Distinct malignant behaviors of mouse myogenic tumors induced by different oncogenetic lesions

Simone Hettmer1,2,3,4*, Roderick T. Bronson5 and Amy J. Wagers2,3,4

1 Division of Pediatric Hematology and Oncology, Department of Pediatric and Adolescent Medicine, University Medical Center Freiburg, Freiburg, Germany
2 Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Harvard University, Boston, MA, USA
3 Howard Hughes Medical Institute, Chevy Chase, MD, USA
4 Joslin Diabetes Center, Boston, MA, USA
5 Department of Biomedical Sciences, Tufts University Veterinary School, North Grafton, MA, USA

INTRODUCTION
Rhabdomyosarcomas (RMS) are heterogeneous cancers with myogenic differentiation features. The cytogenetic and mutational aberrations in RMS are diverse. This study examined differences in the malignant behavior of two genetically distinct and disease-relevant mouse myogenic tumor models. Kras; p1619null myogenic tumors, initiated by expression of oncogenic Kras in p16p19null mouse satellite cells, were metastatic to the lungs of the majority of tumor-bearing animals and repopulated tumors in seven of nine secondary recipients. In contrast, SmoM2 tumors, initiated by ubiquitous expression of a mutant Smoothened allele, did not metastasize and repopulated tumors in 2 of 18 recipients only. In summary, genetically distinct myogenic tumors in mice exhibit marked differences in malignant behavior.

Keywords: rhabdomyosarcoma, myogenic differentiation, metastasis, transplantation

MATERIALS AND METHODS
MICE
R26-SmoM2 (mixed genetic background including 129/Sv and Swiss Webster as main components) (11), CAGGS-CreER (11), and NOD.CB17-Prkdcsid/J (NOD.SCID) mice were purchased from The Jackson Laboratory. p16p19null mice (B6.129 background) were obtained from the NIH/Mouse Models of Human Cancer Consortium. Mice were bred and maintained at the Joslin Diabetes Center Animal Facility. All animal experiments were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee.

SARCOMA INDUCTION
Kras; p1619null myogenic tumors were initiated by fluorescence-activated cell sorting of p16p19null satellite cells, followed by lentiviral transduction to introduce oncogenic Kras(G12v) and implantation in the gastrocnemius muscles of NOD.SCID mice as previously described (10). R26-SmoM2;CAGGS-CreER were injected with Tamoxifen (1 mg/40 g) on postnatal day 10 to activate expression of CRE recombinase and SMOM2. R26-SmoM2;CAGGS-CreER spontaneously developed multifocal skeletal muscle tumors (SmoM2 tumors) as previously described (11, 12).

HISTOPATHOLOGY
Tumor tissue was dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin. Standard H&E stained sections were prepared. Staining for Actin (Dako, M0635, 1:200), Desmin (Dako, M0760, 1:50), and Ki67 (Vector Labs, VP-K451, 1:250) was performed as previously described (10). R26-SmoM2;CAGGS-CreER was injected on postnatal day 10 to activate expression of CRE recombinase and SMOM2. R26-SmoM2;CAGGS-CreER spontaneously developed multifocal skeletal muscle tumors (SmoM2 tumors) as previously described (11, 12).

LUNG METASTASES
Tumor-bearing mice were monitored at least twice weekly for health problems, and were sacrificed once tumors reached a volume of 1 cm³ or were ill. Lungs were dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin. Standard
H&E stained sections were prepared and evaluated for the presence of metastases by Roderick T. Bronson.

**TUMOR TRANSPLANTATION**

Tumors were harvested, digested in DMEM + 0.2% collagenase type II (Invitrogen) + 0.05% dispase (Invitrogen) for 90 min at 37°C in a shaking waterbath, triturated to disrupt the remaining tumor pieces, and filtered through a 70 µm cell strainer. Red blood cells were lysed from tumor cell preparations by 3 min incubation in 0.15 M ammonium chloride, 0.01 M potassium bicarbonate solution on ice. Defined numbers of tumor cells were resuspended in 10–15 ml of HBSS with 2% FBS and injected into the gastrocnemius muscles of 1- to 3-month-old, anesthetized NOD.SCID mice using a transdermally inserted dental needle attached to a Hamilton syringe via polyethylene tubing. Recipient muscles were preinjured 24 h before cell implantation by injection of 25 ml of a 0.03 mg/ml solution of cardiotoxin (from *Naja mossambica*, Sigma) in order to enhance cell engraftment. Mice were screened once weekly for the development of tumors at the injection sites.

**STATISTICS**

Differences between *Kras; p16p19null* and *SmoM2* mouse myogenic tumors were evaluated by *T*-test (Ki67 indices), Fisher’s Exact test (prevalence of lung metastases), and Kaplan–Meier analysis (tumor-repopulating activity).

**RESULTS**

*Kras; p16p19null AND SmoM2 MOUSE TUMORS EXHIBIT A MYOGENIC TUMOR PHENOTYPE*

*Kras; p16p19null* mouse myogenic tumors were induced by intramuscular implantation of *Kras(G12v)*-expressing *p16p19null* muscle satellite cells (10). In contrast, *SmoM2* mouse myogenic tumors were initiated by ubiquitous activation of a mutant, constitutively active smoothened (*SmoM2*) allele in R26-*SmoM2;CAGGS-CreER* mice (11, 12). The phenotypes of *Kras; p16p19null* and *SmoM2* myogenic tumors were previously described (10–12). In brief, *Kras; p16p19null* tumors contained bundles of cells with large, atypical nuclei, frequent mitotic figures, and occasional multinucleated giant cells. Subsets of cells (<50% of all tumor cells) expressed terminal muscle differentiation markers such as desmin and actin (Figure 1A), and the proliferative index as evidenced by the percentage of Ki67-expressing nuclei was 41.6 ± 12.5% (range 30.5–59.3%; four tumors evaluated) (Table 1). *SmoM2* tumors contained many multinucleated, elongated cells with abundant cytoplasm interspersed with small round cells. *SmoM2* tumors lacked cellular atypia and diffusely expressed desmin and actin in many tumor cells (more than 75% of all tumor cells; Figure 1B). As previously reported (12), the Ki67 index of *SmoM2* tumors was 19.1 ± 15.9% (range 3.4–41.8%; six tumors evaluated) and lower than that observed in *Kras; p16p19null* tumors (p = 0.05; Table 1).

*Kras; p16p19null AND SmoM2 MOUSE MYOGENIC TUMORS HAVE DIFFERENT METASTATIC POTENTIAL*

The lung is the primary organ affected by distant sarcoma metastases in humans. To assess the metastatic potential of *Kras; p16p19null* and *SmoM2* tumors, random lung sections obtained from tumor-bearing animals were screened for the presence of metastases. Six of seven mice with *Kras; p16p19null* myogenic tumors were found to have lung metastases at the time of death (mice were sacrificed 17–28 days after detection of palpable tumors) (Figure 2). In contrast, 0 of 8 mice with *SmoM2* myogenic tumors had lung metastases at the time of death (mice were sacrificed at 38–55 days of age and 5–21 days after detection of palpable tumors). The prevalence of lung metastases in *Kras; p16p19null* and *SmoM2* myogenic tumor-bearing mice was significantly different (p = 0.001).

**Kras; p16p19null AND SmoM2 MOUSE MYOGENIC TUMORS DIFFER IN TUMOR-REPOPULATING ACTIVITY**

Most malignant tumors contain cells that have the capacity to repopulate secondary tumors when transplanted into a susceptible secondary environment, and this assay has been used as a test of the malignancy of distinct tumors and tumor cell subsets (13). To evaluate the tumor-repopulating activity of *Kras; p16p19null* and *SmoM2* mouse myogenic tumors, viable tumor cells were transplanted into the cardiotoxin-pre-injured gastrocnemius muscles of NOD.SCID mice. The *Kras; p16p19null* tumor cell pool contains approximately 70% GFP+ cells and 30% GFP− cells (10). Because tumor-repopulating activity in *Kras; p16p19null* tumors resides within the Ki67-expressing, GFP− subset of tumor cells descended from virally infected satellite cells (Figure S1 in Supplementary Materials).
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Table 1 | Differences in the malignant behavior of Kras; p16p19null and SmoM2 mouse tumors.

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<thead>
<tr>
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<th>Kras; p16p19null tumors</th>
<th>SmoM2 tumors</th>
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<tr>
<td>Terminal muscle differentiation</td>
<td>Actin/desmin expression in &lt;50% of tumor cells</td>
<td>Actin/desmin expression in &gt;75% of tumor cells</td>
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<tr>
<td>Ki67 index (p = 0.05)</td>
<td>41.6 ± 12.5%</td>
<td>19.1 ± 15.9%</td>
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<tr>
<td>Metastases (p = 0.001)</td>
<td>7 of 9 mice with lung metastases</td>
<td>0 of 10 mice with lung metastases</td>
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<tr>
<td>Transplantation (p &lt; 0.001)</td>
<td>7 of 9 transplanted mice developed tumors (50 cells injected)</td>
<td>2 of 10 transplanted mice developed secondary tumors (100–150k cells injected)</td>
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Kras; p16p19null and SmoM2 mouse myogenic tumors exhibit profound differences in tumor-repopulating activity and metastatic behavior.

**FIGURE 2 | Kras; p16p19null mouse myogenic tumors metastasize to the lungs of tumor-bearing animals.** Random lung sections from Kras; p16p19null tumor-bearing mice show metastases. Tumor cells invade lung capillaries (top panel). Similar to primary tumors arising from GFP+ Kras-expressing; p16p19null satellite cells, lung metastases are GFP+ (bottom right panel). Images were taken at 10× and 20× (scale bars indicate 100 µm).

**FIGURE 3 | Kras; p16p19null tumor cells repopulate tumors in secondary recipients more effectively than SmoM2 mouse tumor cells.** Pi- Ca+ GFP+ Kras; p16p19null tumor cells were sorted independently from two primary tumors and injected into the cardiotxin-pre-injured gastrocnemius muscles of NOD.SCID mice (50 cells per injection). Pi- Ca+ SmoM2 tumor cells were sorted independently from four primary tumors and injected into the cardiotxin-pre-injured gastrocnemius muscles of NOD.SCID mice (100,000–150,000 cells per injection). Recipient mice were monitored for the occurrence of secondary tumors at the injection site for up to 4 months. (%100,000 to 150,000 Pi−, Calcein+ SmoM2 tumor cells per recipient), only 2 of 18 recipient mice developed secondary tumors, which were detected 71 and 127 days after cell injection. These experiments indicate marked differences in tumor-repopulating activity of Kras; p16p19null and SmoM2 tumors (p < 0.001, Figure 3), in terms of both the frequency of tumor-repopulating cells and the latency of secondary tumor formation.

**DISCUSSION**

Our findings highlight differences in the malignant phenotype and behavior of mouse myogenic tumors driven by activation of distinct RMS-relevant oncogenic pathways. Kras; p16p19null myogenic tumors were metastatic to the lungs of the majority of tumor-bearing animals and contained high tumor-repopulating activity. In contrast, SmoM2 tumors did not metastasize and were substantially less effective in repopulating tumors in secondary recipients. These observations indicate that genetically distinct myogenic tumors in mice display marked differences in their malignant behavior.
The two model systems described in this study were induced by different experimental methods. SmoM2 tumors originated from Cre-mediated activation of a conditionally expressed transgene. Kras; p16\textsuperscript{19\textsuperscript{null}} mouse tumors, on the other hand, were initiated by viral transduction and intramuscular implantation of target satellite cells. We note that Kras; Tp53\textsuperscript{-/-} mouse myogenic tumors (14, 15), induced by Cre-mediated activation of oncogenic hits instead of viral transduction, exhibit a phenotype that closely resembles the Kras; p16\textsuperscript{19\textsuperscript{null}} mouse tumors described here. For example, Kras; p16\textsuperscript{19\textsuperscript{null}} share their propensity to metastasize to the lungs of tumor-bearing animals with Kras; Tp53\textsuperscript{-/-} mouse tumors (14). Nevertheless, it is possible that differences in the tumor induction strategy (such as off-target effects of viral transduction) could contribute to the observed differences in malignant behavior between SmoM2 and Kras; p16\textsuperscript{19\textsuperscript{null}} myogenic tumors.

Similar to mouse myogenic tumors, human fusion-negative RMS comprises a group of tumors with clear differences in histology, myogenic differentiation state, oncogenic pathway activation, and genetic background. In recent years, subsets of human RMS tumors that exhibit a combination of specific genetic and phenotypic characteristics were distinguished. For example, a subset of human fusion-negative RMS with spindle cell/sclerosing histology was recently found to exhibit diffuse MyoD expression, carry frequent somatic MyoD mutations, and portend a poor prognosis (16, 17). Also, children with TP53 germline mutations are predisposed to develop anaplastic RMS at a young age (18), and germline mutations in DICER1 were linked to a genetic susceptibility to develop RMS of the genitourinary tract (19). Future extended (epi-)genotype/phenotype correlations might pinpoint clinically/biologically distinct subgroups of human fusion-negative RMS and identify biomarkers to facilitate prognostication and/or stratification of therapy.

AUTHOR CONTRIBUTIONS
SH, RB, and AW conceived experiments, analyzed data, wrote, and approved of the manuscript.

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REFERENCES
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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