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CD133 Marks a Myogenically Primitive Subpopulation in Rhabdomyosarcoma Cell Lines that are Relatively Chemoresistant but Sensitive to Mutant HSV

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Abstract

Background—Rhabdomyosarcoma (RMS) is characterized by features of skeletal muscle and is comprised of two major histological subtypes, embryonal (E-RMS) and alveolar (A-RMS).

Subsets of each RMS subtype demonstrate resistance to multimodal therapy leading to treatment failure. Cancer stem cells or cancer-initiating cells (CIC) represent a theorized population of cells that give rise to tumors and are responsible for treatment resistance.

Procedure—We investigated the ability of CD133, a putative CIC marker, to distinguish a chemoresistant, myogenically primitive population in alveolar (RH30) and embryonal (RD) RMS cell lines. We tested CD133+/- cells for sensitivity to engineered herpes simplex virus (oHSV)

Results—Relative to CD133- cells, CD133+ A-RMS and E-RMS cells demonstrate an enhanced colony-forming ability, are less differentiated myogenically, and are more resistant to cytotoxic chemotherapy but equally sensitive to oHSV oncolysis. Compared to CD133- RD cells, CD133+ cells express relatively high levels of genes typically expressed in skeletal muscle progenitor satellite cells including PAX7, c-MET, and the GLI effectors of the hedgehog signaling pathway. In contrast, CD133+ RH30 cells were not associated with enhanced expression of satellite cell markers or Hh targets.

Conclusions—Our findings demonstrate that CD133+ cells from A-RMS and E-RMS cell lines are characterized by a myogenically primitive phenotype. These cells have the capacity to form colonies *in vitro* and are more resistant to chemotherapy than CD133- cells. CD133 expression may denote a subset of RMS cells with an important role in tumorigenesis and treatment failure. These resistant cells may be effectively targeted by oHSV therapy.

Keywords

Rhabdomyosarcoma; CD133; chemotherapy; cancer stem cells; herpes simplex virus; HSV

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INTRODUCTION

Characterized by features of the skeletal muscle lineage, rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children [1]. There are two main histological subtypes of RMS, embryonal (E-RMS) and alveolar (A-RMS). Most A-RMS tumors possess the t(2;13) PAX3-FOXO1 translocation whereas the majority of E-RMS tumors display loss of heterozygosity (LOH) at the chromosome 11p15.5 locus [2-3]. Despite significant improvement in survival over the past few decades, more than one-third of RMS patients still die of disease [4]. Patients with metastatic or treatment refractory tumors carry a particularly dismal prognosis. Augmentation of conventional chemotherapy regimens has not significantly improved survival [5]. Moreover, the poorly targeted nature of conventional therapies leads to toxicity in benign bystander cells. Interestingly, some patients that ultimately relapse often achieve a complete response to therapy with no remaining radiological evidence of tumor only to suffer recurrence during or after therapy, suggesting a minimal number of tumor cells may be responsible for treatment resistance. Thus, identifying the resistant tumor cell population may allow for the development of effective targeted therapies against those cells, with the goal of substantially increasing survival while decreasing adverse effects to normal tissue.

A subpopulation of cells termed cancer stem cells or cancer-initiating cells (CIC) has been identified in many different types of pediatric solid tumors [6-11]. CIC are theorized to contribute to cancer initiation, propagation, invasiveness, and metastasis. The CIC model recapitulates that of normal developmental hierarchy in tissues: primitive progenitor cells give rise to more differentiated cells that perform specialized functions characteristic of the tissue type. CIC are putatively responsible for the cellular heterogeneity within a neoplasm. Importantly, CIC are generally resistant to conventional chemotherapy and radiotherapy, and therefore, may be the cells responsible for tumor recurrence [12,13]. The cell surface protein CD133 (prominin-1), a pentaspan transmembrane protein expressed on a variety of normal stem cells including mesenchymal stem cells (MSC), is a commonly used marker to identify CIC in pediatric malignancies [14].

We sought to determine if CD133 marks a myogenically primitive, chemoresistant population in both A-RMS (RH30) and E-RMS (RD) cell lines and if those resistant cells can be targeted with engineered herpes simplex virus oncolytic therapy (oHSV), a promising novel therapeutic for pediatric malignancies that does not rely on the cell cycle to infect and kill tumor cells [15]. We examined the expression of CD133 in a variety of RMS cell lines. In CD133⁺/₋ RH30 and RD cell fractions, we determined the degree of myogenic differentiation; the expression of skeletal muscle satellite cell markers, other putative stem cell markers, and effectors of the hedgehog (Hh) embryonic signaling pathway.

METHODS

Cell Lines and Genetically Engineered HSV

The E-RMS cell line RD possesses loss of heterozygosity (LOH) at chromosome 11p15.5, and the A-RMS cell line RH30 contains t(2;13)(q35;q14) PAX3-FOXO1 fusion oncogene [1,3]. RD and RH30 were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). The embryonal cell line Birch and the alveolar cell line RH28 were provided by Frederick Barr, M.D. Ph.D., National Institutes of Health. SMS-CTR was provided by Jiayuh Lin, Ph.D., Ohio State University. Cells were cultured in Dulbecco modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). oHSV M002 is a conditionally replication-competent mutant virus expressing both subunits of murine interleukin-12 and produces bioactive murine IL-12 in physiologic relevant amounts [16].

Fluorescence-activated Cell Sorter (FACS) Analysis

Expression levels of CD133, FGFR3, c-MET and CD111 were obtained by FACS analysis as we have previously described [17]. Adherent cells were harvested from tissue culture using trypsin (Invitrogen, Carlsbad, California). The following antibodies were used: allophycocyanin (APC)-CD133 (Miltenyi Biotec), phycoerythrin (PE)-c-MET (R&D Systems, Minneapolis, MN), PE-FGFR3 (R&D Systems), PE-PPR1 (CD111) (Beckman Coulter, Fullerton, CA). Samples were analyzed using a BD Biosciences FACSCalibur (UAB Flow Cytometry Core Facility). Data were compiled using Flo-Jo software and results expressed as a percentage of gated cells.

To determine co-expression levels of CD133 and PAX-7, adherent cells were prepared as above. Fixed cells were washed with a quenching buffer (Glycine (0.375g) in 50mL Hank's Balanced Salt Solution) and their membranes permeabilized by incubation (room temp; 60 seconds) in 100uL of 0.2% Triton 100-X in PBS. Washed cell pellets were incubated (room temp; 10 minutes) in 70uL MACS buffer and 20uL of FCR blocker, labeled with a rabbit polyclonal antibody to PAX-7 (0.1uL; Abcam, Cambridge, Massachusetts) and incubated in the dark (4°C, 30 minutes). After washing twice, cells were incubated (4°C; 45 minutes) with 0.5uL of PE-labeled donkey polyclonal antibody to detect bound rabbit IgG (Abcam). Cells were analyzed immediately as described above.

Cell Separation

Cultured cells were harvested as above, pelleted by centrifugation (200xg, 8 minutes), washed twice, and separated into CD133+/- fractions (greater than 85% purity and 90% viability) via a magnetic bead separating kit (MACS, Miltenyi Biotec) per manufacturer's protocol.

Colony Focus Formation and Colony-Forming Chemotherapy Assay

5000 cells separated by CD133 expression were plated onto 100mm plates and cultured (37°C, 5% CO₂) undisturbed for 7 days in DMEM supplemented with 10% FBS serum. Medium was replaced at 7 days; at day 14, medium was removed, foci were washed gently with PBS and stained with crystal violet dissolved in 25% methanol. To determine chemoresistance of CD133+/- cells, daunorubicin (dauno; 20-735 nM; Ben Venue Laboratories Inc., Bedford, Ohio) or vincristine (VCR; 2.5-250nM; Mayne Pharma USA Inc., Paramus, New Jersey) was added to plates at inhibitory concentrations estimated at 25% and 75% based on chemotherapeutic inhibitory assay results in CD133+ cells. Media was changed every 3 days and chemotherapy was added twice a week for 4 doses. Seven days after the last dose, plates were prepared as above and stained. Foci were documented by digital photography, and numbers of colonies greater than one mm in diameter and percentages of colony confluency were determined using ImageJ software.

Chemotherapeutic Inhibitory and Virus Cytotoxicity Assay

The alamarBlue assay was used as previously described to determine sensitivity of CD133+/- cells to dauno (0-500 nM), VCR (0-25 nM), or oHSV M002 (0-100 plaque-forming units per cell; PFU/cell) [17]. Wells that received neither drug nor virus were used as a control and relative changes as percent of control were calculated for each graded dose.

Cell Cycle

To determine percentages of CD133+/- cells in G₁, S, and G₂ phase, 10⁶ separated cells were fixed with absolute ethanol (-20°C, 15 minutes) and pelleted by centrifugation. Ethanol was removed and cells were allowed to rehydrate in 5ml of PBS (room temp, 15 minutes). Cell suspensions were centrifuged, supernates discarded, and 1ml of propidium iodide

(Invitrogen) diluted in staining buffer was added per manufacturer's protocol. Data were compiled with Flo-Jo software using the Watson (Pragmatic) model.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

After placing in Trizol (Invitrogen), fractionated cells were subjected to phenol/chloroform extraction followed by ethanol precipitation. RNA was subjected to reverse transcription and the gene of interest and a duplexed 18S rRNA internal control were measured by qRT-PCR on an ABI-Prism 7900HT Sequence Detection System. Quantification of gene expression was calculated by the $\Delta\Delta C_t$ method [18,19]. Calibrator samples for each assay were either unfractionated RD or RH30 cells. Human-specific primer and probe sets for the internal control 18S and experimental genes of interest were purchased from Applied Biosystems (Foster City, California) as described elsewhere (supplemental material).

Statistical Analyses

Student's *t*-test analyses for significance of mean differences were performed using Microsoft Excel. All experiments were performed in triplicate at a minimum. A *p* value of < 0.05 was considered significant.

RESULTS

CD133 and Fibroblast Growth Factor Receptor 3 (FGFR3) Expression

Cell surface expression of CD133 and FGFR3, a reported marker of RMS-initiating cells, in five RMS cell lines was measured (Table I) [20]. CD133 expression ranged from 12.7% to 53.5% of cells. FGFR3 expression was lower than CD133 expression in all cell lines ranging from 1.6% to 7.4%. A significantly higher percentage of CD133+ cells co-expressed FGFR3 as compared to CD133- in all cell lines ($p=0.04$ - 0.002). Using qRT-PCR, the relative expression of FGFR3 was found to be higher in CD133+ RD cells than CD133- cells ($1.7x \pm 0.392$, $p=0.009$). In contrast, CD133+ RH30 cells expressed lower levels of FGFR3 than CD133- cells ($0.676x \pm 0.178$, $p=0.001$) (Figure 2-B).

Colony Focus Formation

CD133+ cells formed more and larger colonies, indicating a more transformed population of cells *in vitro* from a limited number of cells (Figure 1-A). CD133+ RD cells formed 258 ± 43 colonies greater than one mm in size compared to only 54 ± 25 colonies in CD133- RD cells ($p=0.005$); CD133+ RH30 cells formed 210 ± 38 colonies compared to 110 ± 30 colonies in CD133- RH30 cells ($p=0.003$).

Satellite Cell Markers

In RD cells, CD133+ cells expressed higher RNA levels of satellite cell markers PAX7 ($1.57x \pm 0.314$, $p=0.001$) and c-MET ($1.51x \pm 0.282$, $p=0.003$) than CD133- cells (Figure 2-A). In FACS analysis, CD133+ RD cells expressed a higher amount of PAX7 ($52.9 \pm 4.8\%$ versus $13.8 \pm 2.5\%$, $p<0.0001$) and c-MET ($78.7 \pm 4.3\%$ versus $41.0 \pm 1.7\%$, $p<0.0001$) compared to CD133- RD cells. M-Cadherin (M-CAD) expression was equivalent ($0.817x \pm 0.137$, $p=0.161$).

CD133+ RH30 cells expressed lower levels of PAX7 ($0.519x \pm 0.279$, $p=0.029$) and equivalent levels of c-MET ($0.938x \pm 0.1$, $p=0.562$) and M-CAD ($1.13x \pm 0.094$, $p=0.151$). Very high surface expression of c-MET (>99%) was detected by FACS on RH30 cells which was anticipated since c-MET is a known target of PAX3-FOXO1 [21]. PAX3-FOXO1 RNA was more highly expressed in CD133+ cells ($1.1x$ versus unfractionated RH30 cells) than CD133- cells ($0.58x$ versus unfractionated RH30 cells), $p<0.0001$. In

unfractionated cells, PAX7 expression in RD was >2000x higher than in RH30 while c-MET expression was 3x lower in RD than RH30.

Embryonic Factors and Stem Cell Markers

Expression levels were higher in CD133+ RD cells than in CD133- cells for the following: NANOG (5.47 ± 2.458 , $p=0.002$), POU1 (OCT4) (1.705 ± 0.392 , $p=0.008$), CD44 (1.83 ± 0.24 , $p=0.027$), PAX3 (1.62 ± 0.363 , $p=0.036$), and CXCR4 (1.37 ± 0.213 , $p=0.0255$) (Figure 2-B). Of these markers, only NANOG was found to be more highly expressed in CD133+ RH30 cells (1.46 ± 0.072 , $p=0.008$). Other markers were similarly expressed in CD133+/- RH30 cells including POU1 (0.972 ± 0.05 , $p=0.711$), PAX3 (0.77 ± 0.126 , $p=0.001$), and CXCR4 (0.857 ± 0.119 , $p=0.225$), which is a known downstream target of PAX3-FOXO1 [22]. CD44 RNA expression was not detectable in either CD133+/-RH30 cells.

Myogenic differentiation

To determine the degree of myogenic differentiation in each RMS CD133+ fraction, expression of various myogenic regulatory factors (MRFs) was measured by qRT-PCR. CD133+ RD cells demonstrated lower relative expression of the early MRF MYOD (0.81 ± 0.123 , $p=0.075$), the late MRF MYF6 (0.49 ± 0.291 , $p=0.02$), and the terminal differentiation factors myogenin (MYOG) (0.363 ± 0.365 , $p=0.023$), myoglobin (MB) (0.37 ± 0.349 , $p=0.005$), and desmin (DES) (0.49 ± 0.283 , $p=0.003$) (Figure 2-C). CD133+ RH30 cells also demonstrated less myogenic differentiation with decreased relative MYOG expression (0.77 ± 0.1384 , $p=0.05$) and MB (0.44 ± 0.29 , $p=0.016$). Expression of other MRFs was similar between CD133+/- RH30 cells: MYOD (0.95 ± 0.053 , $p=0.32$), MYF6 (1.03 ± 0.09 , $p=0.52$), and DES (0.89 ± 0.17 , $p=0.513$).

Hh signaling

The main effectors of the Hh pathway including the zinc finger transcription factors GLI1 (1.84 ± 0.057 , $p<0.001$) and GLI2 (1.61 ± 0.153 , $p=0.002$) and the key regulator of the Hh pathway feedback loop PTCH1 (1.57 ± 0.042 , $p<0.001$) and PTCH2 (1.56 ± 0.106 , $p=0.001$) were found to be more highly expressed in CD133+ RD cells than CD133- cells (Figure 2-D). In contrast, RH30 CD133+ cells had lower expression of GLI1 (0.517 ± 0.27 , $p=0.009$), PTCH1 (0.387 ± 0.336 , $p<0.001$), and PTCH2 (0.252 ± 0.41 , $p<0.001$). GLI2 expression was not statistically different between CD133 +/- RH30 cells (1.025 ± 0.045 , $p=0.59$).

Chemotherapy resistance

RH30 CD133+ cells had a significantly higher percentage of live cells compared to CD133- cells at all tested concentrations of dauno (half maximum inhibitory concentration (IC_{50}) 86.7 ± 26.7 nM versus 41.7 ± 14.7 nM, respectively, $p=0.04$) and four of five concentrations of VCR (IC_{50} 11.5 ± 2.1 nM versus 4.9 ± 1.3 nM, $p=0.0002$) (Figure 3). Similarly, there were significantly higher numbers of RD CD133+ cells alive compared to CD133- cells after exposure to dauno (IC_{50} 2400 ± 570 nM versus 490 ± 60 nM, $p=0.008$) and VCR (IC_{50} 1930 ± 940 nM versus 280 ± 190 nM, $p=0.006$) at all but one of the concentrations tested. Based on estimated IC_{25} and IC_{75} values for CD133+ cells, fractionated cells were treated with dauno or VCR as described. Colony formation data revealed that CD133+ RD and RH30 cells were more resistant to both chemotherapeutics and formed significantly more viable colonies relative to that of either CD133- RD or RH30 cells (Figure 1).

CD133+ RD cells had a significantly greater percentage of cells in G_1 ($71.9 \pm 3.3\%$ versus $51.4 \pm 0.5\%$, $p=0.005$) and a lower percentage of cells in S ($17.8 \pm 1.8\%$ versus $21.6 \pm 0.8\%$, $p=0.03$) and G_2 phase ($10.3 \pm 1.6\%$ versus $26.9 \pm 1.3\%$, $p<0.0001$) compared to CD133- RD

cells. Similarly, CD133+ RH30 had more cells in G₁ (49.2±2.9% versus 37.4±2.8%, $p=0.007$) and fewer cells in G₂ phase (19.3±1.8 versus 28.0±5.0%, $p=0.048$) than CD133- cells. No difference was seen in RH30 CD133+/- in S phase (31.5±1.5% versus 34.6±2.5%, $p=0.14$). These data suggest that cell cycle differences may be a contributing factor to the chemoresistance of CD133+ cells.

Genetically engineered HSV oncolytic virotherapy

To establish if CD133+/- RH30 and RD cells could be effectively targeted by oHSV, a cell cycle-independent therapeutic, cells were first tested for expression of CD111 (nectin-1), an adhesion molecule widely expressed in a variety of tumors that serves as the key HSV-entry mediator [23]. CD111 was very highly expressed in both CD133+/- RH30 cells (93.3±5.9%; 97.4±1.3 respectively, $p=0.20$) and CD133+/- RD cells (94.2±2.2; 92.9±4.9, $p=0.76$), suggesting that HSV should readily infect these cells. While CD133+ RH30 and RD cells were more resistant to VCR and dauno, CD133+ and CD133- cells from either tumor proved to be equally sensitive to killing by oHSV M002 over a range of doses from 0.1 to 10 PFU/cell (Figure 4). The lethal dose required to kill 50% of cells (LD₅₀) was similar for CD133+/- RH30 cells (0.4±0.2 versus 0.6±0.1, $p=0.23$) and for RD cells (3.1±0.2 versus 3.7±1.4, $p=0.45$). While both cell lines were very sensitive to killing by oHSV, RH30 cells were significantly more sensitive than RD cells ($p<0.003$). At 100 PFU/cell, all cells were killed within 72 hours.

DISCUSSION

The CIC theory provides a useful model for conceptualizing the complex cellular hierarchy of malignant neoplasms and offers a potential explanation for treatment resistance often encountered in many tumor-types, including the mesenchymal tumor RMS. Similar to recent reports, we found that CD133 expression was present in all tested RMS cell lines and was quite variable (12.7 to 53.5%) [11,24]. These expression levels are consistent with that found in other solid tumors; CD133 expression has been correlated with aggressiveness and tumor grade in a number of cancers including breast cancer, glioblastoma multiforme, and more recently E-RMS [11,25,26]. Interestingly, CD133+ A-RMS and E-RMS cells co-expressed FGFR3, a reported marker of RMS-initiating cells, in greater amounts than CD133- cells suggesting a potential association of these markers that requires further study [20].

Confirming the findings of Walter et al., we demonstrated that CD133+ E-RMS RD cells possess the ability to form colonies *in vitro* and are more resistant to chemotherapeutic agents than CD133- cells. A novel finding in this report includes the extension of enhanced colony-formation and chemoresistance of CD133+ cells to include A-RMS cells. Moreover, cell cycle differences seen in RD and RH30 CD133+/- cells offer an explanation for chemoresistance of CD133+ cells. These findings may have critical therapeutic implications; targeting the CD133+ resistant fraction may improve outcomes in difficult-to-treat RMS tumors.

An entirely different therapeutic strategy to attack and kill resistant CD133+ RMS cells is oHSV, which has shown promise preclinically in treating human RMS tumors [27]. oHSV works in a cell-cycle independent manner infecting cells leading to cellular destruction and release of progeny virus able to infect more tumor cells. Specific mutations have been created in the wild-type virus that deletes the $\gamma_{134.5}$ diploid gene, rendering the virus unable to replicate in normal cells but readily so in cancer cells. The virus enters cells most efficiently through the adhesion molecule CD111 (nectin-1), which is expressed in similar amounts on the surface of CD133+/- cells in other tumor types [17,23]. We demonstrate for the first time that CD133+/- A-RMS and E-RMS cell lines expressed very high levels of

CD111 and were extremely sensitive to killing by the oHSV M002. Importantly, the chemoresistant CD133+ fractions were equally as sensitive to killing by the virus as the CD133- populations, which is likely in part due to their similar expression of CD111. The human IL-12 version of M002 (M032) will be studied in an upcoming phase I trial in adults with recurrent/progressive high-grade gliomas. The first pediatric trial investigating an oHSV injected intratumorally in extracranial solid tumors including RMS is ongoing (ClinicalTrials.gov identifier NCT00931931).

Another unique finding in this study is that both RH30 and RD CD133+ cells are less differentiated myogenically than their respective CD133- counterparts. Such a myogenically primitive phenotype suggests that RMS tumors arise from a pluripotent MSC or an immature yet myogenically committed cell such as a skeletal muscle satellite cell. The different expression patterns of muscle satellite markers, embryonic factors, stem cell markers, and Hh effectors in CD133+ RD versus RH30 cells lends support to the theory that E-RMS and A-RMS tumors may arise from distinct cell types [28,29]. Marked by the expression of the transcription factor PAX7, the membrane receptor and proto-oncogene c-MET, and the calcium-dependent cell adhesion molecule M-Cadherin, resting satellite cells function as committed muscle progenitors that contribute to postnatal growth and repair [30]. During skeletal myogenesis and repair, MRFs are expressed sequentially (Figure 5) [31]. Upon muscular injury, satellite cells are activated leading to the expression of early MRFs including MYOD and MYF5. This is followed by intermediate MRFs such as MYF6 (MRF4 or herculin) and myogenin (MYOG). Finally, the expression of terminal differentiation factors including myoglobin (MB) and desmin (DES) is evident [32]. Given their regenerative function, satellite cells that have acquired cancer promoting genetic alterations may represent a potential RMS cell of origin. Since PAX7 is over-expressed by many E-RMS but not A-RMS, satellite cell involvement may be limited to E-RMS [33].

CD133 expression in the E-RMS cell line RD correlated with satellite cell markers PAX7 and c-MET at both the RNA and protein level, whereas PAX7 RNA expression was decreased in RH30. Unfractionated RD cells expressed over 2,000 fold more PAX7 RNA than RH30 cells suggestive of a possible role of satellite cells in the genesis of RD but not RH30. Mouse model data suggests that PAX7 expressing satellite cells are unlikely to function as A-RMS progenitor cells; Keller et al. demonstrated that the expression of PAX3-FOXO1, the fusion oncogene in alveolar RMS, in PAX7+ myoblasts did not lead to the development of A-RMS tumors in conditional mouse models [34].

Together these data suggest that E-RMS may arise from muscle satellite cells whereas we speculate A-RMS tumors such as RH30 may arise from a less differentiated MSC or even a more differentiated muscle cell. If pluripotent MSC are the origin of some RMS, this would provide a rationale for why some RMS tumors arise in tissues devoid of skeletal muscle such as the urinary bladder or bone marrow [35]. Given the substantial clinical and biological distinctions between the two major RMS subtypes, it is plausible that each arises from different cells of origin.

Hh signaling pathway is important in embryonic cell development and is a known regulator of muscle satellite cell fate [36,37]. The pathway regulates myogenesis and angiogenesis after muscle injury. Hh signaling has been implicated in CIC maintenance and self-renewal in a variety of malignancies [38]. As demonstrated by the expression of GLI1, GLI2, PTCH1, and PTCH2, Hh pathway activity was found in CD133+ RD cells which is consistent with previous studies that have demonstrated Hh pathway activity is detectable in a subset of E-RMS tumors [39,40]. In contrast, a relative increased expression of Hh targets was seen in CD133- RH30 cells. While unfractionated RH30 cells express abundant GLI1 and PTCH1, this finding may represent an artifact of *in vitro* cell cultivation. RH30 has an

amplicon on chromosome 12q-13-15 that includes *GLI1*. The amplification of *GLI1* and corresponding overexpression found in RH30 is not typical of A-RMS tumors surveyed [40]. The increased Hh and satellite marker expression as well as decreased expression of myogenic differentiation in CD133+ RD cells, supports the idea that muscle satellite cells may have played a role in the genesis of the E-RMS RD. A variety of other genes with potential roles in myogenesis were found to be more highly expressed in CD133+ RMS cells. *NANOG*, expressed in embryonic stem cells and necessary for maintaining pluripotency, is markedly more expressed in CD133+ RD and RH30 cells [41]. The expression of *NANOG* has been found to inhibit myogenic differentiation [42]. Other factors including the embryonic stem cell factor and myogenic inhibitor *POU1F1*, the cell surface receptor and CIC marker CD44, the myogenic determinant *PAX3*, and *CXCR4*, a chemokine receptor implicated in myogenesis, were more highly expressed in CD133+ RD cells but not CD133+ RH30 cells [43-46]. Along with *FGFR3*, the expression of *NANOG* appears to support the concept that CD133+ RD and RH30 cells maintain a primitive myogenic phenotype with the E-RMS RD characterized by the enhanced expression of embryonic factors.

In summary, our results demonstrate that E-RMS and A-RMS cell lines contain myogenically primitive cells marked by CD133 that have an enhanced colony-forming ability and are chemoresistant compared to CD133- cells. Our data suggest that A-RMS and E-RMS may arise from distinct cells origin. Importantly, the CD133+ chemoresistant cells can be effectively targeted by oHSV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Colony focus formation of CD133+ and CD133- RD and RH30 cells with and without chemotherapy

The colony-forming ability of RMS cells, fractionated by the expression of CD133, was assessed. **A)** Representative example of plates 14 days after separation (control) and after exposure to daunorubicin (Dauno) or vincristine (VCR) twice weekly for four doses at an estimated 75% inhibitory concentration (IC75). CD133+ RD and RH30 cells formed more colonies than CD133- RD and RH30 cells. **B)** Percent colony formation of RD and RH30 CD133+/- cells treated with daunorubicin (RD IC25=245nM, IC75=735nM; RH30 IC25=20nM, IC75=60nM) relative to control without daunorubicin. **C)** Percent colony formation of RD and RH30 CD133+/- cells treated with vincristine (RD IC25=2.5nM, IC75=250; RH30 IC25=2.5nM, IC75=7.5nM) relative to control without vincristine. Significantly more colonies formed between all treatment groups of CD133+ and CD133- cells (all *p* values < 0.01)

Figure 2. Expression of skeletal muscle satellite cell markers, stem cell markers, MRFs, and Hh effectors

Relative expression of various genes was measured by qRT-PCR and values calculated by the $\Delta\Delta C_t$ method. Values demonstrated in the CD133+ cells are relative to corresponding CD133- counterparts for each cell line which were normalized to a value of 1. RNA expression was considered undetectable if the emitted fluorescence representing the accumulated PCR product did not reach a predetermined threshold level by the completion of 40 PCR cycles. **A)** Relative expression of satellite cell markers PAX7, c-MET, and M-CAD. **B)** Relative expression of various stem cell markers in CD133+ cells. CD44 expression was not detectable in either fraction of RH30 cells (NE-not expressed) **C)** Relative expression of MRFs in CD133+ cells. **D)** Relative expression of hedgehog effectors GLI1 and GLI2 and Hh regulators PTCH1 and PTCH2 in CD133+ RMS cells.

Figure 3. Chemosensitivity of CD133+ and CD133- RMS cells
 Percentage of live cells measured 72 hours after exposure to chemotherapeutic agents. (A) RH30 cells exposed to daunorubicin. (B) RH30 cells exposed to vincristine. (C) RD cells exposed to daunorubicin (D) RD cells exposed to vincristine.

Figure 4. Sensitivity of CD133+ and CD133- RMS cells to genetically engineered oncolytic HSV
Percentage of live cells measured after exposure to engineered HSV M002 for 72 hours.

Figure 5. Myogenic differentiation
As muscle cells differentiate from primitive satellite or stem cells to mature muscle cells, they express unique myogenic regulatory factors. Stem cells commit to become satellite cells which express PAX7 and c-MET. Satellite cells differentiate to form myoblasts which make up myotubes. Following terminal differentiation, myotubes form mature muscle. The relative expression of each gene in CD133+ RD and RH30 cells is depicted at the bottom as follows: 0 to 0.5 (↓↓), 0.5 to 1 (↑), 1 to 1.5 (↓), 1.5 to 2 (↓↓).

Table 1

CD133 and FGFR expression ($\pm$ standard deviation) measured by FACS analysis in RMS cell lines.

Tumor ID	Type	CD133+	FGFR+	CD133+ FGFR+ *	CD133- FGFR+ *
RH30	A-RMS	43.5 $\pm$ 10.5% (n = 10)	2.0 $\pm$ 0.5% (n = 4)	2.6 $\pm$ 0.2%	1.4 $\pm$ 0.5%
RH28	A-RMS	16.0 $\pm$ 0.4% (n = 8)	1.6 $\pm$ 0.6% (n = 4)	5.7 $\pm$ 2.3%	1.0 $\pm$ 0.4%
RD	E-RMS	12.7 $\pm$ 6.9% (n = 10)	1.7 $\pm$ 0.5% (n = 4)	3.7 $\pm$ 1.2%	1.4 $\pm$ 0.6%
Birch	E-RMS	53.5 $\pm$ 7.7% (n = 4)	7.4 $\pm$ 0.6% (n = 4)	9.5 $\pm$ 0.9%	5.4 $\pm$ 0.4%
SMS-CTR	E-RMS	38.1 $\pm$ 4.8% (n = 4)	1.6 $\pm$ 0.2% (n = 4)	2.3 $\pm$ 0.2%	1.1 $\pm$ 0.2%

* Represents the percentage of CD133+ or CD133- cells that are FGFR+.