) (\( R \)), and/or empty vector (\( V \)) were injected s.c., i.v., or i.m. to assay for tumor growth. “Lo” is a cell line expressing low levels of \( \text{H-Ras}^{V12G} \).

Abbreviations: NA, not applicable (as mice injected via tail vein developed lung nodules); SQ, s.c.; IV, i.v.; IM, i.m.

Tumorigenic conversion of skeletal muscle cell precursors by expression of T/t-Ag, hTERT, and \( \text{H-Ras}^{V12G} \). To explore the effects of these genetic changes on the tumorigenic process, the resulting cell populations were assayed \textit{in vitro} for immortalization and anchorage-independent growth, common features of cancer cells, and \textit{in vivo} for tumor growth in immunocompromised mice. As regards immortalization, cells lacking T/t-Ag entered a permanent growth arrest between population doublings 13 and 15 with morphologic features consistent with senescence (Fig. 1C; uninfected, M-VVV, M-VHV), in agreement with the results of others (27). The stable expression of \( \text{H-Ras}^{V12G} \) alone or in combination with hTERT resulted in cell death (Fig. 1C; M-VVR, M-VHR), presumably reflecting a cellular response against Ras upregulation in the presence of an intact p53 pathway (28). As expected (29), expression of T/t-Ag alone or in the presence of oncogenic Ras extended the proliferative life span of the cells by up to 60 population doublings, at which time the cells entered crisis and perished (Fig. 1C; M-TVV, M-TVR). As with other cell types (21), independent of \( \text{H-Ras}^{V12G} \) expression, only T/t-Ag in conjunction with hTERT extended cellular life span (Fig. 1C; M-THR, M-THV).

Thus, combined ectopic expression of T/t-Ag and hTERT endows human SkMCs with an infinite life span, in agreement with the observed dysregulation of the p53, RB, Myc, and hTERT pathways in rhabdomyosarcoma tumors and cell lines (2, 14–16).

As regards transformation, each of the above-described cell lines was also assayed for growth in soft agar, one of the most stringent assays for transformation \textit{in vitro}. Expression of all four transgenes, and not any less, was necessary and sufficient for anchorage-independent growth of SkMCs (Fig. 1D; M-THR). This growth depended upon increased oncogenic Ras expression, as both the number of colonies and the heterogeneity of colony sizes decreased upon lowering oncogenic Ras expression 5-fold (Fig. 1D; other data...
not shown). Thus, Ras expression is required in addition to T/t-Ag and hTERT for anchorage-independent growth of SkMCs.

The most telling assay of tumorigenesis is tumor growth itself. SkMCs expressing all four transgenes, or control SkMCs expressing two or three of these transgenes, were injected s.c. into immunocompromised mice and monitored for tumor growth. As in soft agar, expression of all four transgenes was required for tumor growth (Table 1). This growth was sensitive to Ras expression, as a decrease in expression of this oncogene delayed or abolished tumor growth (Table 1), suggesting an important role for the stimulation of the Ras pathway in rhabdomyosarcoma. In sum, SkMCs minimally must undergo dysregulation of the p53, RB, Myc (and possibly other targets of PP2A), Ras, and hTERT pathways to form tumors in vivo.

SkMC-derived tumors ranged from highly undifferentiated small round blue cell tumors with large nuclei (Fig. 2A) and scant cytoplasm to tumors with foci of spindle-shaped cells (Fig. 2C). No classic embryonal or alveolar histology could be identified by light microscopy, nor were there cytoplasmic cross-striations to confirm skeletal muscle differentiation. Immunohistochemical staining for desmin, muscle-specific actin, and myoglobin, markers used in clinical practice to evaluate for rhabdomyosarcoma (1), showed some biochemical evidence of skeletal muscle differentiation. Staining for these markers ranged from undetectable (Fig. 2B) to positive for all three antigens in the tumor that showed foci of spindle-shaped cells (Fig. 2D-F). Staining for myogenin and MyoD1, muscle-specific transcription factors expressed in the nuclei of rhabdomyosarcomas (1), was negative (data not shown). All tumors showed brisk mitotic activity and areas of necrosis, consistent with high-grade sarcomas. Thus, introducing genetic changes characteristic of rhabdomyosarcoma in cultures of human SkMCs led to a broad spectrum of sarcomas, ranging from undifferentiated small round blue cell tumors to spindle-shaped cell tumors with areas of necrosis.

Figure 3. S.c. tumor xenografts derived from primary human myoblasts (HSMM) stably expressing T/t-Ag, hTERT, and K-RasV12 showed morphologic, immunohistochemical, and ultrastructural resemblance to human embryonal rhabdomyosarcoma. Tumor sections were evaluated using (A-C) H&E for morphology, and immunohistochemical staining for the skeletal muscle-specific antigen markers desmin (D), muscle-specific actin (E), myoglobin (F), MyoD1 (G), and myogenin (H). Images (B) and (C) are from different tumors. I, expression of IGF2 in tumor lysates (MY-THR) was measured by semiquantitative reverse transcription-PCR and compared with IGF2-negative (−) NCI-H460 cells (31), and positive (+) control cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to control for total RNA input. Tumor sections were also imaged by transmission electron microscopy, revealing disorganized cytoplasmic myofilaments (J, 29,400×) and myofilaments attempting to organize into sarcomeres (K, 21,875×; arrowheads). N, nucleus. Light microscopy images are 100× (B, D, and E), 200× (A, C, G, and H), or 400× (G).
round blue cell tumors (sarcomas, not otherwise specified) to
tumors exhibiting some differentiation markers characteristic of
rhabdomyoblasts, but lacking frank histopathologic features of
either embryonal or alveolar rhabdomyosarcoma.

Transformation of human skeletal muscle myoblasts gen-
erates an embryonal rhabdomyosarcoma phenotype. Given the
variable tumor histology resulting from a mixed population of
SkMCs, we hypothesized that the skeletal muscle cell-of-origin
might underlie differences in rhabdomyosarcoma tumor histology.
As recent data suggests that embryonal rhabdomyosarcoma might
derive from satellite cell myoblasts (30), which should be present in
the SkMC population, an enriched population of HSMMs was
infected with retroviruses encoding T/Ag, hTERT, and H-RasV12G,
generating a new polyclonal myoblast MY-THR cell line. These cells
were tumorigenic in mice (Table 1). However, in contrast to SkMC
cells, transformed HSMM cells showed rhabdomyosarcoma mor-
phology, characterized by large numbers of rhabdomyoblasts with
abundant, deeply eosinophilic cytoplasm (Fig. 3A), and spindle-
shaped cells in a myxoid background (Fig. 3B–C). Immunohisto-
chemical staining showed skeletal muscle differentiation, with
diffuse and strong desmin staining, focally positive muscle-specific
actin, and diffusely positive myoglobin (Fig. 3D–F). The tumors
were focally positive for MyoD1 and myogenin (Fig. 3G–H) and
expressed IgF2 (Fig. 3I), a fetal growth factor overexpressed in
rhabdomyosarcoma tumors (reviewed in ref. 2). When examined by
electron microscopy, the most rigorous test for establishing skeletal
muscle origin, these tumors had cytoplasmic myofilaments (Fig. 3J),
and myofilaments with z-band material attempting to form
sarcomeres (Fig. 3K). Thus, genetic changes observed in rhabdo-
myosarcoma can convert HSMMs to tumors resembling human
rhabdomyosarcoma. Moreover, given the presence of embryonal
rhabdomyosarcoma-specific findings (nuclear pleomorphism, myx-
oid change with spindling, focal staining of MyoD1, and myogenin)
and the absence of alveolar findings (nuclear monotony, diffuse
immunostaining with MyoD1 and myogenin, collagenous septae
lined by rhabdomyoblasts with associated nesting of tumor cells),
these tumors are most consistent with an embryonal rhabdomyo-
sarcoma histology.

Genetically defined embryonal rhabdomyosarcoma tumors
are invasive and metastatic. Aside from histologic markers,
human rhabdomyosarcoma tumors are characterized by their
ability to invade adjacent tissue and metastasize. Therefore, the
genetically defined myoblast-derived tumor cells (MY-THR) were
introduced into the systemic circulation, after which four of five
injected mice developed clinically and anatomically apparent lung
metastases by 8 weeks (Table 1), with almost complete obliteration
of normal pulmonary alveolar architecture (Fig. 4A). Tumor cells
tended to localize around pulmonary arterioles, often encasing
pulmonary bronchioles (Fig. 4B). Histologic evaluation was again
consistent with an embryonal rhabdomyosarcoma morphology
(Fig. 4C). Although the tail vein assay evaluates many steps in
metastasis, it does not assay the capacity of tumor cells to be
motile or invasive. In this regard, nodules located at the lung
periphery were noted to invade locally, eroding through the visceral
and parietal lung pleura, and infiltrated through basement
membrane into the adjacent chest wall skeletal muscle and ribs
(Fig. 4D). Moreover, MY-THR cells injected orthotopically into the
gastrocnemius muscle of four mice developed as tumors (Table 1)
that displaced the normal tissue (Fig. 4E) and invaded into the
surrounding native skeletal muscle (Fig. 4F), although not as
extensively as the lung nodules. Intriguingly, local invasion was not
observed in s.c. xenografts (data not shown), suggesting that the

Figure 4. Primary human myoblasts stably expressing T/Ag, hTERT, and H-RasV12G show metastatic and invasive behavior
when introduced systemically through the tail vein or orthotopically
into native skeletal muscle. A, low-power view of mouse lung
largely replaced by tumor nodules (10×). Dashed box, area
magnified for (B). B, single tumor nodule encasing pulmonary
bronchus and vein (100×). Asterisks, areas of remaining intact
alveoli. C, magnification of metastatic nodule showing embroyonal
morphology. D, invasion of adjacent chest wall by peripheral
pulmonary nodules (10×). Closed arrows, area of local invasion.
Rib is cut transversely for orientation. E, low-power view of
gastrocnemius muscle largely replaced by tumor xenograft (10×).
Hind limb is cut transversely for orientation. Dashed box, region
magnified for (F). F, tumor cells (open arrows) streaming into
adjacent native skeletal muscle (200×).
surrounding microenvironment provides factors necessary to support this more aggressive phenotype. Thus, expression of Tgf- 
Ags, hTERT, and H-RasV12G endowed human myoblasts with the invasive and metastatic phenotypes characteristic of rhabdomyo-
sarcoma tumors.

Summary

The presentation of rhabdomyosarcoma is heterogeneous, arguing that treatments may need to be tailored to tumor type. To explore rhabdomyosarcoma etiology, we found that heterogeneous populations of SkMcs could be converted to a broad spectrum of tumors via the corruption of the p53, RB, Myc, telomerase, and Ras pathways, whereas by limiting these changes to committed myoblasts (HSMMs), embryonal-like rhabdomyosarcoma tumors were generated. We therefore argue that cell type may play a key role in rhabdomyosarcoma, and now with the described ability to genetically model rhabdomyosarcoma, it should be possible to dissect the histogenesis and molecular mechanisms underlying this disease using normal primary human cells.

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