# Identifying the functional relevance of CD271 and CD133 in medulloblastoma tumor formation

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Summary: Medulloblastoma (MB) is the most common primary malignant pediatric brain tumor. The relationship between stem cell function and invasiveness has not been investigated in MB. We have dissected MB heterogeneity and compared the capacity for self-renewal and invasion. Our laboratory has identified a combination of 3 cell surface markers that can be used to select for cells with a high self renewal capacity or high invasive capacity. CD271 and CD24 cell surface markers were shown to select for cells that exhibited higher self-renewal capacity whereas CD133 selected for higher invasive capacity. The next step was to confirm these findings *in vivo.* A mouse model was developed and optimized in the laboratory using NOD SCID mice. Initial data has shown mice injected with higher unsorted cell numbers develop tumor symptoms earlier than mice injected with less cells. Additionally, CD271+/133- cells appear to develop tumors at a faster rate than CD271-/CD133- cells.

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## Abstract

Medulloblastoma (MB) is the most common primary malignant pediatric brain tumor. In the last decade, cancer research has seen a large shift towards elucidating the cellular characteristics of cancer stem cells. These cells are thought to initiate and propagate tumors. Although research into CSCs and MB is widely done, the relationship between stem cell function and invasiveness has not been investigated. We have dissected MB heterogeneity and compared the sub-clone capacity for self-renewal and invasion. Our laboratory has identified a combination of 3 cell surface markers that can be used to select for cells with a higher self renewal or invasive capacity. CD271 and CD24 cell surface markers were shown to select for cells that exhibited higher self-renewal capacity whereas CD133 selected for higher invasive capacity. The next step was to confirm these findings *in vivo.* A mouse model was developed and optimized in the laboratory using NOD SCID mice. Intracerebral injections of unsorted Daoy cells using a serial dilution, as well as sorted Daoy cells using CD271+/CD133- and CD271-/CD133- were performed. Initial data has shown mice injected with higher unsorted cell numbers develop tumor symptoms earlier than mice injected with less cells. Additionally, CD271+/133- cells appear to develop tumors at a faster rate than CD271-/CD133- cells. Additional experiments will be done in the future, including serial dilutions of all three cellular marker combinations, and cellular injections with high vs low self-newal capacity. The research has the potential to lead to selective targeting of cellular markers and pathways and a more favorable clinical outcome for MB patients down the road.

## Introduction

Medulloblastoma (MB) is the most common form of primary malignant childhood brain cancer (Louis *et. al.* 2007 and Fastro, M.M. *et. al.* 2011). MB is classified as a Grade IV tumor by the World Health Organization (Louis *et. al.* 2007). There is an annual incidence rate of 0.5 per 100,000 children below the age of 15, which a higher (65%) predominance in males (Louis *et. al.* 2007). It is a malignant and invasive embryonal tumor of the cerebellum with a tendency to spread via the cerebral spinal fluid (Louis *et. al.* 2007). Due to its aggressive nature and propensity to spread, it is difficult to treat. MB has a high rate of morbidity due to the effect of chemotherapy and radiation on the children's developing nervous system. Therefore, understanding the biological mechanisms of propagation, metastasis and invasive capacity will be essential for development of knew therapies to treat this cancer.

Over the last decade, a lot of emphasis has been placed on cancer stem cell populations in both hematological and solid tumors. The currently accepted theory of cancer stem cells (CSC) postulates that there is a small subset of tumor cells that are stem cells. The developmental origin of these cancer stem cells, and the events leading up to tumor formation are not yet fully understood (Werbowetski-Ogilvie, T.E. *et. al.* 2012). CSCs possess the ability to self-renew, undergo multilineage differentiation and to survive adverse tissue microenvironments (Pang, et al. 2010). Self renewal is the ability to indefinitely maintain an undifferentiated state. The rest of the tumor is composed of rapidly proliferating cells and differentiated cells (Clevers, 2011). The stem

cells are thought to be responsible for propagating the tumor, and are therefore known as a tumor propagating cells (TPC). Originally discovered in leukemia, several solid-state tumors have been shown to contain CSCs including breast, colon and brain cancer (Singh et al, 2004) and (Pang et al. 2010).

Brain tumor propagating cells and highly invasive cells share certain important characteristics including increased resistance to chemotherapy and radiation (Singh, A., and Settleman, J. 2010). It is possible that both cell types are molecularly similar. Because of this, current treatments may not be selectively destroying TPCs within the tumor, which would explain why tumors can be eradicated beyond detection, but recur years later. It is possible these CSCs are protected against chemical and radiological insult the same way normal stem cells are, through quiescence, expression of ABC drug pumps, high expression of antiapoptotic proteins and resistance to DNA damage (Clevers, 2011). This phenomenon however does not hold up for all types of cancer stem cells and early undifferentiated tumor initiating cells, such as seen in testicular cancer (Clevers, 2011). More research is needed to determine if cellular markers of CSCs will correlate with therapy and clinical outcome. Enrichment of the TPCs was shown recently in colon cancer tumors with chemotherapy agents 5-fluorouracil by Pang, et al. (Pang, R. 2010). This can also explain how cancer is capable of spreading to other organs in the body, evading chemotherapy and thriving in its new environment.

Evidence for a BTPC marker that is reliable for selecting invasive or metastatic activity is somewhat two sided. Recent work has shown that CD133- cells gives rise to a more invasive and aggressive behavior than the CD133+ cells associated with higher self renewal function (Joo, K.M. *et. al.* 2008 and Chen, R. *et al.* 2010). Chen et al. also showed that CD133- cells give rise to both CD133+ and CD133- cells. On the other hand, other recent research has shown that invasive and core/stationary glioblastoma multiforme (GBM) cells exhibit self-renewal capacity and stem cell markers, however the invasive cells exhibit higher stem cell like properties *(*Molina, J.R. *et. al.* 2010*).* Despite this fact, most work conducted on brain tumor propagating cells (BTPCs) and invasiveness has been mutually exclusive.

The previous work conducted in our laboratory has involved evaluating the association between BTPCs and highly invasive MB cells by deconstructing heterogeneity within the MB cultures. Three cell surface markers have been identified that enable us to select for cells that exhibit higher self-renewal capacity or invasiveness. It was found that cells that exhibit higher CD271/CD24 with lower CD133 levels have a higher self-renewal capacity, whereas cells with lower CD271/CD24 and higher CD133 select for cells with higher invasion capacity. It was also found that the cells with a higher CD271/CD24 level were more primitive and exhibited a less differentiated phenotype. From the collection of findings, our laboratory proposed a model whereby a more primitive, self-renewing CD271/CD24 high, CD133 low cell in the MB tumor helps sustain tumor growth. Once a tumor cell has committed itself to differentiation and migration, the cell up regulates CD133 while down regulating CD271/CD24.

The next step is to establish these findings in vivo. No previous *in vivo* work has been conducted in the laboratory, so it was important to establish a working animal model.

The gold standard for CSCs is their capacity to initiate in vivo tumors that contain all the differentiated cellular populations of the primary tumor, and secondly can be serially re-transplanted in vivo to form additional tumors indicating self renewal capacity (Baiocchi, *et. al*.). We did not do this in our current *in* vivo experiments however will be using this method in further studies. Currently, the standard has been to initiate tumors in immunodeficient mice based on CSCs. Cellular markers were initially demonstrated to mark CSCs in solid tumors in breast cancer using CD44+/CD24- cells (Baiocchi, et. al.). Glioblastoma CSCs with the ability to induce tumors were separated based on CD133 (Baiocchi, *et. al.).* We have shown that in vitro, MB CSCs with a high self renewal capacity can be selected for by increased CD271/CD24 levels. We expect the current in vivo work to confirm this, however the microenvironment and level of mouse immunodeficiency in the NOD SCID mice used strongly affects CSC growth and development.

Here, we dissected MB heterogeneity, and directly compared invasion and self-renewal in MB sub-clones. We were able to identify 3 novel cell surface markers, CD271, CD24 and CD133 that are differentially expressed in high vs. low invasive capacity tumorspheres. CD271 and CD24 both selected for cells that displayed a higher capacity for self-renewal whereas CD133 selected for cells that displayed a higher invasive capacity. In order to confirm these findings, we needed to develop an in vitro model with the laboratory. Initial experiments were performed to optimize the animal work within the lab, and at the same time set up larger more complex experiments in the future. Currently, we have two initial experiments ongoing, a set of mice injected with unsorted Daoy sub-clones with a limited dilution of cells between 200,000 and 10,000. Secondly, we have mice injected with sorted CD271+/CD133- and CD271-/CD133- cells in order to look at the difference in tumor initiating potential. A third experiment has just been started looking at the difference between injection of cells with high vs. low self-renewal capacity. Mice injected with a higher cell number develop tumor symptoms earlier than mice injected with a lower cell number (Figure 9). More specifically, mice injected with CD271+/CD133- develop tumor symptoms earlier than the mice injected with CD271+/CD133- cells (Figure 10). This data is incomplete however and more time is needed to confirm these findings as more mice develop tumors.

## Methods

### Migration vs core/stationary cell dissections

Daoy human medulloblastoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured on adherent plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Ottawa, ON, Canada) to 100% confluence. This medium is collectively known as brain tumor medium (BTM). Cells were removed from plate using Accutase (Invitrogen, Burlington, ON, Canada), spun down, and resuspended in BTM to a volume of 25,000 cells per 20 μl. Hanging drops were made by placing 20 μl drops (25,000 cells) on the roof of a cell culture plate and incubated at 37°C, 5% $CO_2$ for three days. The resultant spheres that formed after three days of incubation were transferred in to a

12 well plate containing BTM, three spheres per well. The spheres were allowed to attach and migrate along the bottom of the plate for two days in the incubator. After two days, the core cells were dissected from the migratory cells. This was done under a dissecting microscope using a 20 μl pipette. The pipette tip was used to cut around the core cell mass, and the mass was then removed. After all core samples were collected, the remaining migratory cells were dissociated and collected from the plate using Accutase.

**Invasion Assays**

To assess invasiveness, a hanging drop collagen invasion assay was used. DAOY medulloblastoma cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) on adherent plates to 100% confluence. Cells were dissociated from the plate and resuspended to a concentration of 25,000 cells per 20 μl. Hanging drops were made by placing 20 μl drops (25,000 cells) on the roof of a cell culture plate and incubated at 37°C, 5% $CO_2$ for three days. The resultant spheres that formed after three days of incubation were transferred in to 48 well plates containing Type I collagen (VWR, Mississauga, ON, Canada) mixed with 10X DMEM (Gibco), one sphere per well. The collagen pH was adjusted using 0.1N NaoH (Fisher Scientific). Upon gelation, another layer of collagen was placed in the well and incubated for 30 minutes at 37 degrees. After gelation, a layer of BTM was placed on the top of the collagen. The invading cells were measured using a Zeiss Primo Vert microscope with micrometer on day 0,1,2 and 3. The diameter was measured twice, both vertically and horizontally and averaged. The measurements were always taken from the cell that had invaded the furthest distance from the core. Invasion measurements were then calculated by subtracting the invasion diameter at day 3 from the sphere diameter at day 0.

**Tumorsphere Formation and Counting**

Aliquots of 2500 Daoy cells from each subpopulation were plated in a 24-well ultra low attachment plate (ULAP) and cultured for 5 days in neuro precursor media composed of Dulbecco's Modified Essential Media F12 (DMEM F12) (Invitrogen) containing 1% B27 (Gibco, Invitrogen, Burlington. ON, Canada), 1% N2 (Gibco), 20 ng/ml EGF (VWR, Mississauga, ON, Canada) and 20 ng/ml bFGF (Fisher Scientific). The tumorspheres were collected, dissociated in Accutase and counted using an automated cell counter (Bio-Rad, Mississauga, ON, Canada) or hemocytometer . 20,000 cells were plated in an ULAP in neural precursor media and incubated for seven days. At day 3 or 4, half of the neural media was removed from the plate and replaced by fresh media. At day 7, tumorspheres were counted manually in each well using a microscope. Cells were then dissociated, and 20,000 cells re-plated in a ULAP in neural precursor media. After seven more days, the tumorspheres were counted.

**Fluoresence-Activated Cell Sorting**

Day 7 passage 2 Daoy tumorspheres were pooled, dissociated, washed and resuspended in DPBS with 0.5% FBS. Cells were counted using an automatic cell

counter, and stained for CD133 and CD271 or CD24 using CD133-PE, CD271-Alexa and CD24-PE respectively. All antibodies were obtained from BD Biosciences, with exception of CD133 (Miltenyi Biotec, Cambridge, MA, USA). Sort samples were also stained with 7AAD viability dye (Beckman Coulter). Cells were sorted based on CD271/CD133 using MoFlo™XDP cell sorter and analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

**Intracerebral Injections**

NOD SCID mice between 5-7 weeks were used for the injections. The mice were anesthetized using an intraperitoneal injection starting dose of 150 mg/kg of ketamine and 10 mg/kg xylazine or later experiments using an induction dose of 50 mg/kg of ketamine and 10 mg/kg xylazine with a surgical plane maintained with isoflurane. Anesthetic plane was assessed using the pedal reflex. Mice were topped up with anesthetic if plane was not reached. Mice were secured in a stereotactic frame by the ear canals and snout (David Kopf Instruments, Tujunga, CA, USA) The surgical area was sterilized using iodine, soap and 70% ethanol. An incision was made from between the eyes to between the ears longitudinally to expose the coronal suture, ensuring the periosteum is scraped away. A burr hole was drilled 2-3 mm anterior to coronal suture, 2 mm lateral to midline. After bleeding has stopped from the burr hole, a 10μl Hamilton syringe (Hamilton Company, Reno, NV, USA) containing the 10 μl of cell suspension is inserted 5 mm into the burr hole, perpendicular to the skull at an anterior angle of 30°. Unsorted or sorted cells (see Fluorescence-activated cell sorting method above), were suspended in PBS according to the number of cells to be injected. The cells are injected into the frontal lobe and needle removed. The incision was closed using three sutures of 4 gauge micron. Subcutaneous injections of 0.9 ml NaCl and 2 mg/kg Meloxicam were used to hydrate and provide analgesia to the mice following surgery. All animals were monitored until they awoke from the anesthetic. 24 hours post surgery, the mice received 2mg/kg of Meloxicam) and 1ml of NaCl Subcutaneous. Mice were monitored daily for signs or symptoms of tumor formation. Signs of tumor formation include a domed head appearance ruffled fur, and lethargy. The end point was determined by either an inability to thrive, characterized by unusual quiescent behavior and severe neurological symptoms, or a loss of 10% body weight from the initial weight when the domed head first appeared.

**Cardiac perfusion and brain fixation**

Once an end point has been established for each individual mouse, the mouse was anesthetized using a cocktail of ketamine and xylazine. The chest wall was cut away revealing the heart. 50 ul of heparnin was injected into the base of the heart and 10 heart beats were counted. The auricle of the right atrium was cut, and a 30ml syringe of saline was then inserted into the base of the heart using a 18g needle. The 0.9% NaCl was injected over approximately 5 minutes until the fluid running from the auricle is clear. 20 ml of formalin was then injected into the base of the heart over approximately 5 minutes. Once the formalin was injected, the head of the mouse was removed and the skull was peeled back using tissue forceps. The brain was removed from the skull and placed in formalin for histopathological analysis. Samples will be sectioned, paraffin

embedded and stained with Hematoxylin and Eosin. We will examine all tumour samples using a variety of phenotypic and immunohistological criteria for MB including the presence of neural rosettes, a dense cell population consisting of a high nuclear-to-cytoplasmic ratio and evidence of tumour cell invasion into the host tissue (ie. well demarcated tumour vs. diffuse infiltration into the mouse brain)

## Results

### Medulloblastoma subclones possess differential morphology, invasion and self-renewal capacity.

To initially evaluate the difference in invasion potential and morphology among MB sub-clones, 21 individual cell sub-clones were cultured and expanded. For sub-clone expansion, Daoy MB cells were dissociated, resuspended and sorted using a MoFlo™XDP (Beckman Coulter, Inc.) cell sorter. One single Daoy cell was deposited into each well of a 96-well plate. These cells were then cultured and expanded. Sub-clones were cultured in a 3-D hanging drop preparation using the previously established methods (Del Duca et. al.). The hanging drops were subsequently implanted into a 3-D type I collagen matrix and invasion was measured after 72 hours. The sub-clones exhibited significantly different invasion capacity (Figure 1), with a six-fold difference between the least and most invasive clones. The most and least invasive were then plated into tumorsphere culture at a concentration of 20,000 cells/well to determine their self renewal capacity. Tumorspheres derived for the most invasive sub-clones exhibited significantly higher self-renewal capacity as compared to the least invasive clones. Together, these results show that MB cultures exhibit morphological and invasive heterogeneity.

### Least and most invasive clones exhibit differential expression of CD271, CD133 and CD24 cellular markers in tumorsphere format.

MB sub-clones exhibit variable self-renewal capacity in tumorsphere format. We next wanted to determine if higher vs. lower self-renewing tumorspheres exhibit different cell surface marker profiles. Tumorspheres from higher vs. lower self-renewing sub-clones were dissociated at passage 1, and screened using flow cytometry for the presence of cells surface markers already known to play a role in neural lineage specification, BTPCs and/or tumor cell metastasis. Analysis showed differential expression of 3 cell surface markers; CD271 (p75 neurotrophin receptor), CD24 and CD133, however only CD271 was significantly different (Figure 2). 4 out of 5 higher self-renewing clones showed elevated levels of CD271, whereas, all 3 lower self-renewing clones expressed negligible CD271.

### Migrating Medulloblastoma cells exhibit decreased CD271/CD24 and increased CD133 cell surface marker expression.

It is unknown from the previous experiments if the difference in the cell surface markers is attributable to the difference in self-renewal between sub-clones or the parent cultures

exhibit different invasive capacities. To shed further light on this, we decided to do dissection assays to determine if changing the biological assay will change the expression profile of CD271, CD24 and CD133. To do this, I developed a manual dissection technique to separate the core spheroid from the migrating cells. We prepared hanging drops from Daoy MB parental cells. The hanging drops were then transferred to a cell culture plate and allowed to adhere and migrate for 48 hours. The core was then manually dissected from the migrating cells under a dissection microscope using a 20µl pipette (Figure 3). The attached migrating cells were then dissociated from the plate. The core and migrating cells were subjected to the same 8-cell surface marker screen conducted in the previous invasion study. The results showed that the same three, CD271, CD24 and CD133, were differentially expressed (Figure 4). CD271 and CD24 were elevated in the core while CD133 was elevated in the migrating cells. qPCR was also used to examine transcript levels of neural lineage markers Otx2, Sox1 and βIII tubulin. The primitive neuroectoderm marker Otx2 was significantly higher in the core cell population, while Sox1 remained the same. βIII tubulin, a marker of higher differentiation was significantly lower in the core population. Taken together, our data suggest that a low CD271/CD24 level and an elevated CD133 level marks a migrating MB cell that may represent a more differentiated cell state. The reverse of this is true for the core MB cells. The differential expression patterns in Figure 2 therefore reflect a change in self-renewal potential in tumorsphere format. Because of these results, the lab decided to further evaluate these cell markers in the additional experiments including the animal models.

**CD271 and CD24 select for MB cells with increased self-renewal capacity while CD133 select for cells with higher invasive capacity**

From the results showing that CD271, CD24 and CD133 are differentially expressed in both tumorsphere and core vs migrating cells, we tested the hypothesis that these cell markers mark functionally distinct cells. Cells from adherent cell cultures or tumorspheres were sorted using fluorescent activated cell sorting (FACS) based on the cell surface marker combinations CD133/CD271 and CD133/CD24 (Figure 5A). The sorted cells were then used for tumorsphere assays, cell growth assays in adherent culture, and invasion assays in collagen. The results demonstrated that CD271/CD24 select fro MB cells with the highest self-renewal capacity *in vitro*, demonstrated by the ability to form a greater number of neurospheres (Figure 5B-D). In contrast to this, CD133+/CD271- and CD133+/CD24- subpopulations demonstrated a higher invasive capacity in collagen invasion assays (Figure 5E-F). As well, invasion was always lowest in subpopulations that demonstrated the highest self renewal capacity. All of this together, this adds to our data that shows CD271 and CD24 select for a cell with higher self-renewal capacity, while CD133 selects for a cell with a higher invasion capacity.

**Testing the results found in vitro using a *in vivo* mouse model**

In vitro, our past experiments have shown that the combinatory expression of CD271/CD24 selects for cells with a higher self-renewal capacity whereas CD133 selects for cells with higher invasive capacity. We have shown that invasion and self-renewal capacity are inversely correlated, but not mutually exclusive. We then wanted to

show see if we can reproduce these findings in a more biologically relevant environment using immunodeficient mice. Figure 6 shows the initial experimental setup used to establish the animal experiments within the laboratory. Before beginning our in vitro work, there was no working animal model being used with the laboratory. It was therefore necessary to start from the beginning, and I was given the task to optimize the techniques used for our experiments. Our goal was to evaluate the cell phenotypes in vivo. As the CD271 results typically yielded the most significant results, we initially sorted Daoy tumorspheres based on CD271/CD133 only. Two separate but related experiments were conducted. The first involved injecting NOD SCID mice with unsorted control cells taken from a sub-clone that exhibited a higher self-renewal capacity in tumorsphere culture in vitro. Limiting dilution analysis was conducted to determine the number of cells necessary to establish a tumor, as well as the latency period between the injection and appearance of tumor symptoms. Tumor signs were characterized by the initial appearance of a domed head due to hydrocephaly, which in all cases came before any other symptom. The domed head is a subjective feature, however the emergence of this phenotype was confirmed by multiple independent observers. The end point was determined by several factors and was determined on a case by case basis. The animal was euthanized if it started showing an ungroomed appearance and lethargy, or lost 10% of its body weight. 8 mice were injected with 200,000 cells, 3 with 50,000 cells, 1 with 25,000 cells and 1 with 10,000 cells. At this point in time, 12 of the 13 mice injected have developed a domed head appearance. Analysis of the latency period between injection and appearance of a domed head revealed a negative correlation between the number of cells injected and time it takes to form a domed head (Figure 8). 9 animals have been euthanized to date and cardiac perfusion performed (Figure 6). The brains were removed after perfusion and are being stored in formalin while we await histopathological analysis. By conducting a limiting dilution, we would expect to find a minimum number of cells needed to form a tumor or to define the tumor initiating cell capacity. Because the unsorted cells have a fairly high self-renewal capacity, it would be expected that all of the mice injected will eventually form a tumor.

The second experiment involved injecting sorted cells based on the cells surface markers into the NOD SCID mice. Following FACS to isolate cells from Daoy tumorspheres based on CD271/CD133 expression, 2 mice were injected with 200,000 CD271+/CD133- cells, 4 mice injected with 200,000 CD271-/CD133- cells and 4 mice were injected with unsorted cells from Daoy tumorspheres. While higher cell numbers would be ideal for decreasing the latency between injection and tumor formation, we are typically limited by the number of cells that are collected from the sorting procedure. In addition, approximately 5-10% of the cells were CD271+/CD133 -, thus further limiting the number of animals we could inject with various sorted cellular phenotypes. At the current time, 5 of the 6 mice injected have developed domed heads. 3 of them were injected with the -/- cells and two with +/- cells. Mice injected with +/- cells develop a domed head in a shorter time, (35 and 36 days), than mice injected with -/- cells, (35, 41 and 42 days). The mice with unsorted Daoy cells injected show a similar latency period as the mice injected with CD271-/CD133- cells (Figure 9).

Based on our in vitro experiments, we would expect that mice injected with CD271+ cells, which have shown in vitro to have a higher self-renewal capacity will exhibit the

higher capacity for tumor formation in vivo. This would be demonstrated by the formation of multiple tumors, or the formations of a tumor in less time than animals injected with CD271-/CD133- cells. However we do expect mice injected with CD271-/133- to also form tumors. It is known that CD271-/133- cells although lower, still have some self-renewal capacity and therefore contains BTPCs. It is thought these mice will take longer to form a solid tumor mass than the CD271+/133- mice. Based on the early appearance of symptoms and the latency period for multiple phenotypes, we predict that our hypothesis is correct.

## Discussion

Medulloblastoma (MB) is the most common primary malignant pediatric brain tumor (Louis, D. *et. al* 2007 and Fastro, M.M. *et. al.* 2011). Although 5-year survival rates have improved to 60-70% (Fastro, M.M. *et. al.* 2011), current treatments include chemotherapy and radiation which have severely toxic effects on a child's developing nervous system. MBs can also recur despite treatment due to tumor invasion, and on occasion metastasis through cerebral spinal fluid (CSF).

Cancer research has shifted in the past decade towards the understanding of cancer stem cells/tumor propagating cells. First discovered in leukemia, several solid tumors including breast, colon, and brain have been shown to contain these cells. The current theory is that these primitive self-renewing cells are responsible for initiating and propagating tumor mass. It is also thought that they are partially responsible for the chemo and radiation resistance and for tumor recurrence years later.

The lab deconstructed the heterogeneity of MB cell cultures. The relationship between invasive capacity, self-renewal and cellular markers was investigated to determine novel cellular markers capable of selecting for either or characteristic. CD271/CD133/CD24 were identified to as markers that can be used to select for cells exhibiting the highest self-renewal capacity and invasion capacity. It was found that cells displaying higher CD271/CD24 markers has a higher self-renewal capacity, whereas cells with higher CD133 surface markers selects for a cell with a higher invasion capacity. A model was developed whereby an inverse but not mutually exclusive relationship exists between self-renewal capacity and invasion. A more primitive, higher self renewing CD271/CD24 high cell in the MB tumor core are responsible for sustaining growth. Once a cell within the core has been committed to an invasive or migratory cell, the cell down regulates CD271/CD24 and up regulates CD133.

In order to confirm these findings, we developed an *in vivo* model using NOD SCID mice and intracerebral injections. Two initial experiments were performed in order to optimize the technique used, as well to set up larger and more complex experiments in the future. Unsorted Daoy tumorsphere cells were injected using a limited dilution to determine the minimum number of cells needed to develop a tumor, as well as the latency period between injection and tumor symptoms. We found that a larger cell number leads to a smaller latency period. This fits with what we expected to see from this experiment and is due to a higher number of tumor initiating cells or tumor propagating cells present in

the injected cell suspension. All of the mice have developed symptoms of a tumor at this time except the mouse injected with 10,000 cells. Sorted Daoy tumorsphere cells were also used in a separate but related experiment. Each mouse was injected with 200,000 CD271+/CD133- or CD271-/CD133- cells. Initial results are showing that mice injected with CD271+ cells are developing tumor symptoms at an early time than mice with CD271- cells. This also fits with what we expected to see and is consistent with our in vitro data. These findings fit with the hypothesis that CSCs or TPCs, which are defined as cells with the ability to self-renew are responsible for the formation and propagation of tumors; however, this remains to be proven in our model system. Pathohistological analysis of the brains is underway but is yet to be completed.

Injections for a third experiment were performed the week this paper was completed. This experiment will compare higher self-renewing vs. lower self-renewing tumorspheres in a limiting dilution assay. The low self-renewing tumorspheres used, when cultured in neurosphere conditions in vitro show a very low capacity to adhere to one another and form spheres, typically breaking apart after 3 passages. We would expect to see a low tumor initiating capacity from these cells in vivo, reflected by the inability to form tumors at higher injected cell numbers than cells with a higher self-renewal capacity.

The above experiments not only established the intracranial transplant model in our laboratory but are also being used to experimentally validate our in vitro data. Down the road, a full spread of experiments will be done using all 3 cellular markers, CD271/CD133/CD24, using a maximum of 1 million cells, with limiting dilutions down to 10,000 cells. It will also be necessary to collect the tumors, conduct FACS and re-inject the tumor cells removed from the mice in a secondary tumor formation assay. This is the gold standard to determine the presence of CSCs within the tumor population, as it is the standard test to ultimately define self-renewal capacity in vivo.

The cell surface markers found to select for phenotypes within MB populations have the potential to have important clinical value. These surface markers, and the cellular pathways they are associated with can potentially serve as targets for drug discovery or targeting. By showing that there is an inverse, but not mutually exclusive relationship between self-renewal and invasiveness demonstrates that it will be important to target both the invasive cells that are responsible for the majority of the damage MB incurs, as well as the cells responsible for propagating the tumor, something that many people believe the current treatment is not accomplishing. All together, this has the potential to make medulloblastoma a more manageable disease.

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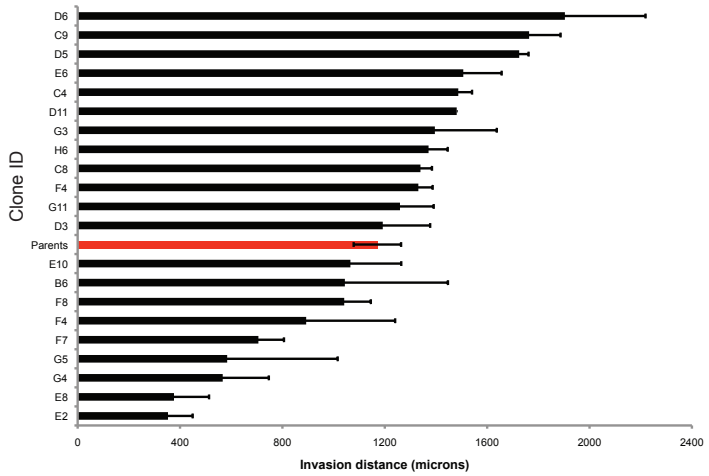


Figure 1 – Graph showing single-cell derived clonal expansion of Daoy cells following fluorescent activated cell sorting (FACS). 21 Expanded clones were cultured as spheroids as a hanging drop for 3 days and placed in a 3-D type I collagen assay for invasion for an additional 3 days. (adapted from Morrison and McClelland *et al.* 2012 unpublished data)

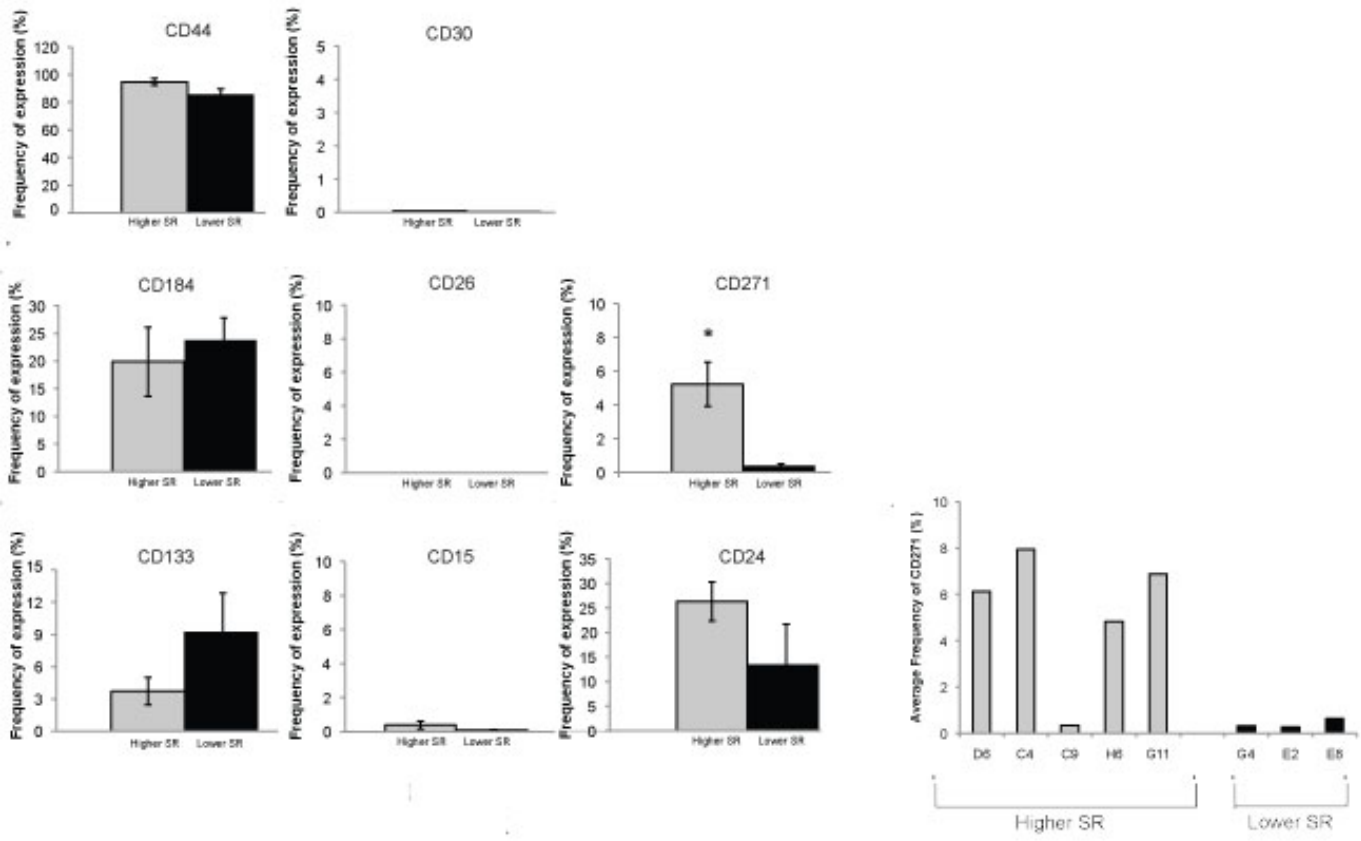


Figure 2 – A. Self-renewal capacity of most vs least invasive derived tumorspheres. In neurosphere culture, the most invasive sub-clones displayed the greatest self-renewal capacity by generating the highest number of tumorspheres. B-I. Quantification of cell surface markers screen on the tumorspheres with the highest vs lowest self-renewal capacity. Cell surface markers were chosen based on their known role they play in neural lineage specification, brain tumor stem cell self-renewal and tumor invasion. Error Bars: SEM. $P<0.05$. N=5 independent “higher SR” subclones, N=3 independent “lower SR” subclones. J. Breakdown of higher SR vs Lower SR subclones derived tumorspheres for CD271(adapted from Morrison and McClelland *et al.* 2012 unpublished data)

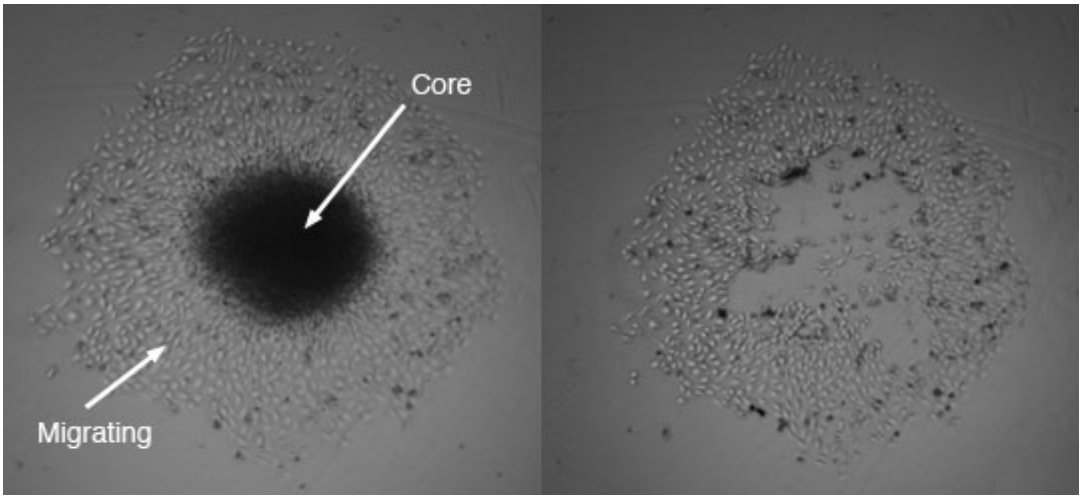


Figure 3 – Magnification of tumorsphere before “core” dissection and after “core” dissection. Tumorspheres were allowed to migrate for 48 hours, followed by manual dissection of the core from the migrating cells. Cells were then dissociated and screen for cell surface markers.

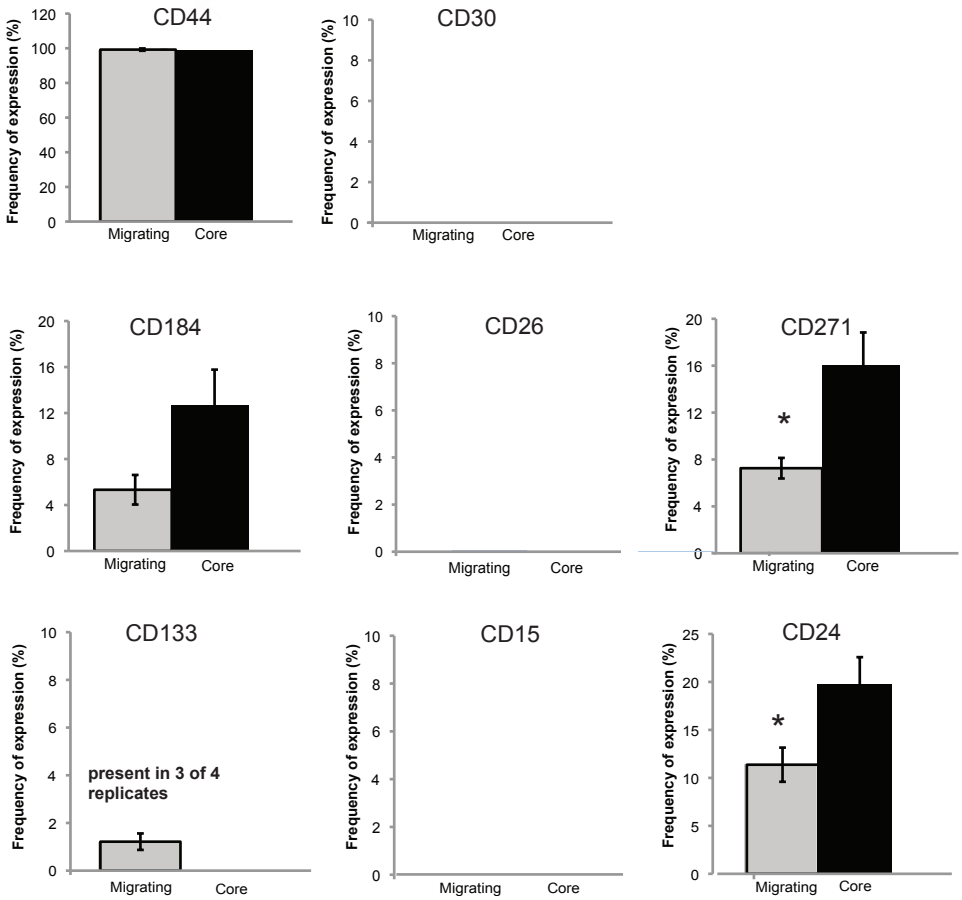


Figure 4 – Cell surface marker antibody screen on the ”core” vs “migrating” cells from Medulloblastoma spheroids 48 hours after attachment. Error bars: SEM. $P<0.05$. N=4 and N=3 independent experiments. (adapted from Morrison and McClelland *et al.* 2012 unpublished data)

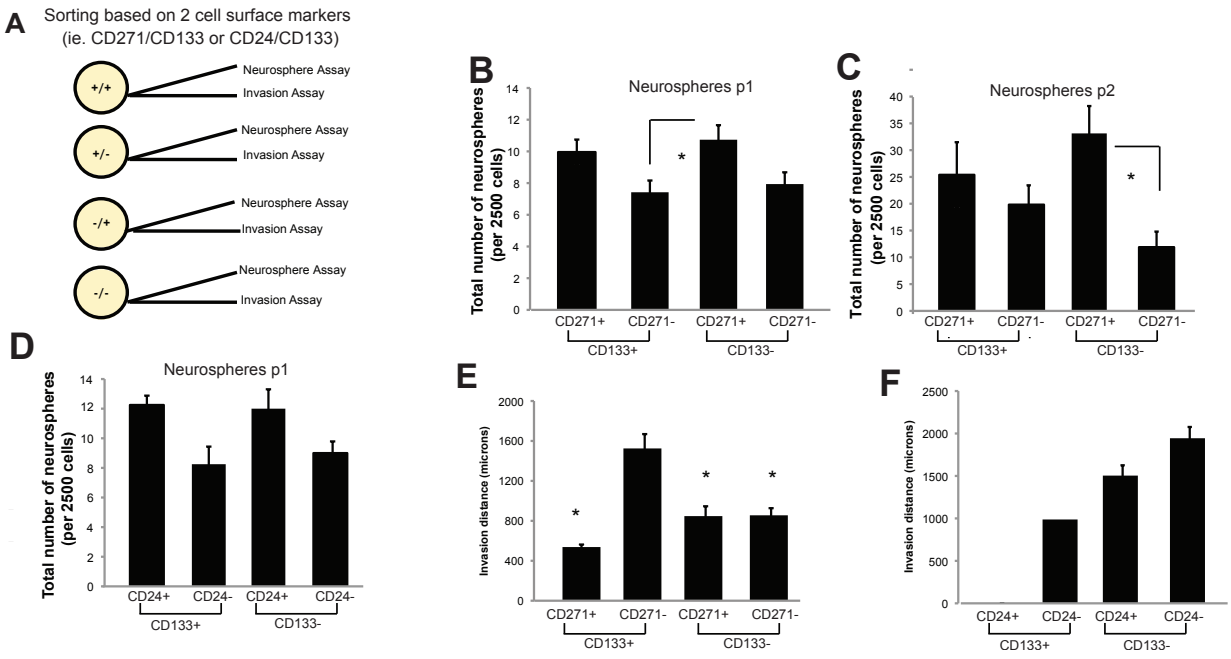


Figure 5 – Sorting of Medulloblastoma neurospheres based on combinatory CD133/CD271 and CD133/CD24 cell surface marker expression reveals an inverse correlation between cell populations with a higher self-renewal capacity and those exhibiting higher invasion. A. Schematic depicting initial 2-marker sorting to obtain 4 subpopulations. B-D. Total number of Tumorspheres formed after cells were sorted based on CD271/CD133 and CD133/CD24, first and second passage. E-F. Quantification of invasion for each subpopulation after being sorted based on CD271/CD133 and CD133/CD24. Cells were sorted based on surface marker combinations, grown in hanging drops and subjected to invasion assays in Type I collagen. Note that subpopulations with highest self-renewal capacity are not the same as those with the highest invasive capacity. (adapted from Morrison and McClelland *et al.* 2012 unpublished data)

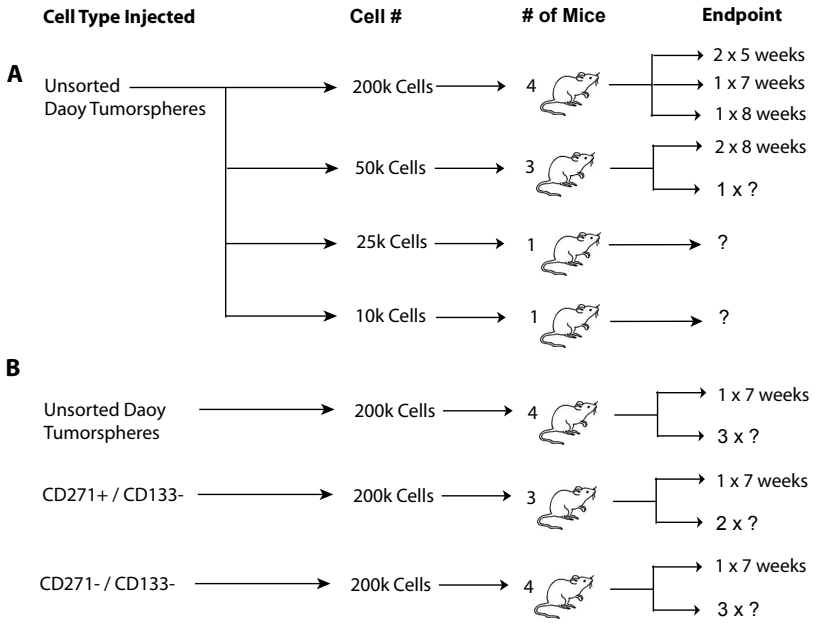


Figure 6 – *In vivo* experimental setup including end-point dates. A. Unsorted limiting dilution Daoy cell experiment. A limiting dilution of 200000, 50000, 25000 and 10000 cells were injected using intracerebral injections into corresponding number of mice. Endpoint indicates the length of time between injection and cardiac perfusion based on endpoint criteria. B. Sroted Daoy cell experiment. Daoy cells derived from tumorspheres were sorted using FACS based on CD271+/CD133- and CD271-/CD133- and injected using intracerebral injections.

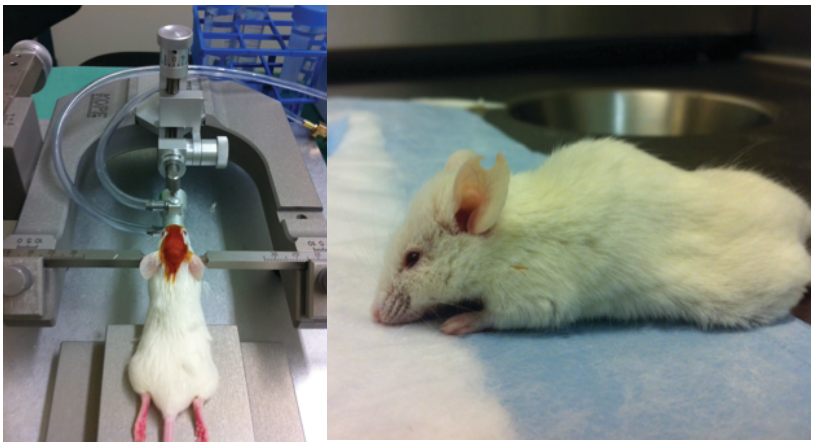

Figure 7 – Image of mouse during surgery. Mouse was induced using ketamine/xylazine and kept under anesthesia using isoflurane. A stereotactic frame was used made by KOPF instruments to allow for immobilization of the head. Mouse showing characteristic domed head appearance on day of cardiac perfusion. Note ruffled appearance.

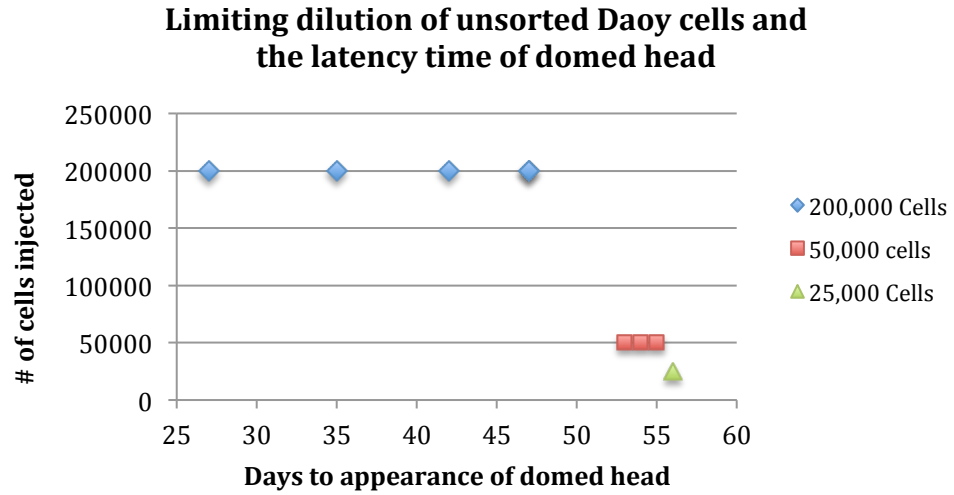


Figure 8 – *in vivo* limiting dilution of Daoy tumorsphere cells shows reduced latency period between injection with cells and appearance of domed head in regards to injected cell numbers. When injected with a higher cell number, the latency period in shorter when compared to lower cell numbers injected.

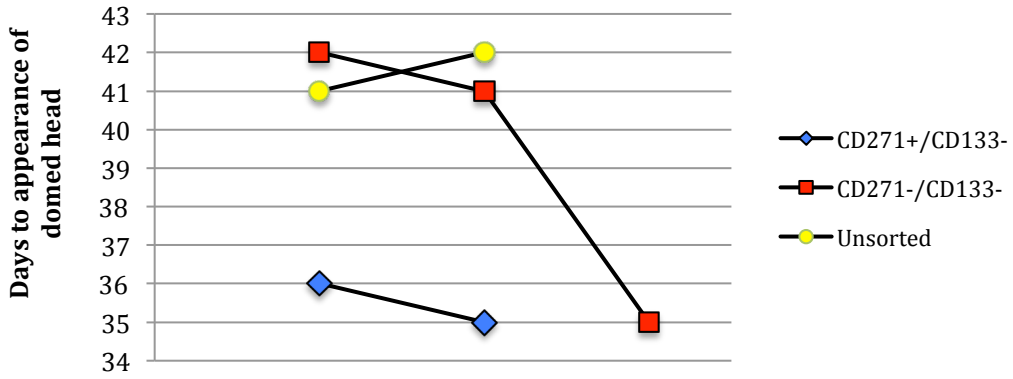


Figure 9 – CD271+/CD133- cells show shorter latency period between injection with cells and appearance of domed head when compared to CD271-/CD133- cells. Cells were sorted using FACS based on CD271 and CD133 surface markers. All mice were injected with 200,00 cells. Not unsorted cells have similar latency period as CD271-/CD133-.