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***In vivo* imaging of tumor-propagating cells, regional tumor heterogeneity, and dynamic cell movements in embryonal rhabdomyosarcoma**

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Summary

Embryonal rhabdomyosarcoma (ERMS) is an aggressive pediatric sarcoma of muscle. Here, we show that ERMS-propagating potential is confined to *myf5*⁺ cells and can be visualized in live, fluorescent transgenic zebrafish. During early tumor growth, *myf5*⁺ ERMS cells reside adjacent normal muscle fibers. By late stage ERMS, *myf5*⁺ cells are reorganized into distinct regions separated from differentiated tumor cells. Time-lapse imaging of late stage ERMS revealed that *myf5*⁺ cells populate newly formed tumor only after seeding by highly migratory *myogenin*⁺ ERMS cells. Moreover, *myogenin*⁺ ERMS cells can enter the vasculature, whereas *myf5*⁺ ERMS-propagating cells do not. Our data suggests that non-tumor propagating cells likely have important supportive roles in cancer progression and facilitate metastasis.

Introduction

Rhabdomyosarcoma (RMS) is a pediatric malignancy that shares common features with skeletal muscle arrested in embryonic development (Xia et al., 2002). The two main subtypes of pediatric rhabdomyosarcoma, embryonal RMS (ERMS) and alveolar RMS (ARMS), differ in their clinical, biological, and molecular characteristics. For example, ERMS and ARMS can be distinguished based on histology and have different long-term prognosis with ERMS patients having better overall outcome than ARMS. These divergent

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clinical features likely reflect the use of different molecular programs that lead to transformation. For example, we have identified that the RAS pathway is active in a majority of human ERMS (Hettmer et al., 2011; Langenau et al., 2007). By contrast, 85% of ARMS have recurrent chromosomal translocations that juxtapose PAX3 or PAX7 with the forkhead transcription factor (FKHR) (Xia et al., 2002). Finally, it is likely that ERMS and translocation-positive ARMS arise in different cell types that eventually undergo transformation. Keller et al. found that PAX3-FKHR⁺ ARMS can arise from Myf6 expressing myoblast cells but not dermamyotome or satellite cells that express Pax7 (Keller et al., 2004). By contrast, ERMS can arise from either satellite cells or myoblasts that eventually reinitiate molecular programs found in satellite cells (Rubin et al., 2011). Despite elegant studies defining possible cells of origin in RMS, identification of an ERMS-propagating cell that is required for continued tumor growth *in vivo* has not been described in mice or humans.

Tumor-propagating cells have been characterized in many malignancies, and in some tumors, this potential is confined to a molecularly definable cell population that can be enriched by cell surface markers. For example, in AML a rare CD34⁺CD38⁻ cell enriches for leukemia-propagating potential while in breast cancer CD44⁺CD24^{low/-} expression is associated with tumor-propagating potential (reviewed in Dalerba et al., 2007). Molecularly defined, rare CD133⁺ tumor-propagating cells have also been identified in subset of gliomas and exhibit striking differences in response to nitric oxide and hypoxia inducible factor (HIF) signaling when compared to more differentiated tumor cells (Eyler et al., 2011; Li et al., 2009). Thus, it is likely that many tumors contain hierarchically organized cell subpopulations that retain the capacity to remake tumor and yet give rise to differentiated tumor cell progeny. One might expect that selection would favor the evolution of tumors with high numbers of tumor-propagating cells at a cost of differentiated cell types. Yet paradoxically, in most malignancies, tumor-propagating cells are far less abundant than differentiated tumor cells that are incapable of remaking tumor. These data suggest that differentiated tumor cells may provide important supportive roles in overall growth and maintenance. To date, a role for differentiated, non-tumor-propagating ERMS cells has yet to be fully explored.

Stem cells often reside in distinct niches in normal tissue and their functions are exquisitely controlled by local factors secreted by supporting cells. For example, hematopoietic stem cells (HSCs) have been shown to home to niches within the calvarium that are tightly associated with osteoblasts (Lo Celso et al., 2009). These and other niche-associated cells presumably provide paracrine-signaling factors to recruit and maintain these cells in a specific niche. Unlike other tissues, the muscle stem cell niche is defined by juxtaposition of satellite cells next to differentiated muscle fibers, and their numbers and differentiation capacity are controlled by complex signaling pathways regulated by mature muscle cells (reviewed in Bentzinger et al., 2012). Despite a large body of data defining stem cell niches in normal tissue, few studies have identified tumor-specific niches and/or regions of compartmentalized tumor cell function and less have used microscopic imaging to directly visualize tumor-propagating cells within live animals. In one example, Sipkins et al used a combination of multiphoton and confocal microscopy to image the HSC niche in the calvarium of mice and demonstrated that these sites can attract multiple tumor cell types (Sipkins et al., 2005); however, it is unknown if these malignant cells are capable of reinitiating tumors. In ERMS, as with most solid tumors, it is unknown if tumor-propagating cells reside in distinct regions within the tumor mass and if the more differentiated cells play a role in promoting tumor progression.

Here, we utilize a transgenic zebrafish model of embryonal rhabdomyosarcoma to identify the tumor-propagating cell in this disease and to define the functional consequences of tumor cell heterogeneity within live animals. Because ERMS cell subpopulations can be

fluorescent-labeled based on myogenic factor expression, ERMS cell subtypes can be visualized in live animals and the processes of cell growth, division, and local dissemination can be visualized as dynamic processes in live animals. Our data provide an explanation for the large number of non-tumor propagating cells in established cancers and reveals an important supportive role for differentiated tumor cell types in local dissemination and metastasis.

Results

Imaging distinct stages of ERMS growth

Externally-visible ERMS can develop as early as 10 days of life in zebrafish injected with *rag2-KRASG12D* (Langenau et al., 2007) and >80% of ERMS develop in the tail musculature (n>50). To assess how tumors initiate and evolve in zebrafish ERMS, *alpha-actin-GFP* transgenic zebrafish were injected at the one-cell stage of development with *rag2-dsREDexpress* and *rag2-KRASG12D* (Figure 1A-F), facilitating imaging of ERMS cells in relation to normal muscle. Microinjection of multiple transgenes into one-cell stage animals leads to co-integration and co-expression in animals that develop ERMS (Langenau et al., 2008). This approach provides a robust method to create mosaic transgenic animals with fluorescently-labeled ERMS cell subpopulations (Langenau et al., 2007).

Sequential confocal imaging over several days showed that ERMS forms in a choreographed and stereotypical manner (Figure 1A-F, J-M). Specifically, dsRed+ ERMS mononuclear cells arise at the extreme outer borders of the myotome segments and move toward the midline where they are initially unable to bypass the horizontal myosepta - a single cell layer that separates myotome segments (Figure 1A, Stage 1, n=7). After several days, a subset of cells cross the horizontal myosepta and take up residence between normal muscle fibers within the newly colonized myotome segment (stage 2, Figure 1B-C, n=5). Differentiated ERMS cells that express both *rag2-dsREDexpress* and *alpha-actin-GFP* can move laterally into neighboring muscle segments by transiting through the collagen matrix of the myoseptum (n=6, Figure 1D-F, Movie S1) or stream into new myotome segments by growing past the edge of myoseptum (n=3, early Stage 3). The collagen matrix of the muscle myoseptum is a cell-impermeable barrier that is the site of muscle attachment in teleost fish and is similar in function to tendons in mouse and humans. Late stage ERMS undergo rapid loss of fibers, breakdown of normal muscle architecture including collagen remodeling, and development of mononuclear tumor cells, reminiscent of the spindle variant of human embryonal rhabdomyosarcoma.

Neovascularization is a hallmark of cancer and an ideal surrogate for assessing tumorigenicity. To assess when KRASG12D-expressing cells are transformed, neovascularization was monitored in *fli1-GFP* transgenic animals that were injected at the one-cell stage of life with *rag2-KRASG12D* and *rag2-dsREDexpress*. Animals were monitored for tumor growth by confocal microscopy beginning at 10 days of life (n=22, Figure 1G-I). ERMS at stage 1 failed to recruit new vasculature (n=0 of 3), but stage 2 and early stage 3 ERMS had begun to recruit new vasculature (n=8 of 8, Figure 1H) with new branches arising from both the intersegmental vessels and vertebral artery. By late stage 3, ERMS developed intricate networks of new vessels (n=11 of 11, Figure 1I). Our imaging studies define distinct stages of ERMS growth and suggest that RAS-expressing cells become fully transformed by stage 2 of tumor development (Figure 1J-M).

Identification of molecularly distinct fluorescent-labeled ERMS cell subpopulations

Previous experiments in zebrafish have identified an ERMS cell subpopulation that had superior tumor-propagating potential when compared to other tumor derived cells. This

ERMS-propagating cell was *rag2-dsREDexpress+/alpha-actin-negative* and expressed high levels of *myf5*, *c-Met*, and *m-cadherin* – markers of satellite and early muscle progenitor cells (Langenau et al., 2007). *MYF5* is highly upregulated in human ERMS compared to both translocation positive ARMS and normal muscle (Zibat et al., 2010) and in comparing zebrafish ERMS to normal muscle (Langenau et al., 2007). To directly assess whether *myf5* labels distinct ERMS cell subpopulations, *myf5-GFP/myosin light chain 2-mCherry* (*mylz2*) syngeneic animals were created by four rounds of out crossing to CG1 syngeneic animals (Smith et al., 2010) and injected at one-cell stage with *rag2-KRASG12D*. *myf5-GFP* transgenic animals exhibit GFP expression in early somitogenesis and later in satellite cells and early muscle progenitor cells (Chen et al., 2007; Seger et al., 2011) while the *mylz2* promoter drives expression in differentiated muscle cells (Ju et al., 2003; Smith et al., 2010). Fluorescent-labeled ERMS cell subpopulations were isolated from double transgenic animals by fluorescence-activated cell sorting (FACS). Reanalysis of sorted cells by FACS confirmed that ERMS contained four distinct populations of cells (purity >87%, and viability >97%).

To verify that discrete fluorescent-labeled ERMS cell subpopulations were molecularly distinct, sorted cell populations were assessed for gene expression differences based on microarray (Figure 2A, Table S1) and real-time PCR (Figure 2B, Figure S1). Gene expression analysis was completed on FACS sorted ERMS cells derived from serially passaged tumors, ensuring that fluorescent-labeled cells were tumor derived. Microarray analysis confirmed that each cell subpopulation exhibited wide differences in gene expression. Subsequent real-time PCR analysis established that the *myf5-GFP+/mylz2-negative* cells expressed high levels of *myf5*, *cMet*, and *m-cadherin* but not *pax7a*, *pax7b*, or differentiated markers (Figure 2B and Figure S1). By contrast, *mylz2-mCherry+* ERMS cells expressed high levels of mature muscle markers including *myoD*, *myogenin*, *troponin I fast-twitch isoform 2* (*tnni2a*), *alpha-actin 1b* (*acta1b*), *ventricular myosin heavy chain-like* (*vmhcl*), *actin related protein 2/3 complex subunit 5B* (*arpc5b*), *carboxypeptidase vitellogenic-like* (*cpvl*), and *chemokine (C-X-C motif) receptor 4b* (*cxcr4b*). Finally, the double-negative cell population was comprised of predominantly blood cells that express *myeloid-specific peroxidase* (*mpx*) and *lymphocyte-specific protein tyrosine kinase* (*lck*). Our data confirms that fluorescent-labeled ERMS cell subpopulations can be prospectively isolated to relative purity following FACS and are molecularly distinct.

Given that FACS could identify unique ERMS cell subpopulations that exhibited wide differences in gene expression, we questioned if these cells also differ in rates of proliferation and cellular turnover. Proliferation was assessed at 6 hours following intraperitoneal injection of EDU into ERMS-affected animals (Figure 2C-E and Figure S1). 24.1 +/- 4.8% *myf5-GFP+/mylz2-mCherry-negative* cells incorporated EDU over a 6-hour pulse, whereas differentiated ERMS cells that express *mylz2-mCherry* were far less proliferative (8.9 +/- 5.0%, $p < 0.00001$). By contrast, following 3 day administration of EDU, all fluorescent-labeled ERMS cell sub-fractions exhibited equal proliferative capacity, suggesting that *myf5-GFP+/mylz2-mCherry-negative* cells divided and differentiated over this time (data not shown). In addition to striking differences in cell proliferation between ERMS cell subpopulations, *myf5-GFP+/mylz2-mCherry+* cells had higher levels of apoptotic cellular turnover when compared with *myf5-GFP+/mylz2-mCherry-negative* and *myf5-GFP-negative/mylz2-mCherry+* cells ($p < 0.01$ Fisher exact test, Figure 2F-J). Taken together, our fluorescent transgenic approach identifies unique ERMS cell subpopulations that have different fluorescent reporter expression, divergent gene expression profiles, and varied capacities for proliferation and apoptosis.

***myf5-GFP+* cells are the ERMS-propagating cell population**

To assess if *myf5-GFP* transgene expression enriches for ERMS-propagating potential, cells were isolated from transplant animals that developed *myf5-GFP+/mylz2-mCherry+* ERMS (Figure 3A-G) and subjected to two rounds of FACS in the presence of propidium iodide or DAPI to isolate highly purified and viable cells (Figure 3H-K, 87.7%-99.7% purity and >98% viability). ERMS cell subpopulations were introduced into CG1 syngeneic recipient animals at limiting dilution (Figure 3L-R), and animals assessed for engraftment from 10 to 120 days post-transplantation (Table 1). All animals developed ERMS before 45 days post-transplantation, confirming that slower cycling ERMS-propagating cell types would not be missed in our analysis. In three ERMS tested, the tumor-propagating activity was confined to the *myf5-GFP+/mylz2-mCherry-* negative cell subpopulation (Table 1) with an average frequency of 1 in 146 cells capable of reinitiating tumors in recipient animals (range 1 in 87-245, 95% confidence interval). By contrast, only 1 in 4,206 *myf5-GFP+/mylz2-mCherry+* cells were capable of inducing tumors (range 1 in 1,550 to 11,409, $p=3.38e-15$ when compared to ERMS-propagating activity in *myf5-GFP+/mylz2-mCherry-negative* cells). 0 of 61 animals engrafted disease from terminally differentiated *myf5-GFP-negative/mylz2-mCherry+* cells (lower bound for ERMS-propagating potential was 1 in >5,969 cells). In total, we observe a remarkable 28 to >40 fold enrichment of tumor-propagating potential within our *myf5-GFP+/mylz2-mCherry-negative* cell type when compared with other sorted ERMS cell subpopulations. Similar results were also observed in primary ERMS. Specifically, three primary ERMS were isolated from 20 to 30-day-old larval zebrafish, pooled, and fluorescent-labeled ERMS cell subpopulations isolated by FACS. 3 of 8 animals engrafted disease from 1×10^2 *myf5-GFP+/mylz2-mCherry-negative* cells whereas the remaining ERMS cell subpopulations could not transfer disease at this cell dose (0 of 23, purity 83-98% and viability >98.6%, $p=0.012$ Fisher Exact Test). These results further support our finding that the *myf5-GFP+/mylz2-mCherry-negative* population is highly enriched for ERMS-propagating activity.

To assess the long-term tumor-propagating potential of the *myf5-GFP+/mylz2-mCherry-negative* ERMS cells, cells were re-isolated from transplant recipient animals (Figure 3N) and introduced into CG1, syngeneic recipient animals (>78.9% purity and 96% viable, Figure 3O-R). Again, the *myf5-GFP+/mylz2-mCherry-negative* cell subpopulation was capable of remaking ERMS (Figure 3S-U, Table 1). Histological analysis showed that primary and serially transplanted ERMS arising from *myf5-GFP+/mylz2-mCherry negative* cell populations have similar morphology and overall proportions of fluorescent-labeled ERMS cell subpopulations (Figure 3C, D, F, G, M, N, T, U).

Visualizing *myf5-GFP+* ERMS-propagating cells *in vivo*

To assess if *myf5-GFP+* ERMS cells could be directly visualized in live animals, *rag2-dsREDexpress* and *rag2-KRASG12D* were co injected into one-cell stage *myf5-GFP* transgenic animals and assessed by confocal microscopy. Discrete *myf5-GFP+* tumor cells could be readily identified by confocal imaging with a majority of *myf5-GFP+* ERMS cells co-expressing both GFP and dsREDexpress (97.5+/-2.9%, n=568 cells counted in 3 animals). Moreover, *myf5-GFP+* early muscle progenitor cells from control animals were relatively rare (n=3 animals, 2.3 +/-2.3 cells/ imaging field) whereas *myf5-GFP+* cells were abundant in ERMS (n=3 animals, 194.2 +/-23.7 cells/field, T-test $p=0.0002$). Taken together, our data suggests that a vast majority of *myf5-GFP+* cells contained within the boundaries of the ERMS mass are tumor-derived.

To further refine the ERMS cell subpopulations for imaging studies, triple fluorescent transgenic ERMS animals were created by microinjecting *myogenin-H2B-RFP*, *mylz2-lyn-cyan*, and *rag2-KRASG12D* into one-cell stage *myf5-GFP* transgenic animals (Figure 4A-

C). Histone fusion proteins are long-lived and confined to the nucleus, whereas lyn-cyan encodes for membrane localized blue fluorescent protein. Because transgenes co-integrate as concatamers (Langenau et al., 2008), ERMS cells co-express all three transgenes and label distinct tumor cell compartments associated with stages of muscle development (Figure S2). In normal development, *myf5* is expressed in satellite cells and early muscle progenitor cells, *myogenin* is expressed in committed, mid-differentiated muscle myoblasts, and *mylz2* is expressed in differentiated myoblasts. Transgenic reporters have been described for all three of these promoters and each drives expression within the correct cellular compartments during normal muscle development (Chen et al., 2007; Du et al., 2003; Ju et al., 2003). Moreover, gene expression studies confirm that these promoters drive correct tissue specific gene expression in ERMS (Figure S2) and additional cell transplantation experiments establish that *myf5-GFP+/myogenin-negative* cell types exclusively retain ERMS-propagating potential (Figure S2). For example, 4 of 18 animals engrafted ERMS from 1×10^2 *myf5-GFP+/myogenin-H2B-RFP-negative* sorted ERMS cells (68.5% purity, 99.8% viable) whereas *myogenin-H2B-RFP+* cell types could not induce tumors, irrespective if they expressed *myf5-GFP* (n=0 of 32, p=0.013, Fisher Exact Test).

Confocal imaging of fluorescent-transgenic ERMS fish that express *myf5-GFP/myogenin-H2B-RFP/mylz2-lyn-cyan* easily identified *myf5-GFP+* cells of which a small subset co-express *myogenin-H2B-RFP* (Figure 4C). Some *myogenin-H2B-RFP+* cells with nuclear fluorescent protein expression fail to express either *myf5-GFP* or *mylz2-lyn-cyan*, indicating that these cells are most similar to mid-myoblast stages. Gene expression studies confirm that myogenin-promoter expression drives H2B-flourescent protein expression in a subset of myosin heavy chain expressing muscle cell populations, implying that these represent differentiated cell types (Figure S2). Nearly all *mylz2-lyn-cyan* positive cells co-express *myogenin-H2B-RFP* (99.5 % +/- 1%, n=8 ERMS, N>1700 cells counted) reflecting that H2B-flourescent protein expression persists in more differentiated ERMS cells.

***myf5-GFP+* cells are reorganized into discrete compartments during late stage tumor growth**

We next wanted to define the location of ERMS cell subpopulations during various stages of tumor growth. *myf5-GFP* transgenic animals were injected with *rag2-KRASG12D*, *myogenin-H2B-RFP*, and *mylz2-lyn-cyan* and imaged by confocal microscopy starting at 10 days of life. Stage 1 ERMS exhibited greatly expanded numbers of *myf5-GFP+* cells when compared to control animals and were confined to regions immediately adjacent muscle fibers (Figure 4D-G, K). Mononuclear *myogenin-H2B-RFP+* and double positive *myogenin-H2B-RFP+/mylz2-lyn-cyan+* ERMS cells were not observed in stage 1 ERMS; however, they were detected by stage 2 and increased in number as tumors progressed to stage 3 (Figure 4F-I, L). By late stage 3 ERMS, *myf5-GFP+* cells lost fiber contacts and began to populate discrete portions of the tumor that were physically separated from more differentiated *myogenin-H2B-RFP* and *mylz2-lyn-cyan* expressing ERMS cells (Figure 4H-J, M). The *myf5-GFP+* cells were often located within different myotome segments compared to differentiated ERMS cell subpopulations; however, regional partitioning of cells based on differentiation status was also observed within a single myotome segment and in transplanted animals (Figure S2), confirming that compartmentalization did not result from physiological constraints imposed during development but rather was an intrinsic property of ERMS growth.

Human ERMS cells are also compartmentalized based on myogenic factor expression

To assess whether human RMS also contain distinct regions of tumor cells based on myogenic factor expression, primary human ERMS and xenografted human ERMS derived from RD and SMS-CTR cell lines (Linardic et al., 2005) were assessed for myogenic marker

expression, including Myogenin, PAX7 and MYOD. Distinct regions of high and low Myogenin-expressing cells were seen in a vast majority of primary tumor samples (n=12 of 14, $p<0.03$, T-statistic) and were present in all xenograft tumors (n=7, six regions/tumor, $p<0.02$, T-statistic, Figure 5 and Figure S3, Table S2). By contrast most ARMS cells expressed Myogenin and its expression was not confined to specific areas within the tumor mass (n=10, range 79-99%), suggesting that regional partitioning of tumor cells based on Myogenin expression is specific to ERMS. We also stained four primary human ERMS tumors for PAX7 and identified regions of high and low expression in two of the four tumors. In one ERMS sample, expression was diffuse while the other tumor was PAX7 negative, indicating that not all primary ERMS express PAX7. Unfortunately, MYF5 antibodies have not been developed to detect human protein within paraffin embedded sections, precluding analysis of less-differentiated regions contained within the tumors.

***myogenin+* ERMS cells are highly migratory and precede the recruitment of *myf5+* ERMS-propagating cells into newly colonized areas of growth**

Having established that the *myf5-GFP*-expressing ERMS cell population contains tumor-propagating activity, we wanted to assess if these cells also promote invasive tumor growth. Multiphoton intravital microscopy recordings from *myf5-GFP/myogenin-H2B-RFP* or *myf5-GFP/myogenin-H2B-Amcyan* transgenic tumor zebrafish revealed that *myf5-GFP+* single-positive ERMS cells were largely stationary and displayed only confined crawling motion (Figure 6A-F, Figure S4, and Movies S2-5). In contrast, *myogenin+* ERMS cells were robustly migratory and had the ability to invade across myotome segments through a normally impenetrable collagen matrix. Cells that expressed lower amounts of the H2B-fluorescent fusion protein, were orderly arranged along the direction of muscle fibers, had uniform nuclear shape, and did not show any motility (Movies S2 and S3), suggesting that these were differentiated tumor cells of which a subset had undergone fusion (Figure 6F, right).

ERMS cell subpopulations also differ in their proliferative capacity. Primary ERMS from *myf5-GFP+/myogenin-H2B-RFP+* animals were pulsed with EDU for 6 hours and then sectioned and assessed for EDU incorporation (Figure 6G-I). *myf5-GFP+* ERMS cells were highly proliferative (39.4 \pm 9.4%, n=3) whereas *myf5-GFP-negative/myogenin-H2B-RFP+* cells rarely proliferated (2.6 \pm 3.8%, n=3, $p=0.0001$). *In vivo* multiphoton imaging of transplant and primary ERMS confirmed that *myf5-GFP+/myl2-negative* ERMS-propagating cells are highly proliferative with 27 of 90 GFP+ cells dividing into two daughter cells (n=3 tumors). Multiphoton imaging revealed that resting *myf5-GFP+* ERMS cells are elongated (Figure 6J) and then round up in shape just prior to cell division (Figure 6K). Following this dynamic shape change, *myf5-GFP+* ERMS cells quickly divide into two GFP-labeled daughter cells (Figure 6L-M). Subsequently, these daughter cells begin to reacquire parental morphology (Movie S6), reminiscent of normal *myf5-GFP+* muscle precursors. By contrast, 0 of 90 *myl2+* cells proliferated over this time, irrespective of whether they expressed *myf5-GFP*.

To further visualize the dynamic movements of ERMS cells *in vivo*, late stage 3 triple transgenic ERMS affected animals were serially imaged over 16 hours to capture cell movements within ERMS (Figure 7A-H and Movie S7). As was seen in our multiphoton imaging, *myf5-GFP+* cells that lack differentiated marker expression move only locally within the tumor and exhibit regional crawling movements whereas *myogenin-H2B-RFP+/myl2-lyn-cyan-negative* cells are highly motile and could be easily visualized migrating into adjacent non-affected normal tissue (Figure 7A-D and Movie S7). By contrast, differentiated ERMS cells that express *myogenin-H2B-RFP* and *myl2-lyn-cyan* are largely stationary.

To investigate which ERMS cells were the first to migrate into unaffected tissue, serial imaging experiments were conducted over longer observation intervals, focusing on regions that were adjacent to expanding tumor. Serial confocal imaging of fluorescent ERMS fish over several days revealed that *myogenin-H2B+* ERMS cells precede the recruitment of *myf5-GFP+* cells into newly colonized areas of tumor growth (n=7, Figure 7I, J and Figure S4). Not only do fluorescent-labeled *myogenin-H2B+* ERMS cells move locally within the tumor, but they also enter the vasculature (Movie S8). A small portion of *myogenin-H2B-RFP+/myf5-GFP-negative* cells were associated with vasculature and could invade neovascular beds in *fli1-GFP* transgenic animals (Figure 7K, L). To verify the fidelity of H2B-fluorescent labeling of ERMS cell sub-fractions and to directly visualize if *myf5-GFP+* ERMS cells can enter the vasculature, ERMS was induced in stable transgenic animals that express *myf5-GFP/flk-mCherry* by co-injecting both *rag2-KRASG12D* and *myogenin-H2b-Amcyan*. As was seen using the H2B-RFP transgenic reporter, we find that *myogenin-H2B-Amcyan+* cells are highly migratory (Movie S4 and S5), were the first cell type to colonize new areas of tumor growth, and could be observed transiting the vasculature (Figure 7M, N, Movie S5 and S9). By contrast, *myf5-GFP+* ERMS cells exhibited reduced motility when compared with *myogenin+* ERMS cells and were never observed entering the vasculature (n= 10 animals). Again, slow moving *myf5-GFP+* cells were found in newly colonized regions only after initial invasion by *myogenin+* ERMS cells.

Discussion

Myf5 as a marker of ERMS-propagating cells

The limiting dilution cell transplantation studies outlined here confirm the existence of a highly purified and molecularly definable ERMS-propagating cell that expresses *myf5*, *m-cadherin*, and *c-met* but not differentiated muscle markers. The *myf5-GFP+* ERMS-propagating cell gives rise to all the other differentiated ERMS cells contained within the tumor mass and exhibits enhanced proliferative capacity as assessed by EDU incorporation and direct *in vivo* cell imaging. These results are in keeping with our previous work showing that ERMS-propagating activity was largely confined to the *rag2-dsRED+/alpha-actin-negative* ERMS cell population that preferentially expressed *myf5* and other activated satellite cell markers (Langenau et al., 2007). However, *rag2-dsRED+/alpha-actin-negative* ERMS cells exhibited only a modest 3-fold enrichment for tumor-propagating potential when compared to *rag2-dsRED+/alpha-actin+* ERMS cells (Langenau et al., 2007). By contrast, experiments outlined here using new fluorescent transgenic reporter lines and syngeneic zebrafish show that the *myf5-GFP+/myf5-negative* ERMS cells exhibit a remarkable 28 to >40-fold enrichment of tumor-propagating potential when compared to other ERMS derived cell populations.

Myf5 is a myogenic regulatory factor related to MyoD and has important roles in muscle development. For example, MyoD/Myf5-deficient mice lack muscle, while deficiencies in only one of these genes does not affect muscle specification (Rudnicki et al., 1993), suggesting important and yet redundant functions of these genes in development. It has also been shown that *Myf5* is highly expressed in activated satellite cells and has important roles in postnatal muscle regeneration in response to injury (Cooper et al., 1999; Gayraud-Morel et al., 2009; Ustanina et al., 2007), suggesting that Myf5 may regulate self-renewal in normal muscle satellite cells. Microarray analysis and cross-species comparisons have shown that *MYF5* is upregulated in both zebrafish and human ERMS, but not translocation-positive ARMS (Langenau et al., 2007; Zibat et al., 2010), and recent work from Rubin et al has shown that Myf5 is differentially expressed in murine ERMS regardless of which muscle cell subpopulation is initially targeted for transformation (Rubin et al., 2011). These results suggest that Myf5 gene programs are likely re-initiated in transformed cells and may have important roles in driving ERMS growth. By contrast, translocation-positive ARMS

fail to express MYF5, precluding MYF5 marker expression as an identifying characteristic of ARMS-propagating cells and raising the interesting possibility that the molecular mechanisms regulating tumor-propagating potential differ between molecular subtypes of rhabdomyosarcoma. Given the critical roles of the Myf5 transcription factor in muscle development and regeneration in mice, it will be important to assess if *myf5* is a marker of ERMS-propagating cells or if it plays a regulatory role in ERMS self-renewal and growth.

Regional partitioning of ERMS cells based on differentiation status

Evidence in solid tumors to support a discrete, specialized microenvironment that augments tumor growth and proliferation is now just beginning to emerge. For example, tumor-propagating cells, including those of glioblastomas, have been shown to reside in a vascular niche that promotes both their maintenance and their ability to divide and produce daughter cells capable of inducing tumors (reviewed by Gilbertson and Rich, 2007). In other solid tumors arising in skin, prostate and breast, tumor stromal fibroblasts also serve an essential role in maintaining a favorable microenvironment for tumor growth and expansion. For example, work by Orimo et al has shown that stromal fibroblasts associated with invasive breast carcinoma cells can promote tumor growth and angiogenesis through secretion of SDF-1 (Orimo et al., 2005). In normal muscle, stem cell numbers are exquisitely regulated by paracrine factors like *wnt5a* (Poleskaya et al., 2003), myostatin (McCroskery et al., 2003), and Notch ligands (Conboy et al., 2003). Many of these factors are secreted by normal fibers that can sense injury and elicit recruitment and expansion of muscle progenitors that are required for regeneration. Thus, mature muscle provides a supportive microenvironment that facilitates homeostatic regulation of muscle stem cells. In our zebrafish ERMS model, we document that *myf5-GFP+* ERMS-propagating cells are initially juxtaposed to muscle fibers in an expanded muscle satellite/progenitor cell niche, suggesting that early stage ERMS cells cannot escape the constraints of muscle architecture or are held in check by local secreted factors emanating from normal muscle. By late stages of ERMS growth, ERMS-propagating cells are reorganized and take up residence in defined regions within the tumor mass. Following this regional partitioning of ERMS cells, mid-differentiated myogenin+ ERMS cells show enhanced migratory capability and move away from ERMS-propagating cells from which they had arisen. These mid-differentiated myogenin+ ERMS cells are highly migratory, seed new areas of tumor growth, and cease to move once they turn on differentiated muscle markers including muscle myosin-light chain. Such biologically constrained characteristics of ERMS cells would ensure that tumor-propagating cells remain confined to regionally defined areas and do not compete with differentiated ERMS cell types for local resources including growth factors and oxygen. The extent to which regional partitioning of tumor cells occurs in other solid tumors is unknown; however, assuming this phenomenon is found in diverse cancer types, it will be important to determine if regional partitioning of tumor cells provides protective advantages to tumor-propagating cells, facilitating the retention of a small number of cancer cells that evade treatment and eventually give rise to disease relapse.

A role for differentiated, non-tumor-propagating cells in facilitating tumor growth and metastasis

Myogenin immunohistochemical reactivity found in >80% of RMS cells distinguishes patients with poor clinical outcome (Heerema-McKenney et al., 2008), suggesting that *myogenin+* cells have a unique role in RMS progression and metastasis. In our model, *myogenin-H2B+* cells arise from *myf5-GFP+* ERMS-propagating cells, lack tumor-propagating potential, and are the first cell type to migrate into new areas of tumor growth. A subset of *myogenin+* ERMS cells infiltrate blood vessels – a first step toward metastasis – and are also the first to colonize new areas of tumor growth, only to be infiltrated latter by slow-migrating *myf5+* ERMS-propagating cells. Our work raises the interesting possibility

that differentiated, non-ERMS-propagating cells may create a supportive environment that augments growth and is responsible for local tumor invasion. For example, it is possible that once mid-differentiated myogenin+ cells infiltrate new areas of growth that they secrete factors that recruit slow moving *myf5*+ ERMS-propagating cells, facilitating tumor spread. Alternatively, it is possible that myogenin+ cells breakdown collagen and cell-cell contacts, acting as trail-blazers to establish migratory tracks that allow slow moving *myf5*+ ERMS propagating cells to transit into newly forming tumor. Our work also highlights that metastatic capacity and tumor-propagating potential need not be confined to the same tumor cell subpopulations, but rather that local infiltration and metastasis may be facilitated by differentiated, non-tumor-propagating cells. We expect that these same principles may be more broadly applicable to a diversity of cancers, accounting for why tumors retain large numbers of differentiated cell types which themselves are incapable of re-constituting tumor.

Our findings of a *myf5*+ ERMS-propagating cell population and a myogenin+ migratory population both contributing to tumorigenesis may have profound therapeutic implications. Instead of targeting only tumor-propagating cells for destruction, drug design should also take into account the mechanisms regulating the homeostasis of more differentiated tumor cells and their non-proliferative roles in regulating growth. Moreover, therapies that focus on modulating the differentiation status of ERMS cells should attempt to force the conversion of tumor-propagating cells into cells with terminally differentiated myoblast characteristics that are incapable of re-creating tumor, cannot migrate, and fail to enter into the vasculature.

Experimental Procedures

Study Approval

These studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care under protocol #2011N000127 (zebrafish), the Duke University Institutional Animal Care & Use Committee under protocol A 036-03-02 (mouse), and the Partners Human Research Committee under protocol #2009-P-002756 (human). Samples were obtained from the Pathology Department of Massachusetts General Hospital. Use of decoded, paraffin embedded human tissue samples does not require informed consent.

Animals

CG1-strain (Smith et al., 2010), *α-actin-GFP* (Higashijima et al., 1997), *myf5-GFP* (Chen et al., 2007), *fli1-GFP* (Lawson and Weinstein, 2002), *flk1-mCherry* (Wang et al., 2010), and *mylz2-mCherry* transgenic zebrafish (Smith et al., 2010) have been reported previously.

The *rag2-KRASG12D*, *rag2-dsRED* express, myogenin-H2B-RFP, myogenin-H2B-Amcyan, and *mylz2-lyn-cyan* constructs were micro-injected into one-cell stage zebrafish singly (*rag2-KRASG12D* injected into *myf5-GFP/mylz2-mCherry* syngenic zebrafish, 60 ng/microliter) or as combinations with linearized DNA at a final combined concentration of 120 ng/microliter essentially as described (Langenau et al., 2008).

FACS and ERMS Cell Transplantation

FACS analysis and ERMS cell transplantation were completed essentially as described (Smith et al., 2010; Langenau et al., 2007). Sort gates were placed based on wild-type control fish, and *myf5-GFP+*, *mylz2-mCherry+*, or *mylz2-lyn-cyan+* ERMS. Dapi, propidium iodide or TOPRO3 was used to isolate viable cells. ERMS tumors were double sorted to obtain pure, viable cell populations. Sort purity was assessed after two rounds of sorting when possible. Following limiting dilution cell transplantation into non-irradiated

syngeneic CG1-recipient animals, fish were analyzed for fluorescent tumor engraftment from 10 to 120 days post-transplantation. Tumor-propagating potential was quantified using the Extreme Limiting Dilution Analysis software (<http://bioinf.wehi.edu.au/software/elda/>). A subset of transplanted fish were sectioned and stained with hematoxylin and eosin to confirm the presence or absence of ERMS.

Immunohistochemistry, EDU and Annexin V staining

Paraffin embedding and sectioning, cryostat sectioning, and immunohistochemical analysis were performed essentially as described (Langenau et al., 2007 and Supplemental Experimental Procedures). EDU staining was performed using the Click-iT Alexa Fluor 647 imaging kit, from Invitrogen. Annexin analysis for apoptotic cells was performed via FACS using annexin V conjugated to Alexa Fluor 647 (Invitrogen).

Gene Expression Analysis-

Total RNA was isolated from 6- and 24-hour post-fertilization AB-strain embryos and FAC sorted ERMS cell subpopulations (TRIZOL, GIBCO/BRL) in the presence of glycol blue. Quantitative real-time PCR utilized gene-specific PCR primers (Supplemental Experimental Procedures) and expression was normalized to 18s and β -actin controls to obtain relative transcript levels using the $\delta\delta$ CT method. Relative gene expression was normalized within individual samples and cumulative transcript expression across the four ERMS cell subpopulation was set to 25. Samples were assessed in relation to 6 and 24-hour embryos to ensure that $\delta\delta$ CT for any given gene were not lower than 10-fold expression found in normal development. Microarray experiments were completed essentially as described (Fold change cut off >1.5 fold log scale, Langenau et al., 2007). Microarray data has been deposited into the GEO database (GSE32425).

Laser Scanning Confocal Microscopy and Dual Photon Imaging

Larval zebrafish were anesthetized in Tricaine and embedded in a single drop of low melt 1% agarose on a glass bottom petri dish (No 1.5, Mat Tek Corporation). Each petri dish was supplemented with fish water and imaged using an inverted Pascal or LSM510 Zeiss laser scanning confocal microscope or an upright Ultima IV multiphoton microscope (Prairie Technologies). Quantification was completed by counting the total numbers of fluorescent-labeled ERMS cell subpopulations contained in two 250 \times 150 μ m areas per animal (Figure 4K-L; control, n=7; Stage 1, n=3; Stage 2, n=4; Stage 3 n=7). Because regional niches can be compartmentalized within a single myotome segment, a smaller area was assessed for total numbers of *myf5-GFP+* and *myogenin-H2B-RFP+ ERMS* cells (50 \times 50 μ m² area, n=6 areas/tumor, Figure 4M).

For cell tracking, sequences of image stacks were transformed into maximum intensity-projected movies using Imaris 7.1 software (Bitplane) and exported as Quicktime movies. Manual 2-D or 3-D tracking was performed using the manual tracking plugin in ImageJ or using Imaris 7.1. Annotation and further processing of movies was completed using ImageJ and Quicktime 7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

Tumor-propagating potential is not found in all malignant cells, and in most cancers, cells with more differentiated features are largely incapable of remaking tumor and yet comprise a majority of the tumor mass. A role for differentiated malignant cells in tumor growth, including dissemination and metastasis, has not been fully explored. We find that mid-differentiated myogenin-positive ERMS cells lack tumor-propagating potential, yet are responsible for local invasion and can enter the vasculature. Slow moving myf5+ ERMS-propagating cells are recruited to new sites of tumor growth after seeding by differentiated ERMS cells. This finding may explain the clinical observation that Myogenin positivity correlates with poor clinical outcome in human ERMS and suggests that differentiated tumor cells play critical roles in metastasis.

Highlights

- Functional heterogeneity of ERMS cells can be visualized in live zebrafish.
- Tumor-propagating potential is restricted to *myf5+* ERMS cells.
- *myogenin+* ERMS cells are highly invasive and colonize new areas of growth.
- Metastatic and ERMS-propagating potential reside in distinct cell subpopulations.

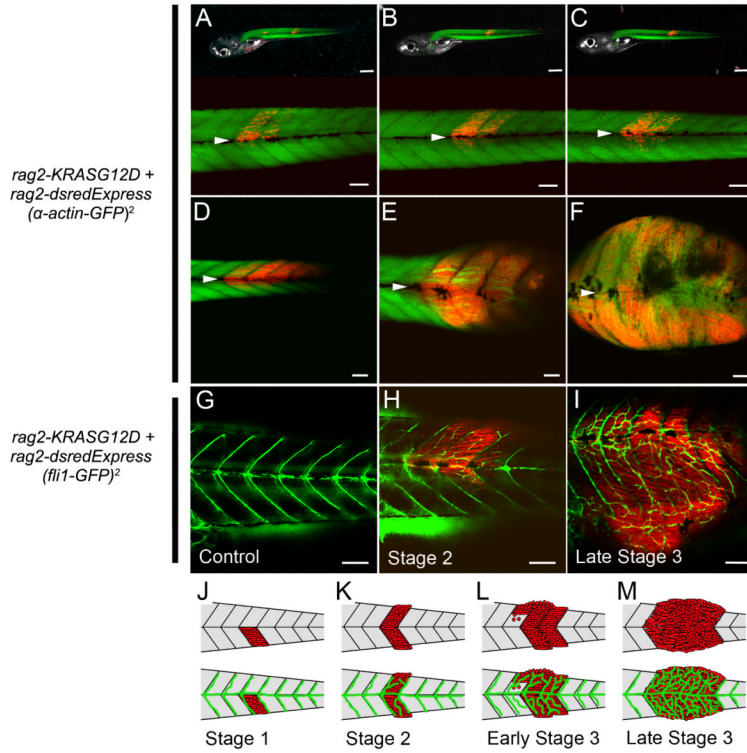


Figure 1. Visualizing distinct stages of embryonal rhabdomyosarcoma growth
 (A-F) *rag2-dsRED*-labeled ERMS arising in *alpha-actin-GFP* transgenic zebrafish. (A-C) The same animal imaged at 6, 9, and 12 days post-fertilization (dpf). (D-F) A representative zebrafish where dsRED+ ERMS cells have already bypassed the horizontal myoseptum and migrated into new segments that were previously free of tumor (F, stage 3) at 13, 18, and 24 dpf (D-F, respectively). The horizontal myoseptum is denoted by white arrows in A-F. (G) *fli1-GFP* transgenic control animal compared with a *rag2-dsRED*-labeled ERMS arising in *fli1-GFP* transgenic zebrafish at early stage 2 (H) or a late stage 3 (I). (J-M) Schematic of stages of ERMS growth.
 Scale bar A-C upper panel 500 μm, A-C lower panel and D-I 100 μm.
 See also Movie S1.

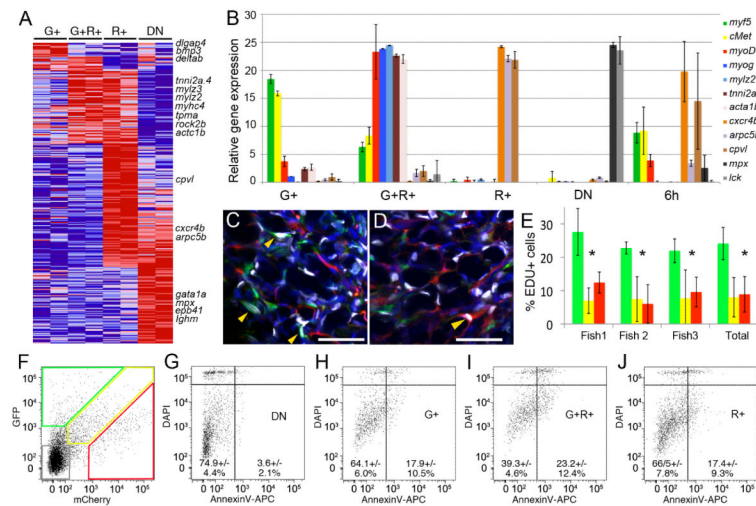


Figure 2. Fluorescent transgenic approaches identify discrete and molecularly definable ERMS cell subpopulations in *myf5-GFP/myl2-mCherry* transgenic fish

(A) Heat map showing differential gene expression between FACS sorted ERMS cell subpopulations isolated from serially-passaged *myf5-GFP/myl2-mCherry* ERMS (microarray log fold-change >1.5). *myf5-GFP+/myl2-mCherry-negative* (G+), *myf5-GFP+/myl2-mCherry+* (G+R+), *myf5-GFP-/myl2-mCherry+* (R+), and double negative (DN).

(B) Quantitative real-time PCR of sorted ERMS cell subpopulations. Expression values \pm 1 STD.

(C-D) Confocal images of EDU stained sections from serially passaged *myf5-GFP+/myl2-mCherry+* ERMS. Tumor regions with large numbers of either *myf5-GFP+* (C) or *myl2-mCherry+* ERMS cells (D). Blue denotes DAPI+ nuclei and white labels EDU+ nuclei. Yellow arrows indicate EDU-labeled cells. Scale bar is 25 μ m.

(E) Quantification of EDU-incorporation over a 6 hour EDU pulse. Data for *myf5-GFP+/myl2-mCherry-negative* ERMS cells denoted by green bars, *myf5-GFP+/myl2-mCherry+* by yellow, and *myf5-GFP-/myl2-mCherry+* cells by red. Three individual tumors shown as well as cumulative data across all tumors (Total). Asterisk denotes $p < 0.00001$ and error bars \pm 1 STD.

(F) FACS plot of serially passaged *myf5-GFP/myl2-mCherry* ERMS.

(G-J) Gated ERMS cells assessed for DAPI and AnnexinV-APC staining (double negative (DN), G; *myf5-GFP+/myl2-mCherry-negative* (G+), H; *myf5-GFP+/myl2-mCherry+* (G+R+), I; *myf5-GFP-/myl2-mCherry+* (R+), J). Live cells are shown the DAPI-negative/AnnexinV-negative gates.

See also Figure S1 and Table S1.

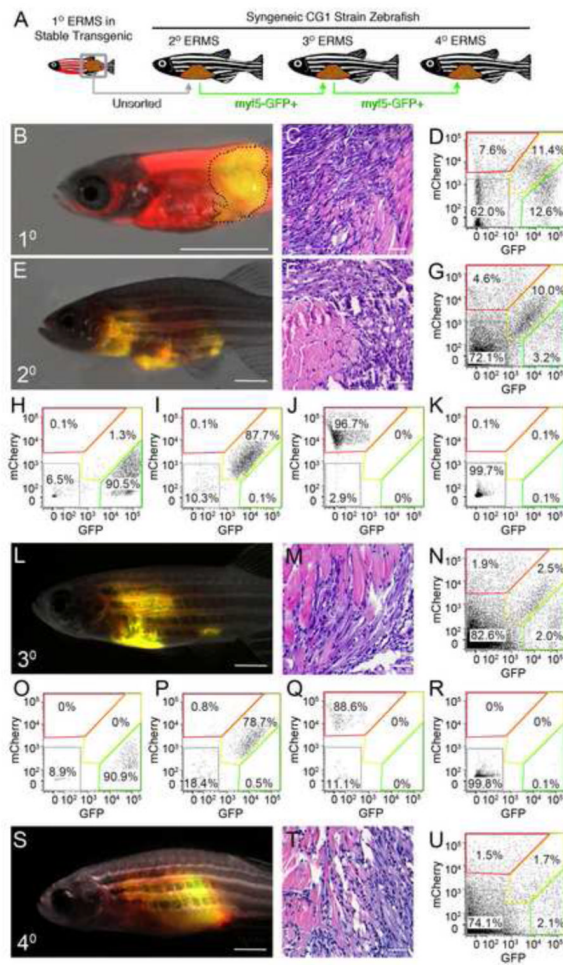


Figure 3. ERMS-propagating cells express *myf5-GFP* but not the *mylz2-mCherry* differentiated muscle marker

(A) Schematic of experimental design.

(B-D) A primary ERMS arising in syngeneic *myf5-GFP/mylz2-mCherry* transgenic zebrafish (35 dpf). Broken black line denotes tumor area.

(E-G) Fluorescent-labeled ERMS engraft into syngeneic secondary recipient animals when transplanted with unsorted primary ERMS cells.

(H-K) FACS plots of fluorescent-labeled ERMS cells isolated from secondary recipient fish following two rounds of FACS.

(L-R) Transplantation of *myf5-GFP+/mylz2-mCherry-negative* FACS sorted cells induced ERMS in tertiary transplant animals and (S-U) quaternary recipients. Hematoxylin and eosin stained sections (C,F,M,T) and FACS (D,G,N,U) of primary and serially passaged ERMS. Scale bars equal 2 mm (B, E, L and S) and 100 μ m (C, F, M and T).

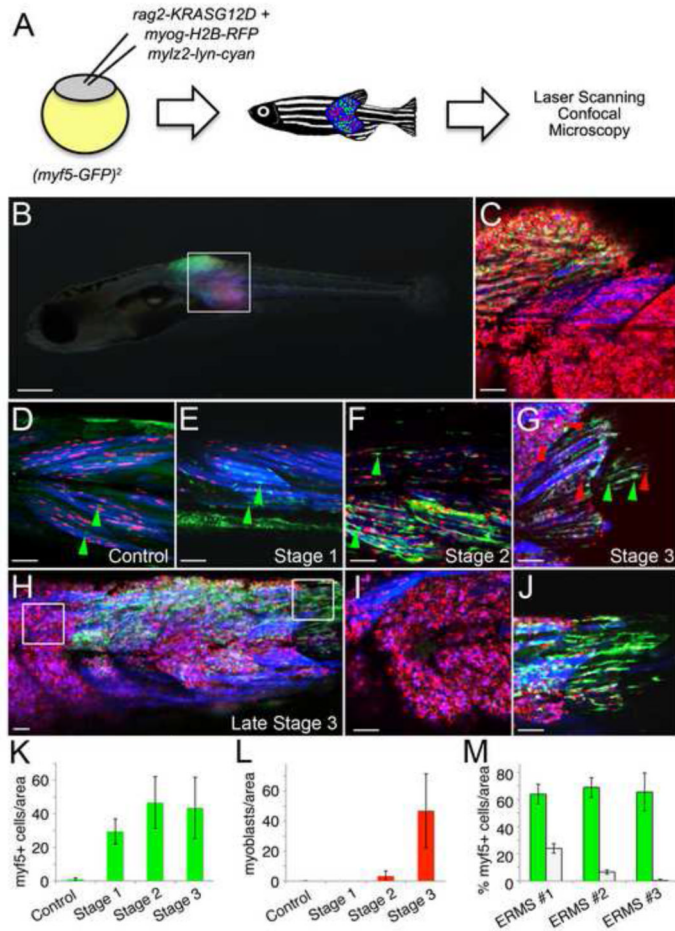


Figure 4. *myf5-GFP*⁺ ERMS-propagating cells are dynamically reorganized during tumor growth

(A) Schematic of the experimental design.
 (B) A *myf5-GFP* transgenic animal injected at the one-cell stage of life with *rag2-KRASG12D*, *myogenin-H2B-RFP*, and *mylz2-lyn-cyan* with triple fluorescent-labeled ERMS at 16 days of life.
 (C) A merged confocal image of the boxed region shown in B.
 (D) Control *myf5-GFP* transgenic animal injected with *myogenin-H2B-RFP* and *mylz2-lyn-cyan*. *myf5-GFP*⁺ muscle precursor cells are denoted by green arrowheads.
 (E-G) Representative image of an ERMS-affected zebrafish labeled with *myf5-GFP*, *myogenin-H2B-RFP* and *mylz2-lyn-cyan* at stage 1, 2 and 3, respectively. Green arrowheads denote *myf5-GFP*⁺ cells whereas red arrowheads denote mononuclear *myogenin-H2B-RFP*⁺ ERMS cells.
 (H) Late stage 3 ERMS from a triple fluorescent-labeled animal.
 (I-J) Boxed regions in H imaged at higher magnification show regional partitioning of differentiated cells (I) compared with *myf5-GFP*⁺ ERMS-propagating cells (J).
 (K) Quantification of *myf5-GFP*⁺ cells during stages of ERMS growth when compared to control animals.
 (L) Quantification of mononuclear *myogenin-H2B-RFP*⁺ cells during stages of ERMS growth when compared to control animals.
 (M) Quantification of regional compartmentalization of ERMS cells based on differentiation status in late stage 3 tumors (n=3). Green bars denote regions that contain higher

percentages of *myf5-GFP+* ERMS-propagating cells compared to white bars where *myf5-GFP+* cells are less abundant and conversely more differentiated. Error bars in K-M \pm 1 STD. Scale bar is 500 μ m (B) and 50 μ m (C-J). See also Figure S2.

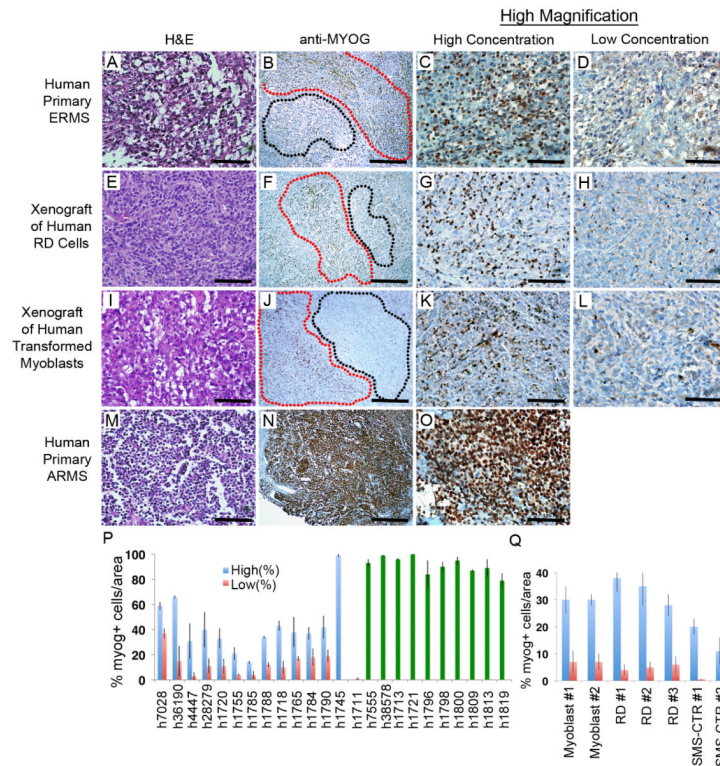


Figure 5. Human embryonal rhabdomyosarcoma exhibit regional portioning of cells based on myogenic factor expression

(A-D) Primary human ERMS.

(E-H) RD human cell lines or (I-L) human RAS-transformed myoblasts introduced into SCID/*beige* mice.

(M-O) Primary human ARMS. Hematoxylin/Eosin stained sections (A, E, I, M) and anti-myogenin immunohistochemistry performed on adjacent sections (B, F, J, N). Regions containing high numbers of myogenin+ cells are denoted by red outline while regions with low numbers of myogenin+ cells are denoted by black outline (B, F, J). ARMS did not show regional portioning based on myogenin staining (N). Magnified views of areas with high concentrations of Myogenin+ cells (C, G, K, O) or areas with low or absent expression (D, H, L).

(P-Q) Quantification of regional compartments in primary and metastatic human RMS (P) and in mice xenografted with human RD and SMS-CTR ERMS cells and human RAS-transformed myoblasts (myoblasts, Q). Numbers in panel Q denote tumors arising in separate animals. Blue bars denote areas with high percentages of Myogenin+ cells compared to areas with low numbers of cells (red). Green bars denote diffuse and ubiquitous expression of Myogenin within ARMS. Error bars +/- 1 STD. Scale bars are 50 μ m (A, C, D, E, G, H, I, K, L, M, O) and 200 μ m (B, F, J, N)

See also Figure S3 and Table S2.

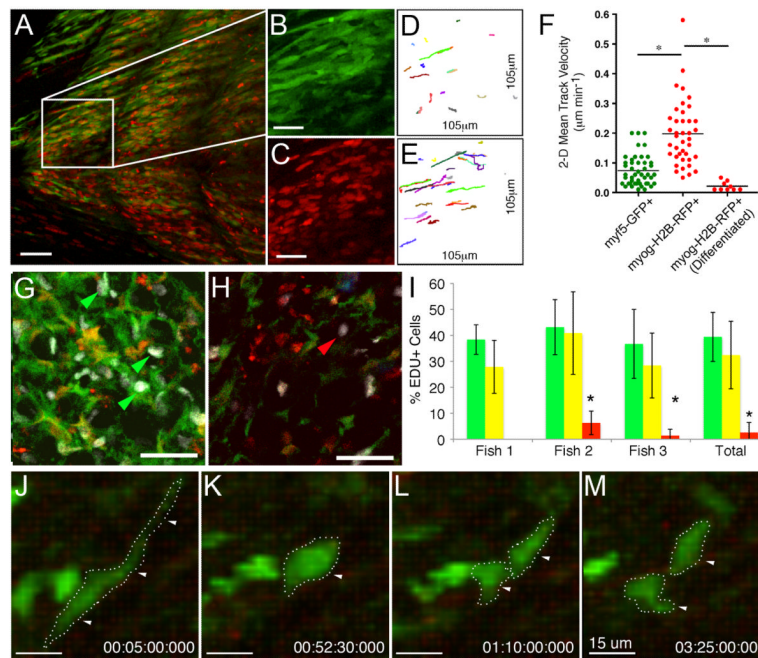


Figure 6. *myf5-GFP+* ERMS-propagating cells are slow moving but highly proliferative while *myogenin-H2B-RFP+* cells do not divide but are highly migratory
 (A-E) Multiphoton recording of a stage 3 ERMS arising in *myf5-GFP/myogenin-H2B-RFP* transgenic zebrafish (B, C) Magnified view of the boxed region in panel A showing *myf5-GFP+* (B) or *myogenin-H2B-RFP+* ERMS cells (C).
 (D, E) Tracks of cell movement over the 6.7h observation period. The same areas are shown as in panels B and C, respectively.
 (F) Mean track velocities of representative cell types contained within the tumor mass. Asterisk denotes $p < 0.001$.
 (G-H) EDU staining of double transgenic *myf5-GFP+/myogenin-H2B-RFP+* primary zebrafish ERMS (35 dpf). Confocal image of a tumor section with high numbers of *myf5-GFP+* ERMS cells (G) compared to a section with high numbers of *myogenin-H2B-RFP+* cells (H). White denotes nuclei that have incorporated EDU. EDU incorporation into *myf5-GFP+* or *myogenin-H2B-RFP+* ERMS cells denoted by green or red arrows, respectively.
 (I) Quantification of proliferation over the 6 hour EDU pulse. *myf5-GFP+/myogenin-H2B-RFP-negative* (green bars), double positive (yellow bars), and *myf5-GFP-negative/myogenin-H2B-RFP+* (red bars). Error bars ± 1 STD. Asterisk denotes significant differences with $p=0.0001$.
 (J-M) Static images of a *myf5-GFP+* ERMS cells dividing. Scales bar is 50 μm (A, G, H), 25 μm (B, C, D and E), and 15 μm (J-M).
 See also Figure S4 and Movie S2-6.

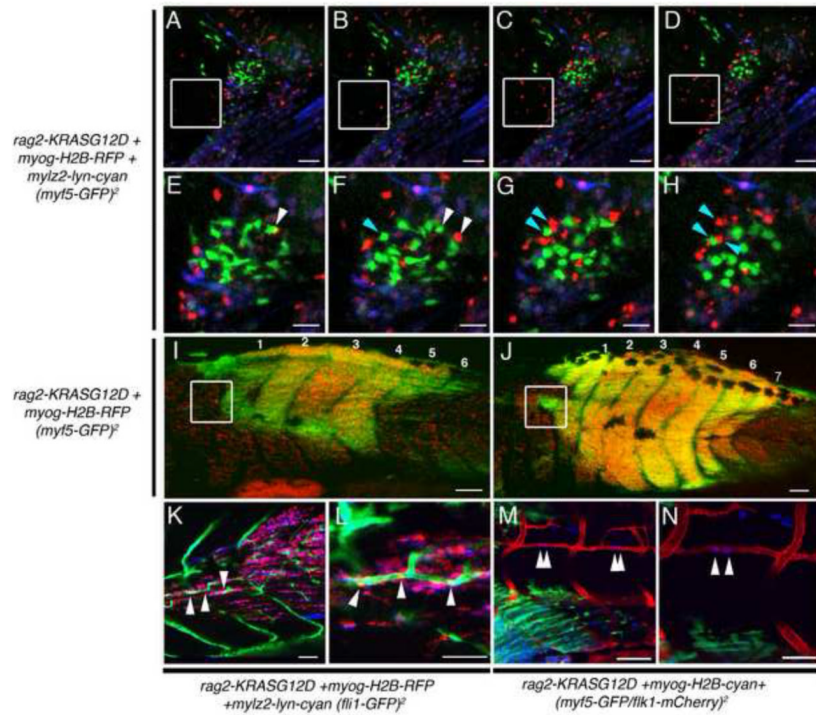


Figure 7. *myf5-GFP*+ ERMS-propagating cells are recruited to new areas of tumor growth only after seeding by *myogenin*+ ERMS cells

(A-H) Time-lapse images of *myf5-GFP* transgenic animal injected with *rag2-KRASG12D*, *myogenin-H2B-RFP* and *mylz2-lyn-cyan*. Panels are merged image planes taken every hour. *myogenin-H2B-RFP*+ cells migrate into normal tissues over time (white boxed region). Magnified views of time-lapse images documenting that *myf5-GFP*+ cells are largely stationary while *myogenin-H2B-RFP*+ cells are highly migratory and migrate away from GFP+ cells (denoted by arrows in E-H).

(I, J) Serial imaging of a *myf5-GFP* transgenic animal injected with *rag2-KRASG12D* and *myogenin-H2B-RFP* shown at 14 and 17 dpf, respectively. White boxes demark a region that initially contained only *myogenin-H2B-RFP*+ cells (I) but was later colonized by *myf5-GFP*+ cells (J).

(K-L) ERMS developing in a *fli1-GFP* transgenic animal injected with *rag2-KRASG12D*, *myogenin-H2B-RFP*, and *mylz2-lyn-cyan*. (K) Merged z-stacks showing three *myogenin-H2B-RFP*+ cells associated with and inside *fli1-GFP*+ vessels, which was confirmed by imaging a single image plane at higher magnification (L, white arrow head).

(M-N) ERMS developing in a *flk1-mCherry* transgenic animal injected with *rag2-KRASG12D* and *myogenin-H2B-cyan* showing four cells entering the vasculature (White arrowheads, M) and a single plane image showing two cells transiting into the vasculature at higher magnification (White arrowheads, N).

Scale bar is 50 μm (A-H, K, L, N) and 100 μm (I, J, M).

See also Figure S4 and Movie S7-9.

Table 1
Limiting dilution cell transplantation identifies that *myf5-GFP*⁺/*myl2- mCherry-negative* cells are the ERMS-propagating cells

ERMS #1-2° Transplants				3° Transplants			
Cell#	G±	G+R±	R±	Neg	G±		
1000	6 of 6	2 of 7	0 of 6	0 of 7	6 of 6		
10	5 of 9	0 of 9	0 of 8	0 of 10	7 of 8		
10	0 of 8	0 of 8	0 of 9	0 of 7	0 of 10		
TPC #	1 in 140**	1 in 3461	NA	NA	1 in 67		
95% CI	59-329	872-13740	NA	NA	31-143		
ERMS #2-2° Transplants							
Cell#	G±	G+R±	R±	Neg			
1000	6 of 6	0 of 6	0 of 6	0 of 6			
10	4 of 7	2 of 10	0 of 10	0 of 10			
10	1 of 8	0 of 9	0 of 10	0 of 8			
TPC #	1 in 109**	1 in 3495	NA	NA			
95% CI	44-270	808-15120	NA	NA			
ERMS #3-2° Transplants							
Cell#	G±	G+R±	R±	Neg			
1000	2 of 3	0 of 2	0 of 3	0 of 4			
10	8 of 9	0 of 8	0 of 8	1 of 8			
10	1 of 8	0 of 9	0 of 9	0 of 9			
TPC #	1 in 159**	NA	NA	1 in 4840			
95% CI	63-401	NA	NA	632-37094			

Tumor-propagating cell number (TPC#) and 95% confidence interval (95% CI). Asterisks denote significant differences in TPC# between *myf5-GFP*⁺/*myl2-mCherry-negative* and double positive ERMS cells (** p<0.00001).

** p<0.00001