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Research Article

Hedgehog-driven myogenic tumors recapitulate skeletal muscle cellular heterogeneity

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ABSTRACT

Hedgehog (Hh) pathway activation in *R26-SmoM2*;*CAGGS-CreER* mice, which carry a tamoxifen-inducible activated Smoothened allele (*SmoM2*), results in numerous microscopic tumor foci in mouse skeletal muscle. These tumors exhibit a highly differentiated myogenic phenotype and resemble human fetal rhabdomyomas. This study sought to apply previously established strategies to isolate lineally distinct populations of normal mouse myofiber-associated cells in order to examine cellular heterogeneity in *SmoM2* tumors. We demonstrate that established *SmoM2* tumors are composed of cells expressing myogenic, adipocytic and hematopoietic lineage markers and differentiation capacity. *SmoM2* tumors thus recapitulate the phenotypic and functional hetereogeneity observed in normal mouse skeletal muscle. *SmoM2* tumors also contain an expanded population of PAX7+ and MyoD+ satellite-like cells with extremely low clonogenic activity. Selective activation of Hh signaling in freshly isolated muscle satellite cells enhanced terminal myogenic differentiation without stimulating proliferation. Our findings support the conclusion that *SmoM2* tumors represent an aberrant skeletal muscle state and demonstrate that, similar to normal muscle, myogenic tumors contain functionally distinct cell subsets, including cells lacking myogenic differentiation potential.

1. Introduction

Adult striated muscle is composed of highly organized bundles of multinucleated myofibers and a variety of functionally heterogeneous mononuclear cells [1–3], including myogenic (muscle-forming) and non-myogenic elements such as fibroadipogenic precursors (FAPs) and immune/inflammatory cells of hematopoietic lineage. Within the myogenic cell compartment, cytoplasmic filaments such as Desmin, Actin and Myosin mark terminal myogenic differentiation, whereas the transcription factor PAX7 identifies satellite cells within the heterogenous pool of myofiber-associated mononuclear cells [2]. Upon injury, satellite cells proliferate, differentiate and fuse to generate new myofibers in a process that is governed by sequential expression of a series of myogenic regulatory factors including MyoD and Myogenin [4,5]. These myogenic regulatory factors (MRFs) are generally silent in mature, resting muscle.

Skeletal muscle differentiation features can be found in a number of neoplastic conditions, including rhabdomyosarcomas, a varied group of soft-tissue sarcomas, and rhabdomyomas, benign

Abbreviations: FAP, fibroadipogenic progenitors; PAX7, paired box 7; MRF, myogenic regulatory factor; Hh, Hedgehog; MyoD1, myogenic differentiation 1; Smo, Smoothened; Gli1, Gli family zinc finger 1; Ptch1, Patched 1; rRNA, ribosomal ribonucleic acid; CAGGS, CMV early enhancer/chicken beta actin; ER, estrogen receptor; FABP4, fatty acid binding protein 4; DMEM, Dulbecco's Modified Eagle Medium; DM, differentiation medium; CM, conditioned medium; HBSS, Hank's Balanced Salt Solution; FBS, fetal bovine serum; bFGF, basic fibroblastic growth factor; FACS, fluorescence activated cell sorting; APC, allophycocyanin; Cy7, cyanine 7; PE, phycoerythrin; PI, propidium iodide; Ca, Calcein; GFP, green fluorescent protein; PCR, polymerase chain reaction; RT-PCR, real time polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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tumors of striated muscle. These conditions have previously been linked to activation of certain oncogenic pathways, including activating mutations in Hedgehog (Hh) pathway genes, detected in fusion-negative human rhabdomyosarcomas [6,7] and fetal rhabdomyomas [8,7]. These tumors express both terminal muscle differentiation markers (e.g. Actin) and myogenic regulatory factors (e.g. MyoD), and they represent an abnormal state of muscle differentiation [8,9]. This study sought to examine cellular heterogeneity in myogenic tumors. We demonstrate that tumors arising in mouse skeletal muscle following induction of hyperactive Hh signaling [8,9] recapitulate normal skeletal muscle cellular heterogeneity and contain an expanded pool of PAX7+, MyoD+ satellite-like cells.

2. Material and methods

2.1. Mice

R26-SmoM2(+/−) and *R26-SmoM2(+/+)* (mixed genetic background including 129/Sv and Swiss Webster as main components) [9] and *R26-SmoM2(+/−);CAGGS-CreER* [9] were bred at the Joslin Diabetes Center Animal Facility. Throughout this manuscript, *R26-SmoM2(+/−)* or *R26-SmoM2(+/+)* skeletal muscle is referred to as "control" muscle, and *R26-SmoM2(+/−); CAGGS-CreER* skeletal muscle as "*SmoM2*" muscle. C57BL6 mice were purchased from the Jackson Laboratory. Tamoxifen (Sigma, St. Louis, MO) at a dose of 1 mg/40 g body weight was administered to *R26-SmoM2(+/−);CAGGS-CreER mice* intraperitoneally on postnatal day 10 (P10) to activate CreER-mediated recombination at transgene-encoded loxP sites. High rates of recombination in skeletal muscle were previously documented [9]. *R26-SmoM2; CAGGS-CreER* mice were monitored once weekly for the onset of soft-tissue tumors or other health problems, and they were sacrificed once they were ill. All animal experiments were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee.

2.2. Histopathological evaluation of skeletal muscle and tumors

Skeletal muscle and tumor tissue was dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin. Standard H&E stained sections were prepared. Staining for Myogenin (Dako, M3559, 1:100), MyoD1 (Dako, M3512, 1:50), Desmin (Dako, M0760, 1:50), FABP4 (Cell Signaling, D25B3, 1:200), CD45 (Abcam, ab10558, 1:4000) and PAX7 (DSHB, 1:5) was performed as previously described [2].

2.3. Muscle and tumor dissociation

Upper extremity, lower extremity and pectoralis muscles from 4–8 week-old C57BL6/J wild-type, 4-9 week-old *R26-SmoM2* mice and 3–9 week-old, tamoxifen-induced *R26-SmoM2*;*CAGGS-CreER* mice were harvested. Isolation of myofiber-associated cells was performed by two-step enzymatic digestion and mechanical dissociation as previously described [1]. Isolation of *SmoM2* tumor cells was performed by one-step enzymatic digestion and mechanical dissociation as follows: Tumors were harvested, digested in DMEM+0.2% collagenase type II (Invitrogen)+0.05% dispase (Invitrogen) for 90minutes 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.

2.4. Fluorescence activated cell sorting (FACS) of myofiber-associated and tumor cells.

Phenotypically distinct muscle and tumor cell subsets were sorted according to protocols that were previously established to isolate functionally discrete subsets of myofiber-associated cells [10,11,1]. In brief, cells were suspended in HBSS supplemented with 2% FBS. Antibody staining was performed for 20 min on ice. The following primary and secondary antibodies were used: APC-CY7-conjugated anti-mouse CD11b (1 in 200, BD Pharmingen, 557657), APC-CY7-conjugated anti-mouse CD45 (1 in 200, eBioscience, 557659), APC-CY7-conjugated anti-mouse TER119 (1 in 200, BioLegend, 116223), APC-conjugated anti-mouse Sca1 (1 in 200, eBioscience, 17-5981-82), PE-conjugated anti-mouse/rat CD29 (1 in 400, BioLegend, 102207), biotin rat anti-mouse CD184 (1 in 100, BD Pharmingen, 551968), PECY7-conjugated Streptavidin (1 in 200, eBioscience, 25-4317-82). Antibody staining was performed for 20minutes on ice. Prior to FACS sorting, cells were suspended in 1 μg/ml propidium iodide and 10 μM calcein blue (Invitrogen) to identify viable cells ($Pi^{-}Ca^{+}$). Cells were sorted twice to maximize purity.

2.5. RT PCR

Tumor samples were FACS sorted to isolate GFP+ cells. mRNA was isolated by TRIzol extraction and reverse transcribed using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR was performed using Taqman PCR reagents and the following primers: Taqman Gene Expression Gli1 (Mm00494654_m1), Ptch1 (Mm00436026_m1), Pax7 (Mm01354484_m1), 18s rRNA (Mm03928990_g1). For qRT-PCR, relative expression of *Gli1*, *Ptch1* and *Pax7* per sample was determined by normalization against the quantity of 18s rRNA within each sample.

2.6. Clonal myogenesis assays

Cells were sorted at 1 cell per well into 96 well plates coated with 1 μg/ml rat-tail collagen (Sigma) and 10 μg/ml natural mouse laminin (Invitrogen). Cells were expanded for 5–7 days in growth medium (GM) composed of Ham's F10+20% horse serum+1% penicillin/streptomycin+5 ng/ml bFGF (Sigma). bFGF was replaced daily. Wells were screened for the presence of cell clones at 3, 5 and 8 days post sorting. The number of cells per individual clone was counted at the same time points.

2.7. Chemical modulation of Hh signaling

Chemical modulators of Hh signaling, including Cyclopamine (Toronto Research Chemicals) at a final concentration of 1000 nM and Hh-conditioned medium (kindly supplied by JM), were added to culture medium. Medium and chemicals were exchanged every 3 days in proliferation assays and every 2 days in differentiation assays.

2.8. Myogenic differentiation assays

Freshly sorted cells were expanded as described above. After 5–7 days, cells were passaged by aspiration of medium, re-plated onto 0.2% Matrigel (Fisher) coated chamber slides, cultured in growth medium for 2 days, and transitioned into differentiation medium (DM) consisting of Ham's F10+2% horse serum+1% penicillin/streptomycin. Alternatively, after 5–7 days of expansion, cells were transitioned into DM in 96 well plates. After 4 days in DM, medium was aspirated and cells were fixed with 4% paraformaldehyde and processed for immunofluorescence.