CD133 Positive Embryonal Rhabdomyosarcoma Stem-Like Cell Population Is Enriched in Rhabdospheres

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Abstract
Cancer stem cells (CSCs) have been identified in a number of solid tumors, but not yet in rhabdomyosarcoma (RMS), the most frequently occurring soft tissue tumor in childhood. Hence, the aim of this study was to identify and characterize a CSC population in RMS using a functional approach. We found that embryonal rhabdomyosarcoma (eRMS) cell lines can form rhabdomyosarcoma spheres (short rhabdospheres) in stem cell medium containing defined growth factors over several passages. Using an orthotopic xenograft model, we demonstrate that a 100 fold less sphere cells result in faster tumor growth compared to the adherent population suggesting that CSCs were enriched in the sphere population. Furthermore, stem cell genes such as oct4, nanog, c-myc, pax3 and sox2 are significantly upregulated in rhabdospheres which can be differentiated into multiple lineages such as adipocytes, myocytes and neuronal cells. Surprisingly, gene expression profiles indicate that rhabdospheres show more similarities with neuronal than with hematopoietic or mesenchymal stem cells. Analysis of these profiles identified the known CSC marker CD133 as one of the genes upregulated in rhabdospheres, both on RNA and protein levels. CD133+ sorted cells were subsequently shown to be more tumorigenic and more resistant to commonly used chemotherapeutics. Using a tissue microarray (TMA) of eRMS patients, we found that high expression of CD133 correlates with poor overall survival. Hence, CD133 could be a prognostic marker for eRMS. These experiments indicate that a CD133+ CSC population can be enriched from eRMS which might help to develop novel targeted therapies against this pediatric tumor.

Rhabdomyosarcoma (RMS) is the most common soft tissue tumor in childhood representing 5 to 8% of all pediatric malignancies [13]. RMS is a member of the small blue round cell tumors additionally comprised of neuroblastoma, non-Hodgkin’s lymphoma, Ewing’s sarcoma and Wilm’s tumor [14]. It occurs in most parts of the body, but more frequent sites are spaces surrounding the brain, the trunk and genitourinary tract [15]. It has been suggested that mesenchymal stem cells (MSCs) might be the origin of rhabdomyosarcomas and accordingly the origin of a potential rhabdomyosarcoma stem cell might also be a mesenchymal one [16,17]. However, some reports indicate that also neuronal cells can transform into malignant myogenic cells after activation and a large number of neuronal genes are expressed in RMS. Hence the origin of potential RMS stem cells remains to be determined [18,19].

CD133, also known as Prominin1, is a five transmembrane protein with eight potential N-glycosylation sites. It was first described in murine neuroepithelial cells and was recognized as a human hematopoietic SG marker, because hematopoietic CD34+ progenitor cells express CD133 [20]. CD133 has been suggested
as CSC marker in brain tumors [21], breast [3], colon [22], pancreatic [23], liver [24], skin [25], prostate cancers [26] and Ewing’s sarcoma [27]. Furthermore, CD133+ glioma stem cells are more resistant to chemotherapy and radiation than bulk and the CD133 negative population [8]. Moreover, CD133 downregulation induced differentiation in neuroblastoma cell lines and thus increased sensitivity to drug treatment [28]. Therefore, CD133 could by itself also represent a potential marker for targeted therapy. Nevertheless, CD133 positive CSC populations in melanoma and prostate cancer are still controversially discussed [29,30].

Here, we enriched for a CSC population in rhabdsphere cultures which are 100 fold more tumorigenic than adherent cells in xenograft experiments. This subpopulation expressed the stem cell genes oct4, nanog, c-myc and pax3 to significantly higher levels and retains the capability to differentiate into adipocytes, myocytes and neuronal cells. Furthermore, the known stem cell marker CD133 was upregulated in rhabdspheres. CD133+ cells characterize a subpopulation which is more tumorigenic and resistant to chemotherapy than the negative population. In addition, high CD133 expression in human eRMS samples correlated with a poor overall survival.

Thus, our study demonstrates for the first time that rhabdspheres can be formed from eRMS cells which are enriched in a CD133+ CSC population.

Materials and Methods

Cell culture methods

Rh36 (kindly provided by Peter Houghton (St. Jude Children’s Hospital, Memphis, TN, USA)), RD, U87MG and MRC5 (purchased from the American Type Culture collection (LGC Promochem, Molsheim Cedex, France)) and Ruch2 (established in house) were cultured in Dulbecco’s modified Eagle medium containing 10% fetal calf serum (FCS).

Sphere cultures were derived from and enriched over several passages by seeding the cell lines in a defined serum free medium (SC medium) consisting of Neurobasal medium (Invitrogen) supplemented with 10ng/ml EGF (R&D Systems), 20ng/ml b-FGF (R&D Systems) and 2x B27 (10ml; Invitrogen) [31]. Adipogenesis was induced as described [32,33]. Briefly, after preparing spheroids, cells were seeded into chamber slides and treated with or without 0.1% DMSO for 3 days. After 8 days in differentiation medium, containing 85nM insulin, 2nM triiodothyronine (T3) and 10% FCS, cells were stained with OilRedO (ThermoScientific) [34]. Neurogenesis and Myogenesis were assayed as described [35]. Briefly, cells were seeded into 6 well plates and treated with different concentrations of retinoic acid (RA; 1nM, 10nM, 300nM). After 24 days in differentiation medium containing RA and 0.5% FCS, cells were fixed in 4% paraformaldehyde (PFA) and stained for differentiation markers. Resistance to chemotherapeutics was tested by seeding 2000 cells in a 6-well plate 48 hours before treatment. The cells were treated twice a week with different concentrations of cisPlatin (Sigma; 10 μM and 50 μM) and Chlorambucil (Sigma; 6.45 μM). Twice a week, colonies were counted and documented. For visualizing the colonies, we stained them with crystal violet according to Franken, et al. [36].

Immunofluorescence, immunohistochemistry and flow cytometry/sorting

For immunofluorescence staining, cells were fixed in 4% PFA and blocked in medium containing 10% FCS and 0.5% Triton. Cells were stained over night at 4°C for CD133 (1/100) (polyclonal antibody, Abcam), GFAP (1/300) (monoclonal antibody, R&D Systems), myogenin (1/2) (F5D; monoclonal antibody, Developmental Studies Hybridoma bank) and N-CAM (1/2) (5.H11; Developmental Studies Hybridoma bank). Alexa Fluor 488 or 594 (1/200) (Invitrogen) antibodies were used as secondary antibodies. All stainings were analyzed with an Axioskop2 mot plus microscope (Zeiss). Xenograft tumors were embedded in paraffin, fixed and analyzed for H&E, Myogenin (1/20) (My4, monoclonal antibody, Novoceastra Laboratories Ltd) and desmin (1/20) (monoclonal antibody; Dabo) by immunohistochemistry. As secondary antibody a horseradish peroxidase (HRP) labeled rabbit anti-mouse antibody (Epitomics) was used. Stainings were visualized with the Refine DAB-Kit (Leica).

For flow cytometry, cells were trypsinized, washed and stained (1/10) with a fluorochrome labeled antibody (CD133/2-APC, Miltenyi). All samples were measured with a BD FACSCanto II flow cytometer (BD Bioscience) or MoFlo high speed cell sorter (DakoCytomation) and analyzed with the software FlowJo.

Molecular methods

RNA was extracted using RNAplus Mini Kits (Qiagen). Reverse transcription was carried out using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. RNA and cDNA concentrations were measured with a Nanodrop ND1000 spectrometer. Quantitative Real-Time PCR was performed using validated TaqMan Gene Expression Assays (Applied Biosystems) for POU5F1/OCT3-4 (Hs02397400_g1), NANOG (Hs023874-00_g1), SOX2 (Hs01053049_s1), CMYC (Hs00153408_m1), PAX3 (Hs00992437_m1), NMYC (Hs0032074_m1), PROM1/ CD133 (Hs01009261_m1) and GAPDH (Hs99999905_m1) as an endogenous housekeeping gene for normalization. Reactions were run using the standard conditions on an ABI 7900HT Fast Real-Time PCR machine. Relative fold difference was calculated using the −ΔΔCt method. Gene expression profiling of different RNA samples from different sphere passages (early = passage 3; intermediate = passage 5 to 7; late = passage 10) and their corresponding adherent control was performed by an Affymetrix Exonmiarray (HuEx-1.0-st-v2). The samples were analyzed with the Genespring10 and Ingenuity IPA software and compared with published data sets (hematopoietic (GSE2666), FM95 (GSE10435), embryonic skeletal myoblast (GSE3230), mesenchymal stem cells (GSE2248), embryonic stem cells (GSE9440), neuronal cells (GSE10691), glioblastoma cells and patient samples (GSE7181), neurospheres (GSE8049) and prostate cancer samples (GSE10832)). The correlation of the samples was analyzed with a script programmed in R (Functional Genomic Center Zurich).

Xenograft experiments

Xenograft experiments were approved by the veterinary office of the Canton of Zurich.

Different amounts of adherent cells and their corresponding sphere cultures were injected intra muscularly into the right leg of NOD.CB17-Prkdc<sup>scid</sup>/J (NOD/Scid) and NOD.Cg-Pkd<sup>cre</sup>/Prkdc<sup>−/+</sup>/SzJ (NSG) mice (The Jackson Laboratory) and tumor size was determined every 2 to 3 days by measuring two diameters (d<sub>1</sub> and d<sub>2</sub>) in right angles of both legs with a calliper. Tumor volumes were calculated using the following formula V = 4/3 π ½(d<sub>1</sub> + d<sub>2</sub>)³/³−4/3 ½(d<sub>1</sub> + d<sub>2</sub>)²/²d<sub>1</sub>.

Patient characteristics

76 eRMS patients, 43 male and 33 female patients, were included from the CWS95 study. The age of the patients at diagnosis varied from a few months to 22 years.
Figure 1. Cancer stem-like cells are enriched in Rhabdospheres. A) B) Embryonal rhabdomyosarcoma (eRMS) cell lines (RD, Rh36 and Ruch2) were cultured in stem cell medium (SC-medium) over several passages. A glioblastoma (U87MG) and a fibroblast (MRC5) cell line were used as controls. A) Representative phase contrast pictures of cultured RD, Ruch2 and Rh36 sphere cultures (400× magnification). B) Subpopulation taking of spheres containing 20,000 cells.
enrichment over several passages (x-axis) was estimated by counting the obtained spheres per cell (y-axis). C), D) Limited dilution (10⁵, 10⁴ and 10³) of adherent versus sphere cells in vivo. Cells were intramuscularly (i.m.) injected into NOD/Scid (n = 6) and NSG mice (n = 3) (D) at the indicated numbers and tumor growth (y-axis; tumor volume in mm³) was measured over time (x-axis). E) Immunohistochemical (IHC) stainings of xenograft tumor sections on a xenograft tissue microarray (TMA). Adherent and sphere cells were used as controls on the TMA. The TMA was stained for H&E and RMS markers (desmin and myogenin). Representative IHC stainings are shown (400× magnification). The small inserts represent magnifications of positively stained cells.

doi:10.1371/journal.pone.0019506.g001

Statistical analysis

For in vitro experiments, Student’s t test was used on triplicates. P values of less than 0.05 were considered significant.

Results

Rhabdospheres are enriched with cancer stem-like cells

To determine whether RMS cells might contain a subpopulation of CSC cells, we attempted to grow embryonal rhabdomyosarcoma (eRMS) cell lines (RD, Rh36 and Ruch2) as rhabdomyosarcoma spheres (short rhabdospheres) in stem cell medium (SC-medium). A glioblastoma cell line (U87MG) and fibroblast cells (MRC5) were used as positive and negative controls, respectively. Three eRMS cell lines (RD, Rh36 and Ruch2) formed rhabdospheres under these conditions over several passages (Figure 1A). To test, whether sphere cells could be serially enriched, we seeded 20000 sphere cells over several passages into the SC-medium and determined the number of spheres at each passage (Figure 1B). Compared to the positive control U87MG sphere cultures which showed the highest enrichment over 10 passages (up to 1750 spheres per 20000 cells), 1500 spheres were counted for RD cultures after 10 passages, while Ruch2 and Rh36 sphere cultures could also be enriched albeit to a lesser extent (600 counted spheres) indicating that a subpopulation of cells with self renewal property can be enriched from three different eRMS cell lines. To investigate whether this self renewing subpopulation is more tumorigenic than the adherent population, we injected different numbers of RD cells and their corresponding sphere cultures (10⁵, 10⁴ and 10³) intramuscularly (i.m.) into the right leg of NOD/Scid mice (n = 6) and measured tumor growth over several weeks (Figure 1C). Xenograft tumors from sphere cultures started to grow around day 40 after injection, compared to adherent cells where we detected the earliest tumor growth around day 80 post injection. Moreover, tumor growth was observed when we injected 100 fold less sphere cells (10³), whereas no tumor growth was seen using the same number of adherent cells. 125 days after injection, in two out of six NOD/Scid mice injected with 10³ adherent cells, a small tumor was seen, while we detected tumors in every mouse injected with 10⁴ sphere cells already after 60 days. These results were subsequently confirmed in a second mouse strain, namely NSG mice, where tumor growth was observed with 10³, 10⁴ and 10⁵ (one out of three mice) injected sphere cells, but only with 10⁶ adherent cells (Figure 1D). Therefore, in both mouse models from cultures spheres or sphere cultures are more tumorigenic and fewer cells are needed for tumor growth compared to adherent cells. To demonstrate that all xenograft tumors were indeed RMS tumors, we collected tumor samples and constructed a xenograft tissue microarray (TMA) with adherent RD cells and corresponding sphere cultures as controls. Stainings of the TMA with RMS markers, myogenin and desmin, was positive in both adherent cells and spheres (Figure 1E) which were negative for markers of other small blue round cell tumors (CD45, CD99, cytokeratin (CK), S100b, Synaptosin, smooth muscle actin (SMA) and WT1; data not shown). Furthermore, all xenograft tumors displayed typical RMS hallmarks such as multinucleated cells and positive stainings for desmin and myogenin, irrespective of the mouse strain they were grown in. These results confirmed that all xenograft tumors represented RMS tumors with similar features. We conclude from these experiments that a subpopulation of RMS cells can be enriched in sphere cultures over several passages which is more tumorigenic in vivo and therefore could represent a potential CSC population.

Sphere cultures have stem cell characteristics

To further substantiate the notion that rhabdospheres are enriched for CSCs, we quantified the expression levels of several known SC genes like oct4, nanog, c-myc, sox2 and pax3 with real-time PCR in different passages of sphere cultures (passages 3, 7, 10) compared to adherent cells. While oct4 and pax3 showed the highest upregulation in RD sphere cultures (P < 0.0001), also c-myc (P = 0.0016), sox2 (P = 0.0068) and nanog (P = 0.0028) were significantly upregulated (Figure 2A). Similar results were obtained with Rh36 cells with the exception of pax3 and c-myc which did not change significantly (Figure 2B). This could be due to already high endogenous expression levels in the adherent Rh36 cell line when compared to RD adherent cells (data not shown). Therefore, we selected RD cells for all subsequent experiments.

It has been shown that cells with multilineage differentiation potential can differentiate into neuronal cells, myocytes and adipocytes after treatment with dimethylsulfoxid (DMSO) or retinoic acid (RA) [32,35,37,38]. On that basis, we next assessed to which extent RD cells can be differentiated towards these lineages. First, we treated adherent and sphere cultures with different concentrations of RA (1nM, 10nM and 300nM). After 24 days, cells were stained with myogenic (myogenin, N-CAM) and neuronal markers (GFAP, N-CAM) (Figure 2C, D). Although adherent cells expressed low levels of myogenin (3.6% and 11.5%) after treatment (Figure 2C), sphere cultures showed much stronger upregulation of myogenin positivity (~50%) (Figure 2D). The highest expression of N-CAM (52%) was detectable after treatment with 10nM RA. While both spheres and adherent cells were negative for myogenin when treated with 300nM RA, we observed positive stainings for N-CAM and GFAP (9,4%), indicative of neuronal differentiation, only in sphere cultures (Figure 2D) and not in adherent cells (Figure 2C). In contrast, no GFAP positive cell was found after 1 and 10nM RA treatment (data not shown). Untreated controls were negative for all markers analyzed (data not shown).

To differentiate cells towards adipocytes, we treated spheroids from both adherent and sphere cells for 3 days with DMSO. After subsequent cultivation in appropriate differentiation medium for 8 days, around 5% of DMSO treated adherent cells were positive for fatty vacuoles (Figure 2E). However, sphere cultures had positively stained fatty vacuoles in up to 90% (mean 73.75%) of the cells when treated with DMSO (Figure 2E).

In conclusion, sphere cultures had a significantly increased expression level of stem cell genes and regained the capability to differentiate towards neurogenic, myogenic and adipogenic lineages with appropriate stimuli. These results indicate that stem-like cells are enriched in rhabdospheres.
CD133 is upregulated in sphere cultures

To characterize sphere cultures in further detail and to identify marker proteins specifically up- or downregulated, a gene expression profiling was performed with a human exomicroarray (HuEx-1_0-st-v2) for both RD and Rh36 cells and three different passages (early, intermediate and late) of their corresponding spheres.

In Figure 3A, a heat map of all samples is shown which revealed that RD and Rh36 cells cluster with their corresponding sphere cultures indicating that both cell lines are more different from each other than their different passages. Nevertheless, in total 2217 genes (upregulated 1568 genes, downregulated 649 genes) are differentially expressed in RD spheres compared to adherent cells with a fold change of at least two. To restrict the number of genes and to find potential markers characterizing the rhabdospheres, a metanalysis with different microarray samples publicly available (hematopoietic, FM95, embryonic skeletal myoblast, mesenchymal stem cells, embryonic stem cells, neuronal cells, glialbloma cells and patient samples, neurospheres and prostate cancer samples) was implemented. All RMS samples, both adherent and rhabdospheres (red (RD) and pink (Rh36)), clustered together with neuronal and glialbloma cells and their spheres, and patient samples (depicted in green) (Figure 3B). Due to this observation, we searched for genes commonly up- or downregulated in RD and glialbloma sphere cultures compared to their corresponding adherent cells with a fold change of at least two (Table 1). 31 genes were identified and further subgrouped according to their subcellular localization; membrane (8 genes), secreted (1 gene), endoplasmatic reticulum ER membrane (1 gene), golgi apparatus (1 gene), cytoplasm (12 genes) and nucleus (8 genes). In addition, 12 genes are commonly downregulated (membrane (6), secreted (2), cytoplasm (4)). To be able to identify and isolate a putative CSC population, we were interested mainly in membrane proteins of which we identified 14 genes. One obvious candidate gene in this list was CD133 or Prominin1 which is a well described SC and CSC marker. Therefore, we validated CD133 as a potential marker of rhabdospheres at the expression level by performing real-time PCR (Figure 3C). In sphere cultures of both RD and Rh36, CD133 expression was indeed significantly upregulated. To verify these results on protein level, RD cells and spheres were stained for CD133 and analyzed by flow cytometry (Figure 3D), fluorescence microscopy (Figure 3E) and western blotting (Figure 3F). In all experiments, CD133 was upregulated in rhabdospheres compared to adherent cells on protein level.

These experiments suggest that CD133+ cells, a known CSC marker, are enriched in rhabdospheres and CD133 might be a potential CSC marker in RMS.

CD133+ RMS cells are more chemoresistant and tumorigenic

To verify whether a CD133+ subpopulation is more tumorigenic and resistant to commonly used chemotherapeutics in RMS, we sorted RD cells for CD133 positive and negative (CD133+, CD133−) populations (Figure 4A) and performed limiting dilutions by orthotopical injections into NOD/Scid mice using adherent RD cells and unsorted bulk RD cells as controls. In contrast to the control where the highest number of cells injected (105 cells) developed a tumor, mice injected with CD133+ cells (105 – 106) did not develop any tumor after 140 days. In contrast, we could detect at least one tumor in the CD133+ injected mice in three out of four dilutions (Figure 4B). To demonstrate that these tumors are indeed RMS tumors, we analyzed them by immunohistochemistry using known RMS markers as described before. All tumors were positive for desmin and myogenin and histologically identical with RMS tumors (Figure 4C). To investigate potential resistance to commonly used chemotherapeutics, we seeded sorted cells at low density 48 hours before starting treatment with cisPlatin and Chlorambucil. Cells were treated twice a week and colonies obtained were counted after staining with crystal violet (Figure 4D). CD133+ sorted RD cells were more resistant to treatment and formed viable colonies which developed significantly less in the CD133− population.

Therefore, rhabdospheres are enriched for a CD133+ population being more tumorigenic and resistant to cisPlatin and Chlorambucil.

High expression of CD133 correlates with poor overall survival

Finally, we investigated whether a CD133+ subpopulation is also present in human patient material. To this end, we stained a human RMS TMA, first described by Wachtel [19], for CD133. For quantification, we scored for two variables, namely intensity of staining and number of positive cells. The added scores were used to classify the tumors as having negative, low, middle or high expression. ERMS patients showing no or low to intermediate CD133 expression showed an overall survival around 75% which is comparable with the survival rate of translocation negative RMS patients [39]. In contrast, patients with high expression of CD133 had a clearly worse survival (less than 50%, p = 0.0272, Figure 5A). Representative tumor sections of high, intermediate and low CD133 stainings are shown in Figure 5B.

These results therefore indicate that CD133 is a potential CSC marker in cRMS that might identify cRMS patients with a poor outcome.

Discussion

Due to a better understanding of tumor organization, new treatment approaches that target directly a CSC population now seem possible [22]. It has been reported that not only leukemia [2] and carcinomas [3] have a subpopulation of cells with self renewal properties [27], but also some sarcomas such as Ewing’s sarcoma might follow the cancer stem cell model [1]. For the most common sarcoma in childhood, RMS, no clear subpopulation has been identified until now [16,17]. Therefore, we used a functional approach to investigate whether rhabdomyosarcoma tumors might...
have a subpopulation enriched in CSCs and are hierarchically organized.

We first adopted a sphere forming assay to enrich a subpopulation with stem cell properties in vitro. Testing different conditions of growth factor concentrations and media, sphere formation over several passages could be observed only in one condition which was described previously as a neuronal stem cell medium [31]. Several lines of evidence then indicate that these rhabdospheres are enriched for stem-like cells. First, limiting dilution in two different immunosuppressed mouse strains indicate

| Table 1. List of genes up- or downregulated at least two fold in RD rhabdospheres. |
|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| Localization                    | Chosen Gene IDs | Gene Symbol | Chosen Gene IDs | Gene Symbol |
| Membrane                        | 2535            | FZD2        | 2674            | GFRA1        |
|                                 | 7976            | FZD3        | 3778            | KCNMA1       |
|                                 | 8842            | PROM1       | 4907            | NTSE         |
|                                 | 23554           | TSPAN12     | 7010            | TEK          |
|                                 | 51678           | MPP6        | 7057            | THBS1        |
|                                 | 55704           | CCDC88A     | 23768           | FLRT2        |
|                                 | 57633           | LRRN1       |                 |              |
| Secreted                        | 255743          | NPNT        | 4015            | LOX          |
| Golgi apparatus                 | 22836           | RHOBTB3     |                 |              |
| ER membrane                     | 80055           | PGAP1       |                 |              |
| Cytoplasm                       | 2037            | EPB41L2     | 3433            | IFIT2        |
|                                 | 3157            | HMGC51      | 3437            | IFIT3        |
|                                 | 4133            | MAP2        | 9060            | PAPSS2       |
|                                 | 6860            | SYT4        | 10231           | RCAN2        |
|                                 | 9315            | C5orf13     |                 |              |
|                                 | 9456            | HOMER1      |                 |              |
|                                 | 9735            | KNTC1       |                 |              |
|                                 | 54874           | FNBP1L      |                 |              |
|                                 | 55792           | PCID2       |                 |              |
|                                 | 56992           | KIF15       |                 |              |
|                                 | 91057           | CCDC34      |                 |              |
|                                 | 113263          | GLCCI1      |                 |              |
| Nucleus                         | 7552            | ZNF711      |                 |              |
|                                 | 9735            | KNTC1       |                 |              |
|                                 | 10926           | DBF4        |                 |              |
|                                 | 55769           | ZNF83       |                 |              |
|                                 | 64105           | CENPK       |                 |              |
|                                 | 81931           | ZNF93       |                 |              |
|                                 | 84230           | ANKRST32    |                 |              |
|                                 | 90317           | ZNF616      |                 |              |
|                                 | 151648          | SGOL1       |                 |              |

doi:10.1371/journal.pone.0019506.t001
that the rhabdosphere population is at least 100 fold more tumorigenic than adherent cells. In contrast, culturing cell lines representing the alveolar subtype of RMS in the same stem cell medium leads to formation of spheres which surprisingly were not tumorigenic after injection into immunosuppressed mice (data not shown). Hence, it seems unlikely that media conditions themselves were responsible for induction of the observed phenotypes and rather selection of a preexisting subpopulation was occurring specifically in eRMS. Second, our data analyzing expression levels of stem cell genes in rhabdospheres compared to adherent cells demonstrate that the stem cell genes oct4, nanog, sox2, c-myc as well as pax3, are significantly upregulated. While sox2, nanog, and oct4 are required for induction of the pluripotent stem cell phenotype, c-myc expression also correlates with tumor formation and upregulation of this oncogene could trigger the higher tumor initiating potential [40]. Pax3 is a known developmental marker expressed during muscle and brain development, repressed in adult tissue and connected to tumor formation and a poor overall survival [41,42,43]. As an additional hallmark of cancer stem cells [1], we investigated whether rhabdospheres have the potential to differentiate into multiple lineages. Indeed, rhabdospheres treated with DMSO and RA, respectively, differentiate towards adipogenic, myogenic and neurogenic lineages similar to what has been observed in cells with multilineage differentiation potential such as embryonal carcinoma cells [32,33,35]. These data support the concept that rhabdospheres contain cells with stem-like features and that RMS tumors are hierarchical organized [1].
Previous studies have suggested that mesenchymal stem cells could be the origin of RMS [17,44,45]. In contrast, our meta-analysis of exon microarray data with published data sets revealed that RMS samples had an expression profile more similar to neuronal cells and patients than mesenchymal stem cells. Furthermore, expression profiles also detected a large number of neuronal genes being expressed in RMS biopsies [19,46,47,48] such as pax3 which is crucial for the development of both the myogenic and neuronal lineage [42]. Interestingly, it has been demonstrated earlier that a population of myogenic, myf-5 positive cells can be derived from neural tube during mouse development [49]. These myf-5 positive cells co-express both neuronal and muscle markers, raising the intriguing possibility that the cell of origin of our CSC population could also be a multipotential stem cell derived from cells in the neuronal compartment. In support of this, it has also been described that neuronal stem cells can differentiate into malignant muscle cells after activation [18]. However, this issue needs to be addressed further in the future.

Previous studies have shown that CD133 marks hematopoietic stem cells [20] and cancer stem cells [29], in particular neuronal and mesenchymal CSCs [21]. Moreover, a CD133+ population was identified as a CSC population in sarcomas such as Ewing’s sarcoma [27] and osteosarcoma [50] which was more resistant to chemotherapy and radiation [7,8,9,10,11]. It was therefore not surprising that CD133 emerged as a marker for RMS CSC in our study as well. Interestingly, also the fraction of CD133+ cells in both Ewing’s sarcoma and RMS seem to be similar. It has been reported that expression of FGFR3 might mark a tumorigenic subpopulation in RMS. However, we did not find an increase in mRNA expression of this receptor in rhabdospheres (Figure S1). The same report also found that CD133 positive cells were not more tumorigenic than the negative population. However, the discrepancy with our study might be explained by the different CD133 epitopes that were used in the two studies. Here, using CD133 as a marker to sort cells which were then injected orthotopically into mice without prior cultivation in stem cell media, we readily detected tumor growth at lower cell numbers in CD133 positive versus CD133 negative cells. Indeed, in the CD133+ injected group one mouse at every dilution developed a RMS tumor which was not observed in the CD133+ population. The relatively low tumorigenicity detected in the sorted population in general is likely due to impaired viability of the cells by the sorting procedure. Interestingly, CD133+ sorted cells were also more resistant to cisPlatin and Chlorambucil treatment suggesting that anti-apoptotic or mismatch repair proteins are active. Indeed, we observed upregulation of several transcripts encoding mismatch repair proteins in rhabdospheres (data not shown).

Finally, we stained a human RMS tissue microarray (TMA) [19] for CD133 to demonstrate that a CD133+ population is also present in human tumor biopsies. Patients with high positivity for CD133 were found to have the worst overall survival, which could be explained by a higher recurrence. However, in a multivariate analysis using a cox regression model, we were not able to demonstrate that CD133 is an independent prognostic marker for RMS since the number of patients in this group was too low. More patients will have to be included therefore in a future study. Nevertheless, CD133 might represent the first candidate marker to identify RMS patients with poor survival and might be used to stratify patients in the future.

Conclusion
Overall, our results demonstrate that cells with self renewal property that can drive tumorigenesis and have the potential to differentiate into multiple lineages are enriched in rhabdospheres. With CD133, we identified an already known CSC marker in an additional sarcoma [27,50] whose expression also correlated with a poor prognosis in eRMS patients. Further characterization of this CD133 positive CSC population might lead to a better understanding of the development of RMS. It now seems possible to screen directly for therapeutically active substances targeting the CSC subpopulation in eRMS to further advance treatment of this childhood sarcoma.

Supporting Information
Figure S1 Prominin and fibroblast growth factor receptor (FGFR) expression in adherent and sphere cells. A) Prominin1 and Prominin2 gene expression profiles in adherent and sphere cells analyzed by Genespring10 software. Intensity values were normalized to adherent cells. B) FGFR1, FGFR2, FGFR3 and FGFR4 gene expression profiles in adherent and sphere cells. Intensity values were normalized to adherent cells. C) Quantitative Real-time PCR with primers for FGFR3 (Hs00997400_g1) and for GAPDH was done with cDNA of adherent cells and three different passages of sphere cells. Quantitative results are indicated in arbitrary units (AU). FGFR3 was not differentially expressed in sphere cells compared to adherent cells.

References
1. Lobo NA, Shimono Y, Qian D, Clarke MF (2007) The biology of cancer stem cells. Annu Rev Cell Dev Biol 23: 675-699.
2. Bonten D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730-737.
3. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100: 13726–13731.
4. Singh S, Dirks PB (2007) Brain tumor stem cells: identification and concepts. Neurosurg Clin N Am 18: 31–38, viii.
5. Todaro M, Francipane MG, Medema JP, Stassi G (2010) Colon cancer stem cells:承诺s of targeted therapy. Gastroenterology 138: 2151–2162.
6. Boiko AD, Razorenova OV, van de Rijn M, Swetter SM, Johnson DL, et al. (2010) Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. Nature 466: 133–137.
7. Bertolini G, Roz I, Perego P, Tortoreto M, Fontanella E, et al. (2009) Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. Proc Natl Acad Sci U S A 106: 16281–16286.
8. Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. Nat Rev Cancer 5: 273–284.
9. Jiang X, Gwyse Y, Russell D, Gao C, Douglas D, et al. (2010) CD133 expression in chemo-resistant Ewing sarcoma cells. BMC Cancer 10: 116.
10. Todaro M, Perez Alea M, Scopelliti A, Medema JP, Stassi G (2008) IL-4-mediated drug resistance in colon cancer stem cells. Cell Cycle 7: 309–313.
11. Wang J, Wakerman TP, Lahia JD, Hjelmeland AB, Wang XF, et al. (2010) notch promotes radiosensitivity of glioma stem cells. Stem Cells 28: 17–28.
12. Morrison SJ, Spradling AC (2008) Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132: 598–611.
13. De Giovanni C, Landuzi L, Nicoletti G, Lollini PL, Nanni P (2009) Molecular and cellular biology of rhabdomyosarcoma. Future Oncol 5: 1449–1475.
14. Gregorio A, Corrias MV, Castriconi R, Doudero A, Mosconi M, et al. (2008) Small round blue cell tumours: diagnostic and prognostic usefulness of the expression of E7-H3 surface molecule. Histopathology 53: 73–80.
15. McDowell HP (2003) Update on childhood rhabdomyosarcoma. Arch Dis Child 88: 354–357.
16. Hirono J, Sato S, Sasaki H, Nakagawara A, Naka S, et al. (2009) Identification of EWS-FLI1 translocation in a human rhabdomyosarcoma. Hum Pathol 40: 1950–1955.
17. Wachtel M, Runge T, Leuschner I, Stegmaier S, Treuner J, et al. (2004) Subtype and prognostic classification of rhabdomyosarcoma by immunohistochemistry. J Clin Oncol 22: 816–822.
18. Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogasawara M, et al. (1997) CD133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90: 5002–5012.
19. Shu Q, Wong KK, Su JM, Adesina AM, Yu LT, et al. (2008) Direct orthotopic transplantation of fresh surgical specimen preserves CD133+ tumor cells in clinically relevant mouse models of medulloblastoma and glioma. Stem Cells 26: 1414–1424.
20. Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, et al. (2007) Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-2. Cell Stem Cell 1: 313–323.
21. Ding W, Mouzaki M, You H, Laird JC, Mato J, et al. (2009) CD133 suppresses neuroblastoma cell differentiation via signal pathway modification. Oncogene 28: 1275–1279.
22. Robson EJ, He SJ, Eccles MR (2006) A PANorama of PAX genes in cancer and cellular biology of rhabdomyosarcoma. Trends Cardiovasc Med 9: 139–143.
23. Astolfi A, De Giovanni C, Landuzzi L, Nicoletti G, Ricci C, et al. (2001) Identification of new genes related to the myogenic differentiation arrest of human rhabdomyosarcoma cells. Gene 274: 139–149.
24. Miki J, Furusato B, Li H, Gu Y, Takahashi H, et al. (2007) Identification of cancer stem cells and in prostate cancer specimens. Cancer Res 67: 3153–3161.
25. Monzani E, Facchetti F, Galmuzi E, Corini E, Benetti A, et al. (2007) Melanoma contains CD133+ and ABCG2 positive cells with enhanced tumorigenic potential. Eur J Cancer 43: 935–946.
26. Mikl J, Furusato B, Li H, Gu Y, Takahashi H, et al. (2007) Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary normal mulligian and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. Cancer Res 67: 3153–3161.
27. Suwa ML, Ruggi N, Stehle JC, Baumann K, Terrier S, et al. (2009) Identification of cancer stem cells in Ewing’s sarcoma. Cancer Res 69: 1776–1781.
28. Takenobu H, Shimozato O, Nakamura T, Ouchi H, Yamaguchi Y, et al. (2011) CD133 suppresses neuroblastoma cell differentiation via signal pathway modification. Oncogene 30: 97–103.
29. Bidlingmaier S, Zhu X, Liu B (2008) The utility and limitations of glycyslated human CD133 epitopes in defining cancer stem cells. J Mol Med 86: 1025–1032.
30. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, et al. (2008) Efficient tumour formation by single human melanoma cells. Nature 456: 593–598.
31. Babu H, Cheung G, Kettenmann H, Palmer TD, Kempermann G (2007) Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons. PLoS ONE 2: e388.
32. Bouchard F, Pajunen J (2009) Skeletal and cardiac myogenesis accompany adipo genesis in P19 embryonal stem cells. Stem Cells Dev 18: 1023–1032.
33. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, et al. (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143–147.
34. Scientific T (2009) Human Mesenchymal Stem Cell Protocol: Oil Red O Staining of Adipogenic Cultures. Thermo Scientific. https://www.thermo.com/eThermo/CMA/PDFs/Various/File_4336.pdf (13.01.2011).
35. Angello JC, Stern EM, Hasekcha SD (1997) P19 embryonal carcinoma cells: a model system for studying neural tube induction of skeletal myogenesis. Dev Biol 192: 93–98.
36. Franken NA, Rodermond HM, Staj J, Haverman J, van Bree C (2006) Clonogenic assay of cells in vitro. Nat Protoc 1: 2313–2319.
37. Mcllinnery MW (1993) P19 embryonal carcinoma cells. Int J Dev Biol 37: 1429–1437.
38. Cordon-Cardo G, Matsushita I, Ziman M (2009) Alveolar rhabdomyosarcoma: is the cell of origin a mesenchymal stem cell? Cancer Lett 279: 126–136.
39. Davicioni E, Finckenstein FG, Shahbazian V, Buckley JD, Trieste TJ, et al. (2006) Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas. Cancer Res 66: 6936–6946.
40. Larsson LG, Henriksson MA (2010) The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. Exp Cell Res 316: 1429–1437.
41. Muratovska A, Zhou C, He S, Goodyer P, Eccles MR (2003) Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. Oncogene 22: 7899–7907.
42. Davicioni E, Finckenstein FG, Shahbazian V, Buckley JD, Trieste TJ, et al. (2006) Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas. Cancer Res 66: 6936–6946.
43. Davicioni E, Finckenstein FG, Shahbazian V, Buckley JD, Trieste TJ, et al. (2006) Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas. Cancer Res 66: 6936–6946.
44. Larsson LG, Henriksson MA (2010) The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. Exp Cell Res 316: 1429–1437.
45. Muratovska A, Zhou C, He S, Goodyer P, Eccles MR (2003) Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. Oncogene 22: 7899–7907.
46. Davicioni E, Finckenstein FG, Shahbazian V, Buckley JD, Trieste TJ, et al. (2006) Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas. Cancer Res 66: 6936–6946.
47. Larsson LG, Henriksson MA (2010) The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. Exp Cell Res 316: 1429–1437.
48. Davicioni E, Finckenstein FG, Shahbazian V, Buckley JD, Trieste TJ, et al. (2006) Identification of a PAX-FKHR gene expression signature that defines molecular classes and determines the prognosis of alveolar rhabdomyosarcomas. Cancer Res 66: 6936–6946.