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SCHOOL OF MEDICINE

Thesis

THE EFFECT OF GROWTH FACTOR WITHDRAWAL ON SOX2 EXPRESSION IN SHH MEDULLOBLASTOMA STEM CELLS

by

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THE EFFECT OF GROWTH FACTOR WITHDRAWAL ON *SOX2* EXPRESSION IN SHH MEDULLOBLASTOMA STEM CELLS

DANIEL I. PARK

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ABSTRACT

Medulloblastomas are the most commonly occurring group of malignant pediatric brain tumors. Over the years, these cerebellar tumors have been histologically and genetically classified to yield a number of distinct subtypes that confer differing disease progression and prognoses. Previous studies on the SHH medulloblastoma mouse model from Jackson-Grusby laboratory have revealed a cancer stem cell origin that may be responsible for the metastatic and more malignant fraction of this subtype. The lab worked with mice harboring mutations in tumor suppressor genes *Ptch1* and *p53*. A novel discovery from this background was that mouse cerebellum deficient in *p53* and heterozygous for the SHH pathway inhibitor gene *Ptch1* developed an aberrant stem cell population predisposed to malignant progression through *Ptch1* loss-of-heterozygosity (LOH).

To study this tumor initiating population of stem cells, the lab has constructed a *Ptch1* heterozygous, *p53* deficient mouse model which reliably provided mice with aberrant cerebellar stem cells (pre-LOH) and cancer stem

cells (post-LOH). Sox2, a neural stem cell marker and transcription factor for stem cell maintenance, has been indicated as a prospective marker to isolate these different Ptch1;p53 stem cells. To characterize the marker's ability to detect the stemness of the differing SHH medulloblastoma stem cells, previous studies in the lab measured Sox2 antibody levels by inducing differentiation through growth factor withdrawal. The finding was that Sox2 levels were maintained in the cancer stem cell population, indicating a resistance to differentiation, while Sox2 levels dropped in the aberrant stem cell population, indicating the ability to differentiate.

The current study aimed to test whether the results of the growth factor withdrawal experiment were also seen at the transcriptional level of *Sox2*. The study was conducted by inserting a *Sox2-GFP* transgene into the lab's SHH medulloblastoma mouse model and measuring *Sox2* transcriptional activity via GFP fluorescence. The results proposed a progressively heightened resistance to differentiation cues based on malignancy in which wild-type stem cells were least resistant, aberrant stem cells were weakly resistant, and cancer stem cells were strongly resistant. The *Sox2* expression in cancer stem cells measured by GFP fluorescence confirmed the existence of a robustly *Sox2* upregulated population. These strongly GFP+ cells absent in aberrant stem cells, justifies the strong stem-like characteristics associated with the malignant stem cell population. Specifically, the *Sox2* expression is amplified by an auto-feedback loop between Sox2 and the SHH pathway to confer resistance to differentiation.

Altogether these results confirm the effectiveness of Sox2 in detecting the stemness of varying cerebellar stem cell types, proposing Sox2 as a reliable prospective marker for pre-malignant and malignant medulloblastoma stem cells.

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ABBREVIATIONS

bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
Bmp	Bone morphogenetic protein
BSA	bovine serum albumin
DCN	deep cerebellar nuclei
DMEM/F12	Dulbecco's Modified Eagle Medium/Ham's F-12
EDTA	ethylenediaminetetraacetic acid
CGNP	cerebellar granular neuronal precursors
CNS	central nervous system
CPe	choroid plexus
CSC	cancer stem cell
EGF	epidermal growth factor
EGL	external granule layer
FACS	Fluorescence-activated cell sorting
FSC	forward scatter
GABA	γ-Aminobutyric acid
GF	growth factor
GFP	green fluorescent protein
GNP	granule neuron progenitors
IGL	internal granule layer
IsO	isthmic organizer

LOH	Loss-of-Heterozygosity
NS	neurosphere
PBS	phosphate-buffered saline
PCL	Purkinje cell layer
P53	Protein 53
Ptch	Patched
Ptf1a	Pancreatic transcription factor 1 subunit alpha
rh1	rhombomere
RLS	rostral migratory stream
SHH	Sonic Hedgehog
Smo	Smoothened
SSC	side scatter
SuFu	Suppressor of fused homolog
Trp53	Transformation related protein 53
VZ	ventricular zone
WHO	World Health Organization
7-AAD	7-amino-actinomycin D

INTRODUCTION

Medulloblastoma

Medulloblastoma is the most common malignant brain tumor found in children, accounting for 25% to 30% of pediatric central nervous system tumors (1). Adult medulloblastomas occur rarely, and represent 4% to 6% of all primary adult intracranial malignancies (5). The tumor itself arises from the cerebellar vermis and forms lesions along the ventricles (6). As a result, medulloblastoma patients present with cerebellar symptoms that range from truncal ataxia, gait disturbances, and hydrocephalus. Lethargy, headache, and morning vomiting are also symptoms commonly observed (3).

In 2007, medulloblastoma has been histopathologically categorized by the World Health Organization (WHO) into 5 different subtypes: classic, anaplastic, large cell, desmoplastic, and extensively nodular. Classic histology consists of round, hyperchromatic nuclei with little cytoplasm and high cell density. Anaplastic and large cell medulloblastomas are similar in that they have extensive mitotic and apoptotic characteristics while retaining undifferentiated cell types. The desmoplastic/nodular histology is characterized by nodules of reticulin-free zones surrounded by a dense fiber of reticulin containing proliferative cells. In extensively nodular medulloblastoma, neuropil-like tissue filled lobular architecture is the dominant morphology (2,3). For children, the 5-year survival rate for medulloblastoma is, if properly treated, 70-80% with even

the most malignant WHO grade IV lesions (2,19). The treatment options include surgery, chemotherapy, and cranio-spinal radiotherapy. Despite the effectiveness of medulloblastoma treatments, most patients develop long-term side effects due to the toxicity of therapies like chemotherapy and radiotherapy. These side effects include developmental, neurological, neuroendocrine and psychosocial problems which severely depreciates the cognition and intellect of patients (1).

Recently, advancements in molecular characterization through large scale transcriptional profiling of pediatric primary medulloblastomas has led to the identification of four subgroups: WNT, SHH, Group 3 and Group 4. (1,4). Similar studies into the gene expression data on adult medulloblastomas revealed a SHH subgroup majority, but in contrast to childhood medulloblastomas, no Group 3 medulloblastomas were found (5).

Cerebellar development of mouse

The mammalian cerebellum is involved in numerous motor, sensory, and cognitive functions (20). It is formed through a complex interplay of developmental signals (Figure 1). During the cerebellar development as well as the development and maintenance of the central nervous system (CNS), the undifferentiated neural epithelium goes through the process of carefully balancing neurogenesis with the maintenance of neural stem and progenitor cells. This is done via Notch1 signals as well as SoxB1 transcription factors,

which includes Sox1, 2, and 3, repressing proneural genes against differentiation proteins like Sox21 (26).

The gross cerebellum first starts forming through the dorsal area of the hindbrain rhombomere 1 (rh1) by receiving signals from the isthmic organizer and fourth ventricle roof plate (Figure 1a-1). The neurons found in the cerebellum are formed from two distinct embryonic germinal zones within the primordial or cerebellar anlage. The two distinct zones, which are the rhombic lip and the cerebellar ventricular zone, form through an antagonistic balance between Notch1 and Bmp signals (8). Specifically, the rhombic lip is formed through Bmp downstream signals originating from the roof plate while Notch1 counters Bmp to form the cerebellar ventricular zone (Figure 1a-2). These two zones in turn can be marked by two distinct transcription factors. Ptf1a is a basic helix-loop-helix (bHLH) transcription factor found in the cerebellar ventricular zone. Ptf1a+ cells from this zone give rise to Purkinje, Golgi, basket, and stellate cells (Figure 1a-3). Dorsal to the ventricular zone is the rhombic lip which expresses a different bHLH transcription factor, Math1 (Figure 1a-4). Loss-of-function studies on Math1 showed that although Math1 isn't required for the formation of the rhombic lip, they are required to generate all CGNPs and consequently the EGL (7).

There is a time-sensitive migratory pattern arising from the ventricular zone and the rhombic lip. The first is that the differentiating cells from the ventricular zone radially migrate within the anlage. Loss-of-function studies have determined that an absence of *Ptf1a* in the ventricular zone cells leads to the

failure in generating cerebellar Purkinje cells and GABAergic neurons which in turn leads to complete cerebellar agenesis.Cells from the rhombic lip migrate to the anlage that produces a continuously proliferating outer layer. Of these cells, the cerebellar granular neuronal precursors (CGNP) forming the external granule layer (EGL) are induced by Sonic Hedgehog (SHH) signals from the Purkinje cells of the anlage to proliferate (Figure 1b-5). Finally, there is an inward migration of EGL cells that form the internal granule layer (IGL) which together forms the mature cerebellum (Figure 1b-6).

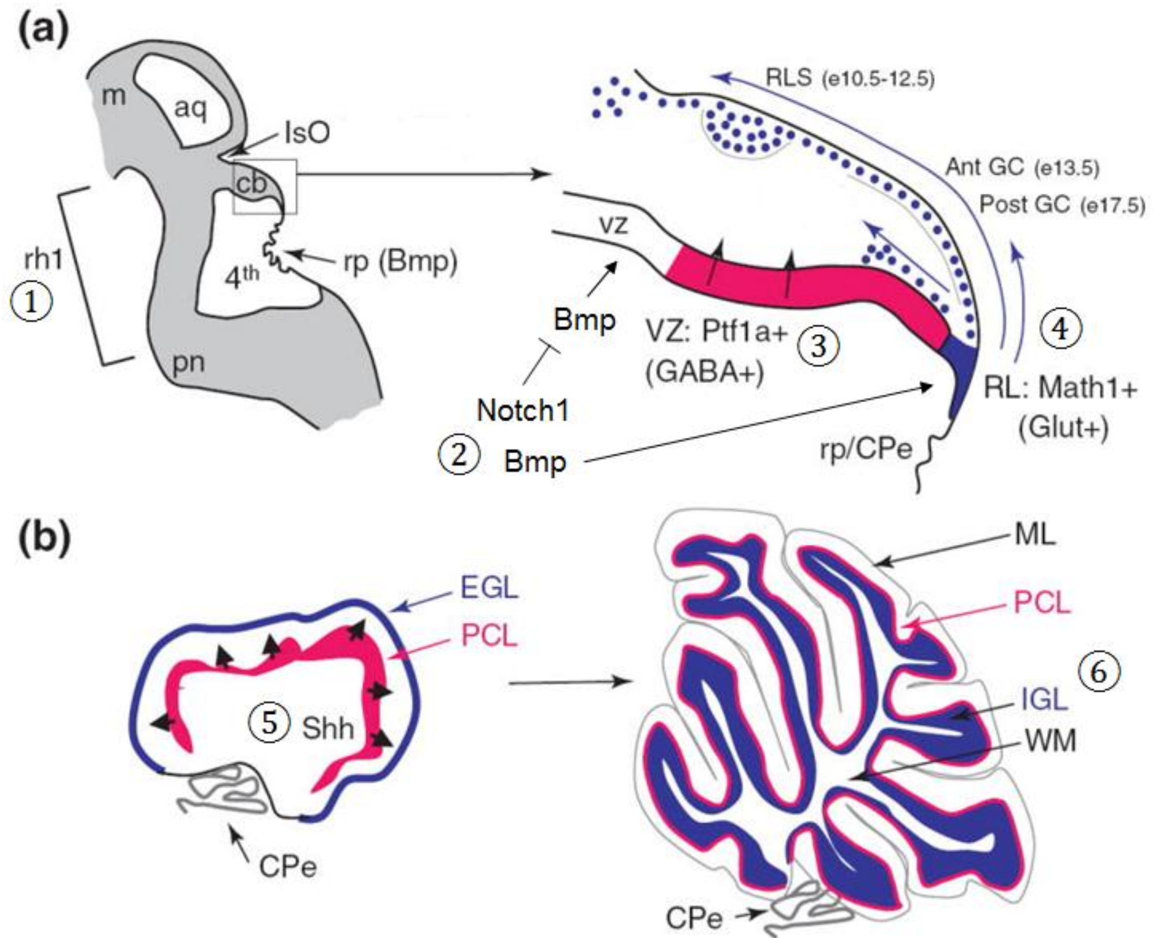

Figure 1. Schematic of mouse cerebellar neurogenesis. (a) Depicted is a parasagital section through the midbrain/hindbrain region of an embryonic day 12.5 neural tube. The hindbrain rhombomere 1 (rh1) forms the cerebellum by receiving signals from the isthmic organizer (IsO) and fourth ventricle roof plate (rp). Within the cerebellar cluster, there are two germinal zones, the rhombic lip (rl) and cerebellar ventricular zone (VZ), and they are distinguished by transcription factors Math1 and Ptf1a. The Math1 expressing glutamatergic progenitor cells from the rhombic lip spread out in waves, and feed into the rostral migratory stream (RLS). The RLS then gives rise to multiple brain stem precerebellar nuclei within the cerebellar anlage such as the pontine nuclei (pn). More importantly, the Math1 expressing progenitors generate cerebellar granule cells (GC) which will eventually form the external granule layer (EGL). The Ptf1a expressing progenitors from VZ spread out radially into the cerebellar anlage to give rise cerebellar interneurons and deep cerebellar nuclei (DCN). M: midbrain; aq: aqueduct **(b)** Depicted is a midsaggital section of a mouse cerebellum at birth. The Purkinje cell layer (PCL) underneath the EGL secretes Shh proteins which are picked up by EGL cells to drive granule cell expansion. As

differentiation occurs, EGL cells cut through the PCL to form the IGL. CPe: Choroid plexus (Adapted from 7).

Sonic Hedgehog Signaling on Cerebellar Development

The Hedgehog pathway is a critical developmental patterning pathway for a variety of tissues. In the cerebellum, the cerebellar granular neuronal precursors (CGNPs) proliferate and initiate migration from the EGL from about P8. The mature cerebellar shape, such as the fold pattern, and its overall function is critically dependent on this expansion phase of CGNPs. Because the Hedgehog pathway is essential for signaling the expansion of CGNPs, it is heavily responsible for proper cerebellar maturation. The developmental importance of this is signified by the fact that Ptc1-null mice do not survive embryogenesis with overgrown neural tubes that fail to close (12). Sonic hedgehog (Shh) secreted from Purkinje neurons bind to a 12 transmembrane receptor, Ptc1, which dissociates it from another transmembrane protein, Smoothened (smo), to disinhibit the Shh signaling pathway. Ultimately, the activation of the pathway leads to the separation of the Gli-SuFu complex to avoid Gli degradation. The released Gli family proteins such as Gli1 and Gli2 then transcriptionally activate granule cell proliferation (Figure 1b & 2). At P21, the CGNPs will eventually move through the Purkinje cell network to reach the IGL and differentiate. Therefore by P21, normal mouse cerebellar development is considered complete (9).

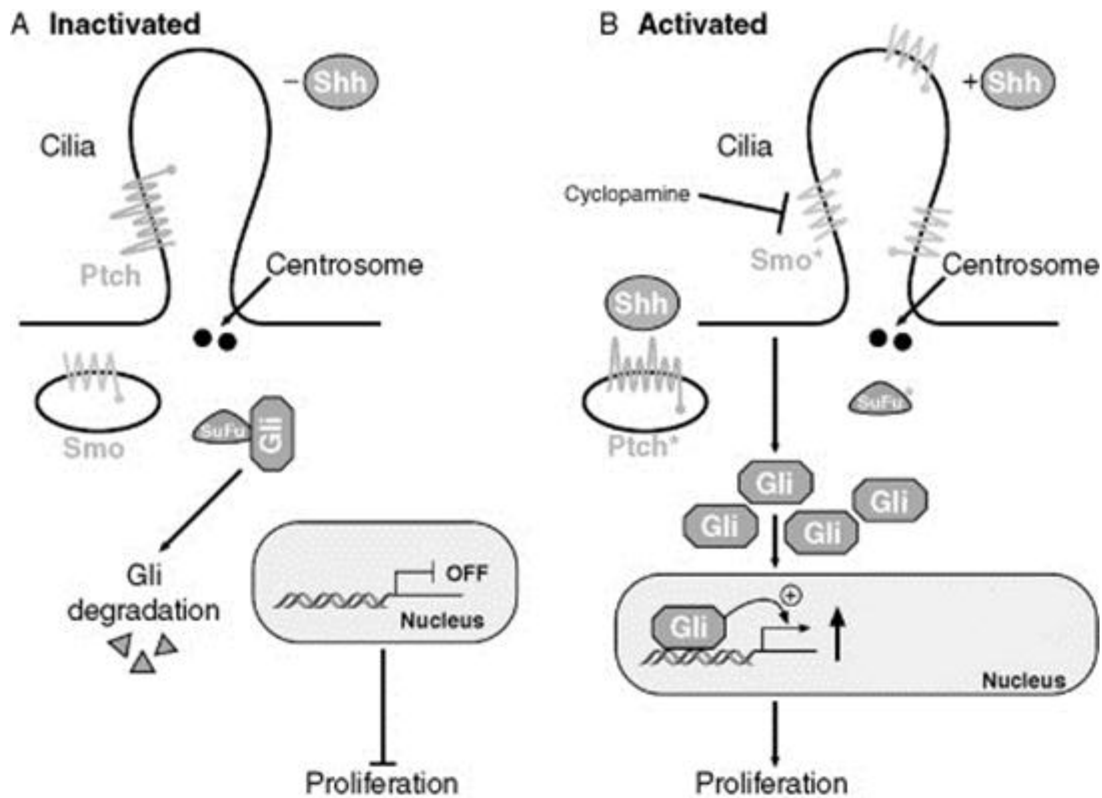

Figure 2. Schematic of the sonic hedgehog signaling pathway in the primary cilium. **(A)** SHH pathway remains inactivated in the absence of Shh, In this scenario, Ptch will be located at the base of the cilium and acts as an inhibitor of Smo located in the cytoplasm. Gli is bound to Sufu which targets Gli for degradation. **(B)** When present, SHH will bind to Ptc1 which leads to a disinhibition of Smo. This leads to the separation of the Gli-SuFu complex. Gli will then act as a transcription factor to drive proliferative transcription of hedgehog target genes (Adopted from 10).

Molecular categorization of medulloblastoma

As mentioned earlier, there are four distinct molecular subtypes of medulloblastoma elucidated through their mRNA expression profiles. These groups are: Wnt, Shh, Group 3, and Group 4.

The WNT-subtype medulloblastomas are driven by *CTNNB1* mutations which drive nuclear accumulation of β-catenin and present with classic histology. Patients with this subtype of medulloblastoma have the best prognosis with more than 95% of the patients surviving. Unfortunately, it is also the least incident tumor, accounting for only about 10% of all medulloblastoma cases. Recent mouse models of *Ctnnb1* transgene with *Trp53* -/- background were shown to develop latent classic medulloblastomas originating from the dorsal brainstem (1, 11).

The SHH-subtype medulloblostoma is driven by aberrant SHH-signaling largely through *Ptch1* mutation and is responsible for the large-cell-anaplastic and desmoplastic presentations of medulloblastoma. The survival rate for patients with SHH medulloblastoma drops down to ~60-80%. A bimodal age distribution of incidence is characteristic in these patients with the susceptibility arising mostly during infancy and adulthood but not during childhood.

Group 3 medulloblastomas have the worst prognosis out of the four subtypes, and is caused by aberrant expression *MYC* which is a proto-oncogene. These medulloblastomas are strictly pediatric and 50% of the cases are shown to be metastatic upon diagnosis. Histologically, they are large-cell or anaplastic.

Group 4 medulloblastomas is the most common subtype accounting for about 40% of the cases. The prognosis is similar to SHH-subtype medulloblastomas. The tumours show classic histology and about one in three patients present with metastases. Proto-oncogenes *MYCN* and cyclin-dependent

kinase 6 (CDK6) amplifications are what drive and distinguish Group 4 medulloblastomas from the rest (1, 4, 11).

With the emergence of molecular markers as powerful prognostic indicators of medulloblastomas, it is now possible to combine both molecular and histopathological stratification for new treatment plans. Around 50% of the SHH medulloblastoma subgroup, for example, uniquely present with a prominent desmoplastic and nodular histology. Even so, certain histological presentations like classic morphology are seen throughout all molecular subgroups and would not provide much information on its own. In contrast, though anaplastic and large cells are present in all four molecular subgroups, its prognostic value is preserved due to the undifferentiated and mitotic nature of the cell types correlating with aggressive tumor. For example, Group 3 tumors which most strongly represent anaplastic and large cell histology have the worst prognosis out of all medulloblastoma subtypes. Despite such indications, the anaplastic and large cell histology hasn't yet been studied thoroughly for each molecular subgroup. Therefore, consolidating molecular and histopathological evaluation of medulloblastomas will allow for a more focused treatment plan through careful risk assessment, not only for prognostic fidelity but also for minimizing side effects (3, 4, 25).

SHH medulloblastoma mouse model

There has been great success in generating the SHH-subtype medulloblastoma mouse model. *Ptch1* mutation as a cause of medulloblastomas was first brought to attention by Gorlin's syndrome patients who exhibited an increased risk of developing medulloblastomas due to an inactivating mutation of *Ptch1.* This led to the development of the first SHH medulloblastoma mouse model heterozygous for *Ptch1*. However, mice that were heterozygous for this gene alone showed low penetrance and high latency in developing medulloblastomas (12). In 2001, it was observed that *Ptch1* +/-; *p53*-null mice (from here on out referred to as Ptch1;p53) had a dramatic rise in medulloblastoma incidence to greater than 95% (13). *P53* is an important tumor suppressor gene that encodes for the transcription factor, Trp53, which inhibits aberrant cell cycle and DNA damage by arresting the cell cycle itself or initiating apoptosis. Because of this, patients with germline *TP53* mutations, such as Li-Fraumeni syndrome patients, exhibit significantly elevated cancer susceptibility (14).

Brain Cancer Stem Cells

Ever since the discovery of stem cells in the haematopoietic system, studies have been made trying to harvest the regenerative capacity that a stem cell therapy could offer. On the other hand, it has also brought into focus the possible role of stem cells in tumor formation. The multipotency of stem cells is

essential to the normal development of organs and tissues in the human body. Their self-renewability is equally important in creating a niche of cells that have the capacity to continually supply and replenish tissues of more committed cell fates. However, if misregulated, the self-renewable nature of stem cells can turn on its biological system to result in tumorigenesis. The implications are quite problematic when considering such small population of freely self-renewing cells could result in the continuous seeding of tumors even upon treatment with conventional bulk cancer therapies such as chemotherapy or radiotherapy (15, 16). To complicate matters further, there have been studies demonstrating how progenitor cells and differentiated cells could acquire stem-like tumorigenic capacities due to accumulated mutations in the stem cells that passed down. What this also implies is that it would be easier for stem cells, which already have self-renewal capacity, to become cancerous through dysregulation rather than having differentiated cells unlocking this potential (15). From these insights, the cancer stem cell (CSC) theory was born.

When stem-like cells in brain tumors were discovered using CD133, a cell surface antigen, the potential for testing the CSC hypothesis on brain tumor arose. A study that eventually stemmed from this idea was the discovery that cancer stem cells in the brain could act as brain tumor initiating cells. Experiments were performed in which CD133+ cells were sorted and isolated from human glioblastomas and medulloblastomas. These cells as well as CD133- brain tumor cells were intracranially xenografted into mice brains and

histologically compared. It was found that CD133+ brain tumor cells recapitulated the heterogeneous patient tumor phenotype while CD133- cells failed to do so, indicating CD133 as a surface marker for neural stem cells (17). While discoveries such as this lend strong support to the CSC theory, it has also opened up the door to the possibility of discovering a potential hierarchy of diverse brain cancer stem cells.

Medulloblastoma brain tumor cells of origin

It has long been established that a subset of SHH subtype medulloblastomas are derived from the external granular layer of the cerebellum and is therefore thought to be caused by CGNP expansion (18). Indeed many studies regarding Shh-pathway mutagenesis in mouse models, such as those involving *Ptc1, SuFu,* and *Smo* show gene expression profiles that support CGNPs as prospective cells of origin (4).

However there is no concrete explanation as to why these progenitor cells are able to become tumorigenic with stem-like characteristics when they are supposedly limited in self-renewal and pluripotency. The fact that there is SHH medulloblastoma incidence for older children and adults who should have depleted their GCPs further highlights this perplexity, and this brought into question whether or not these are true cells of origin (18). As previously discussed, this could be the result of GCPs acquiring a *de novo* mutation that imbues stem-like properties. But it is also likely that there is a population of

aberrant neural stem cells propagating tumorigenesis. Certainly, comparisons between P5 and P7 *Ptch* +/- medulloblastoma gene expression profiles yielded two distinct profiles consistent with this notion. The fact that P5 SHH tumor, which is suggestive of stem cell origin, was highly metastatic while P7 tumors, suggestive of CGNP origin, remained nonmetastatic furthers the validity of this claim (27).

Ptch1 Loss of Heterozygosity and Sox2 expression

Unpublished studies done by ChieYu Lin at Jackson-Grusby's laboratory have suggested that contrary to the two-hit tumor suppressor model in which the first-hit is largely benign, *Ptch1* works synergistically with *p53* to increase SHH medulloblastoma incidence. Upon loss of heterozygosity (LOH) of *Ptch1,* the pre-malignant cells transform into a stem-like metastatic tumor-initiating population. Sox2, an important transcription factor in the maintenance of stem cell identity (28), is vital in the explanation of this observation. Under *in vitro* growth factor withdrawal experiments, epidermal growth factors (EGF) and basic fibroblast growth factors (bFGF) that maintain *Sox2* expression are removed from culture. In this experiment, the aforementioned tumor-initiating population is thought to be able to resist differentiation seen in wild-type cerebellar stem cells under the same condition. This is because Gli2, the downstream effector of the SHH pathway, enhances *Sox2* expression while Sox2 also upregulates *Shh*, creating an auto-feedback loop. The tumor-initiating population is able to resist

differentiation due to the loss of *Ptch1,* the inhibitor of SHH pathway, feeding the auto-feedback loop of *Sox2* expression to maintain stem cell identity (22,23). This highly stem-like tumor-initiating population could therefore retain its stemness and seed metastatic SHH medulloblastomas.

Specific Aims and Objectives

The broad research objective of this study is to determine if the level of GFP expressed by the *Sox2-GFP* reporter transgene represents the self-renewability of isolated cerebellar cell populations. In other words, the overarching goal is to establish a correlation between *Sox2* expression and the stemness of the cerebellar cells. The outcome will determine whether or not Sox2 can be reliably used as a prospective marker for isolating a previously unappreciated cancer stem cell population. This population could be the potential cellular origin of SHH medulloblastomas in Ptch1;p53 mice..

One way to characterize Sox2 as a marker is to compare the different populations of cerebellar cells, particularly the aberrant stem cells and tumor stem cells, under growth factor withdrawal conditions. Growth factors EGF and bFGF are thought to maintain *Sox2* expression by supporting the maintenance of stem cell signaling pathways. Once growth factors are removed, stem cells are unable to maintain their stemness and differentiate.

Preliminary *in vitro* studies from Jackson-Grusby laboratory suggested diminished Sox2 levels upon growth factor removal for 3-week Ptch1;p53 mice

while the same conditions had little effect on Ptch1;p53 tumor Sox2 levels.This proposes that 3-week Ptch1;p53 cells maintain their ability to respond to differentiation cues while Ptch1;p53 tumors resist them. However, this study was done using antibody staining to quantify Sox2 protein levels, and as a consequence lacked the ability to measure *Sox2* expression levels. Therefore the aim of this study is to acquire a higher fidelity measurement of *Sox2* expression through a *Sox2-GFP* transgene mouse model to confirm Sox2 as a functional marker for cerebellar tumor initiating cells.

MATERIALS AND METHODS

Production of mouse chimeras and animal husbandry

Mice harboring the Sox2-GFP reporter were constructed using conventional ES cell technology and crossed to Ptch1;p53 breeding mice. These mice were maintained in pathogen-free conditions at Boston Children's Hospital and procedures were performed following approval by the Institutional Animal Care and Use (IACUC).

Media

Neurosphere media was used to culture cerebellar neurospheres. The media has been optimized for growing and culturing cerebellar stem cells. It contains Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12), basic fibroblast growth factor (bFGF; 20ng/ml), epidermal growth factor (EGF; 20ng/ml), B27 supplement, and penicillin/streptomycin.

Cell isolation from animal tissue

Whole mice cerebellums were individually excised and manually minced with a blade on 100µl NS media. The cells were collected in a 15ml conical vial in 2ml of NS media and dissociated through mechanical trituration media to obtain single cell suspension.

Bulk Culture

5% by volume of the mechanically triturated cerebellum in NS media was plated on to a 60mm petri dish containing 6ml NS media. Passaging of bulk cultures was done by spinning down cell suspension, aspirating media, and mechanically triturating the pellet in 2ml NS media to achieve single cell suspension. Cells were kept in the 37C incubator.

Cell lines

4 different cell lines were selected for the growth factor removal experiment. The P7 WT Sox2-GFP represents a WT cerebellar stem cell population while the non-transgenic P7 WT (-) Sox2-GFP provides a negative control for GFP expression. The 3-week Ptch1;p53 Sox2-GFP will help study the experimental effects on the aberrant stem cell population while the Ptch1;p53 Sox2-GFP tumors can be used as a model SHH tumor stem cell line. Bulk cultures from these representative cell lines were maintained through several passages until ready for growth factor removal study (Table 1).

Table 1. List of cell lines used and their passage history. The number of days indicates how long the cells were in each suspension before passaging.

	1° NS	2° NS	3° NS	4° NS	5° NS	6° NS	7° NS
121022 P7 WT Sox2-GFP	8 days	16 days	11 days	8 days			
121022 P7 WT (-)Sox2-GFP	8 days	16 days	11 days	8 days			
121109 3W Ptch1;p53 Sox2-GFP	7 days	19 days					
120904 Ptch1;p53 Sox2-GFP Tumor	8 days	15 days	6 days	9 days	19 days	29 days	6 days

GF+/-

Growth factor withdrawal

Cells for the growth factor removal experiment were prepared first by genotyping the mice in advance to select desired cell lines. The mice used are outlined in Table 1. The triturated cerebellums were each passaged into bulk neurosphere cultures. Cells were then maintained in NS media through regular passaging until ready for growth factor withdrawal. Cultures were visually confirmed to be fluorescent under the GFP scope before initiating growth factor experiment. A fraction of each bulk culture was FACS analyzed at 0hrs. The rest of each culture was triturated and equal volumes were split into a 15ml conical vial. Both control group suspensions were spun down at 1000rpm for 5 minutes. Supernatants were aspirated and pellets were resuspended with either the growth factor (+) or the growth factor (-) NS media and centrifuged again as a wash step. Finally, the growth factor (+) and (–) cell suspensions were each respectively plated on 60mm petri dishes containing 6ml NS media with or without EGF and bFGF. The cells were then kept in the 37C incubator for 48 hours until ready for FACS analysis (Figure 3).

Flow cytometry

The cerebellar cells were isolated as described above. The cells were then resuspended in FACS buffer, which contains PBS + 2mM EDTA + 0.5% BSA, and centrifuged at 1,000rpm for 5 minutes to wash. The cells were

resuspended in FACS buffer and pipetted through a BD 70 μl cell strainer. Cells were then put on ice until analyzed using a BD LSRII system.

Compensation control was performed using a 121022 P7 WT (-) Sox2-GFP as a negative GFP/unstained control and 120904 Ptch1;p53 Tumor as positive GFP control. To exclude dead cells and debris that inevitably accompany whole cerebellar suspensions, the samples were stained with 7AAD (BD Biosciences) live-dead stain and gated against using forward scatter (FSC)/side scatter (SSC) parameters.

Post-flow cytometry analyses were performed on FlowJo (Tree Star). The program was also used to generate graphs, histograms, and calculate geometric means. FACS analysis experiments were done at HSCI/Joslin Flow Cytometry Core.

Genotyping

Genomic DNAs of mice were obtained by clipping the mice toes at P4-5 for P7 WT mice, P14 for 3-week Ptch1;P53 mice, and P21 for the medulloblastoma mice. The clipped tails were then lysed overnight in lysis buffer + Proteinase K. Genomic DNA was extracted from lysate using standard isopropanol precipitation protocol, and PCR analyzed.

RESULTS

At 48 hours of growth factor withdrawal, the only cell line to show a robust GFP fluorescent population was the Ptch1;p53 tumor line at both (+) and (–) growth factor conditions (Figure 4). This was perplexing since the P7 WT and 3-week Ptch1;p53 bulk cultures contained cerebellar stem cell populations that should be maintaining *Sox2* expression, especially in (+) growth factor NS media.

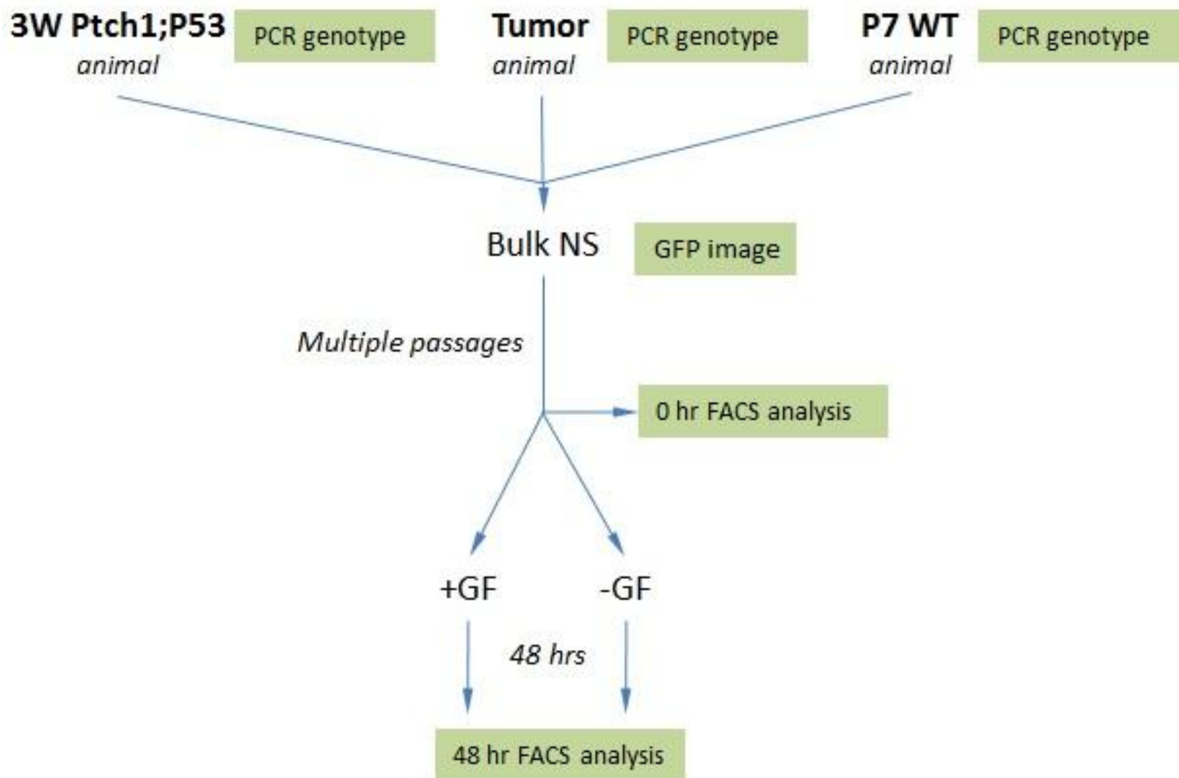

Figure 3. Experimental outline of the 48 hour growth factor withdrawal. 3-week Ptch1;p53 Sox2-GFP, Ptch1;p53 Sox2-GFP tumor, and P7 Ptch1;P53 Sox2-GFP cerebellums were put in bulk culture, passaged regularly, and analyzed before and after 48hr GF withdrawal.

The cell line genotypes were confirmed with PCR genotyping results of mice tail lysates (Not shown). Also, the cultures were visually confirmed to exhibit GFP fluorescence before splitting into growth factor withdrawal treatment groups (not shown). Furthermore, all cell lines were confirmed to be confluent with neurospheres for each passage leading up to the experiment. The fact that genotypically confirmed *Sox2* expressing single cell suspensions were expanding into neurospheres supports the expectation that there are stem-like cells within the culture. With the confidence that there are GFP expressing stem cells represented in the result, alternate means of measuring this population was necessary.

GFP fluorescence histogram reveals distinguishable Sox2-expressing populations

Analyzing the histogram representation of the GFP fluorescence FACS plot provided a competent way of measuring heterogeneous GFP intensities and yielded a surprising discovery on the nature of Sox2 expression. Comparing the GFP fluorescence of the live cell population for P7 WT and 3W Ptch1;p53 under growth factor (+) vs (-) conditions, it was apparent that there is a binary distribution of GFP fluorescence intensity (Figure 5). While P7 WT with growth factors show an appreciable intermediate level of *Sox2* expressing population as well as a low/non-GFP expressing population, the same cell line without growth factors completely lacks the intermediate level population. This suggests that the

removal of growth factors prompted cellular differentiation or apoptosis of the WT

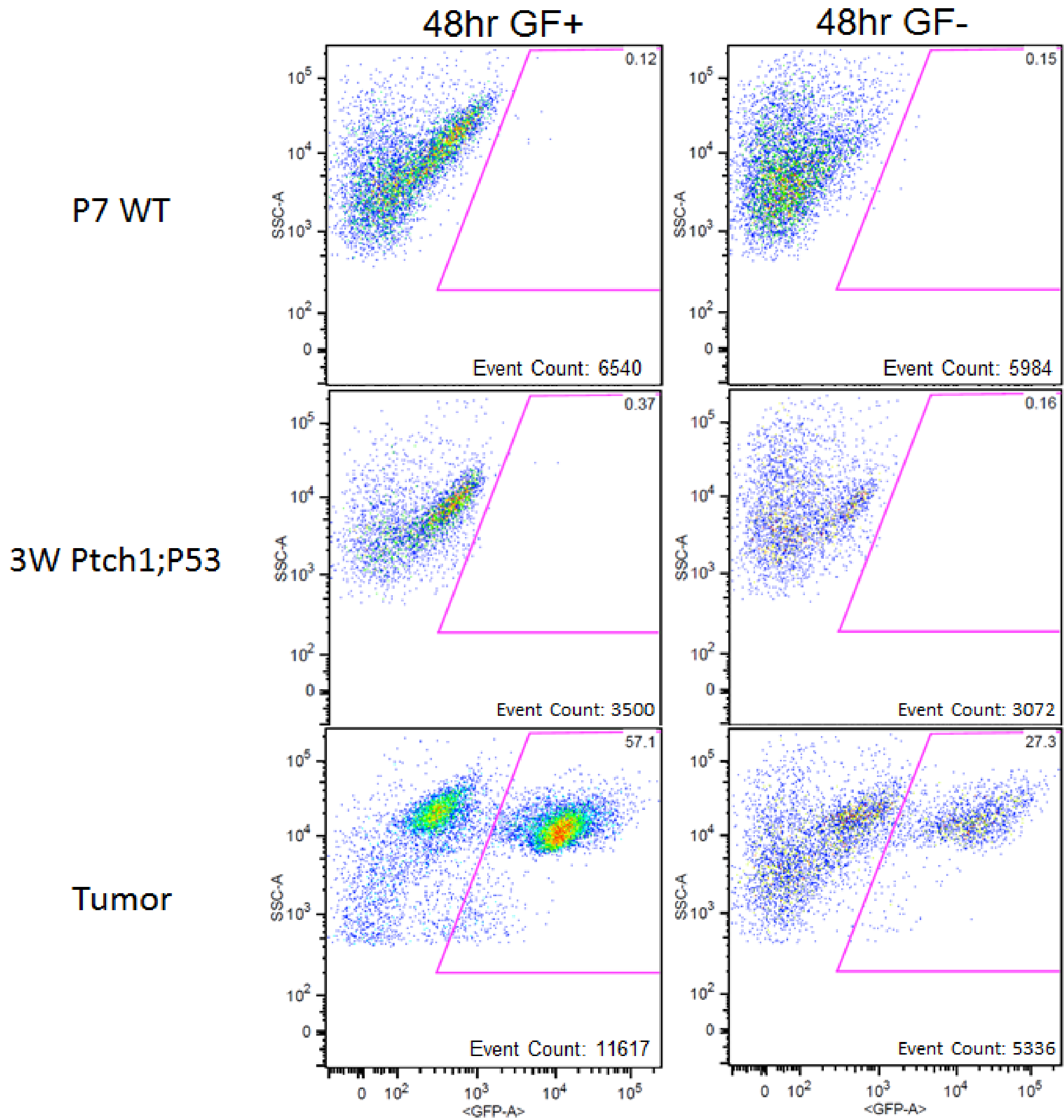

Figure 4. 48hour growth factor withdrawal FACS analysis of GFP fluorescence. Side-by-side comparison of P7 WT, 3W Ptch1;p53, and Ptch1;p53 Tumor under GF(+) and (-) conditions. The graphs represent a spectrum of live cells and their GFP intensity plotted against SSC. The plots have been preliminarily GFP+ gated to visualize strongly GFP expressing cells.

stem cells within the bulk culture. In comparison, the 3W Ptch1;p53, while similar in pattern to the P7 WT, displays a smaller but clear GFP peak under growth factor withdrawal condition. This could indicate that while the aberrant stem cell population retains differentiation capacity, there is subpopulation of these stem cells that might be resistant to differentiation cues or apoptosis upon growth factor removal.

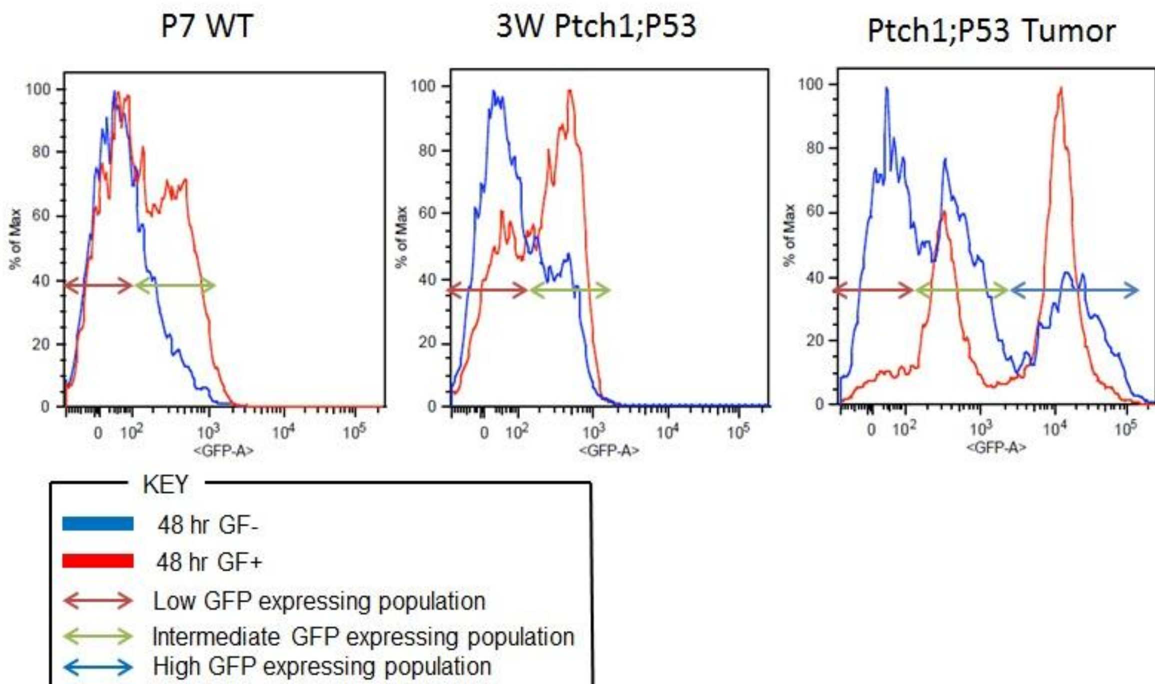

Figure 5. 48 hour growth factor withdrawal GFP fluorescence intensity histogram. Side-by-side comparison of P7 WT, 3W Ptch1;p53, and Ptch1;p53 Tumor under GF(+) and (-) conditions. The graphs represent the GFP fluorescence intensity of live analyzed cell.

Confirming heterogeneity of Sox2-expressing populations

To confirm that the separation of fluorescence peaks represent two distinct population of cells instead of artifacts, the geometric means of the intermediate GFP and low GFP intensity peaks from the 3-week Ptch1;p53 cells were compared (Figure 6). There was over a 7-fold increase in the geometric

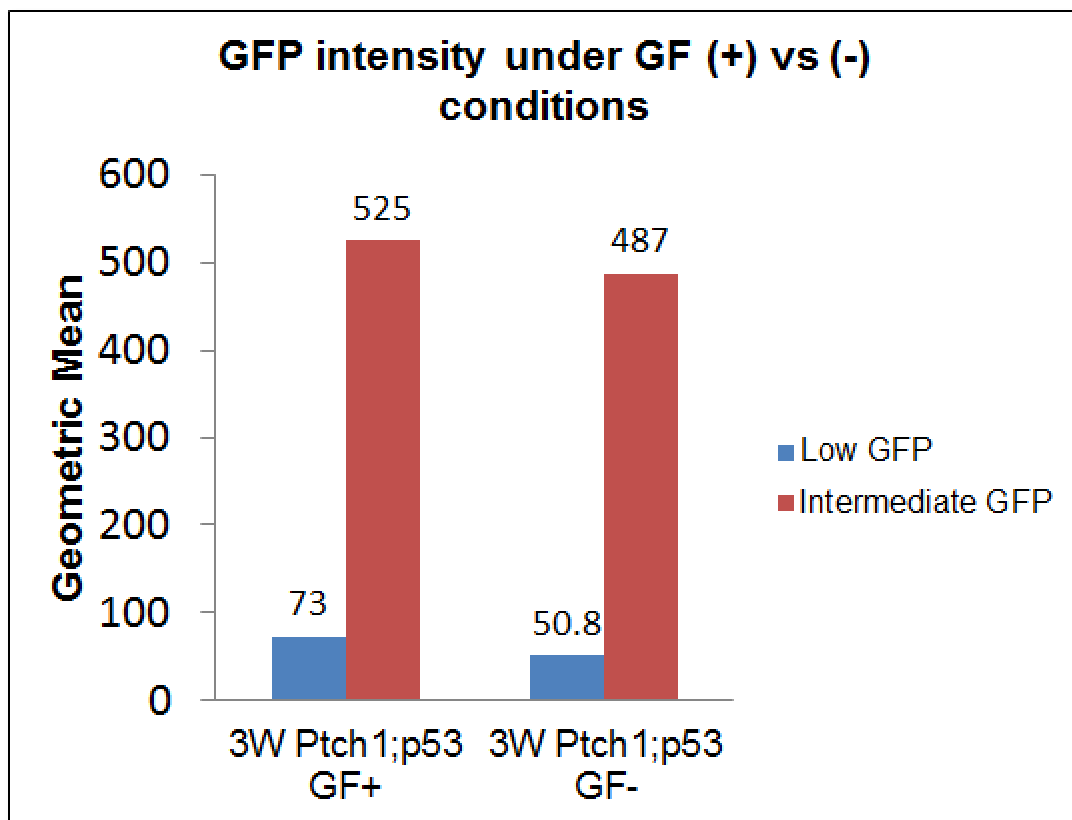

Figure 6. 3W Ptch1;p53 geometric mean comparison of GFP fluorescence intensity between intermediate and low GFP expressing populations.

mean of the intermediate GFP relative to the low GFP fluorescence intensity for the 3-week Ptch1;p53 cells with growth factor. There was also a near 10-fold

increase in geometric mean for 3-week Ptch1;p53 cells without growth factor. The high magnitude separation between the mean GFP fluorescence intensities suggests that there are indeed two distinctly heterogeneous populations of cells differentiated by their GFP fluorescence.

Aberrant stem cells retain differentiation capacity

As mentioned earlier, there was a visible drop of GFP producing 3-week

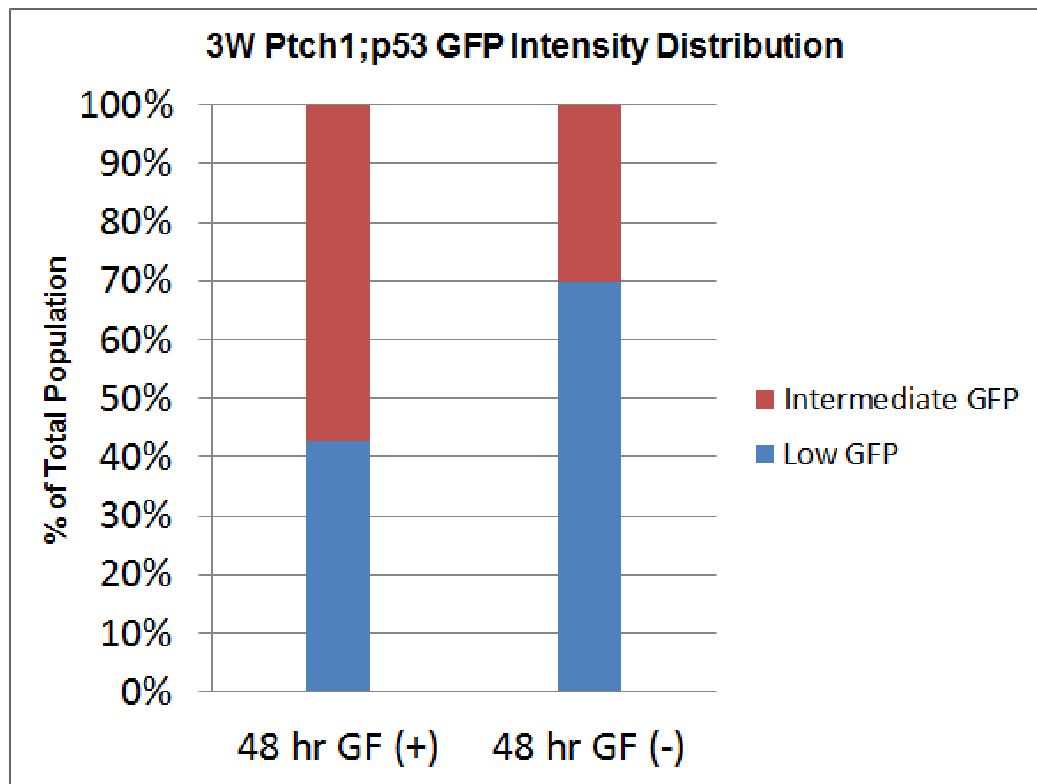

Figure 7. 48 hour growth factor withdrawal GFP fluorescence intensity distribution of 3W Ptch1;p53 cells. The percent total population distribution of low and intermediate GFP fluorescent cells determined by categorizing GFP intensity peaks in the histogram (shown in Figure 5).

Ptch1;p53 cells upon growth factor removal. As a measure of % total population, intermediate GFP expressing cells dropped nearly 30% in growth factor (-) cells compared to (+) cells, suggesting that *Sox2* expression is diminished upon growth factor removal (Figure 7). This reduction in expression is consistent with the fact that growth factor withdrawal cues cerebellar stem cells to differentiate. Thus, the detection of decreased *Sox2* expression in the treatment group coinciding with the decreased stem cell population lends further support to the idea that the Sox2 markers can identify potential medulloblastoma-initiating stem cells. Further, this would also indicate that aberrant stem cells yet retain differentiation capacity although perhaps to a lesser degree than P7 WT.

Ptch1;p53 tumor shows triphasic Sox2 expression pattern and a robustly Sox2 expressing population

The most surprising discovery of this study came from analyzing the GFP fluorescence histogram of the SHH medulloblastoma treatment group (Figure 5). This is due to the clear demonstration of multiple *Sox2* steady state expressions observed between the tumor with and without growth factor. For the (+) growth factor condition, along with the intermediately GFP expressing population there is a strongly GFP expressing population not found in other cell lines. Upon withdrawal of growth factors there's a clear shift of fluorescence from the robustly GFP expressing cells to the to the intermediate GFP expressing cells and to a large population of low/non-GFP expressing cells (Figure 8). The original

prediction that SHH medulloblastoma tumor stem cells are resistant to differentiation cues is partially validated by the persistence of an intermediate and robust GFP expressing population (>60% of the total population) even after growth factor withdrawal.

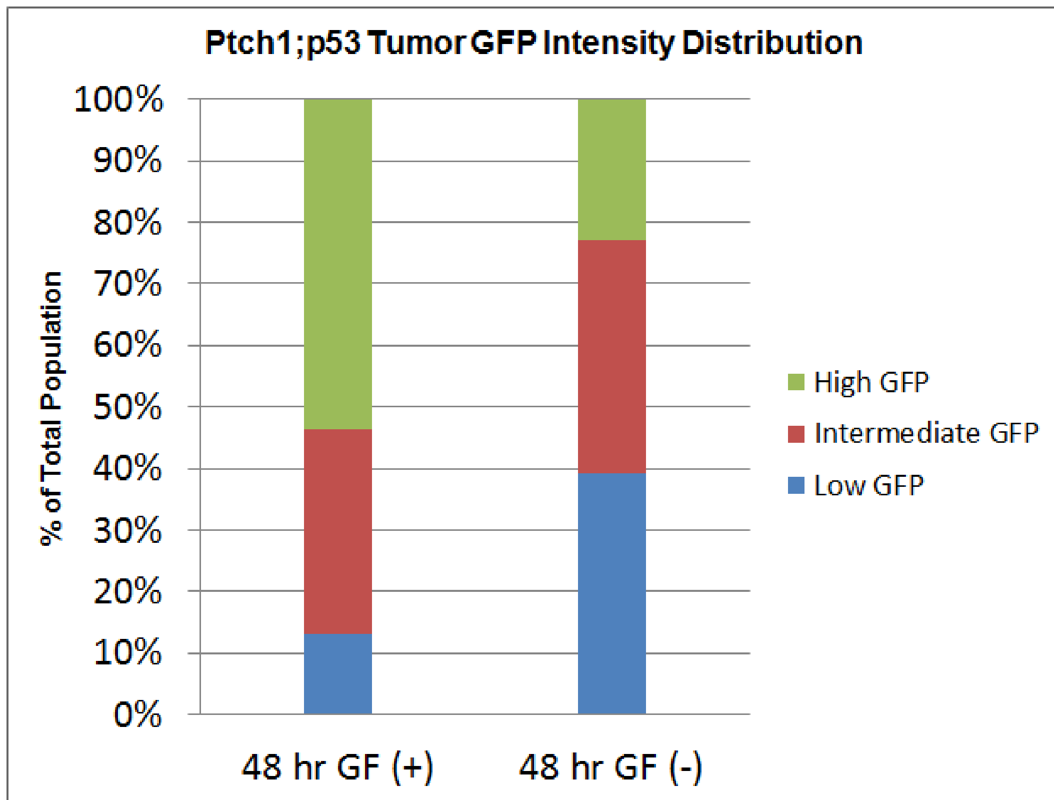

Figure 8. 48 hour growth factor withdrawal GFP fluorescence intensity distribution of Ptch1;p53 tumor cells. The percent total population distribution of low, intermediate, and high GFP fluorescent cells determined by categorizing GFP intensity peaks in the histogram (Figure 5)

A clear triphasic *Sox2* expression pattern also set the tumor GFP histogram apart from the other cell lines. The main indication for this pattern is

that *Sox2* expression isn't binary. Rather, it would be appropriate to view *Sox2* expression as displaying multiple steady states.

Lastly, another unique feature of the tumor stem cells is the presence of a robustly GFP expressing population. The strength of the GFP signal suggests that there might be an amplification of *Sox2* expression. Certainly, recent studies have revealed that Gli2, the downstream effector of SHH pathway, binds to an enhancer that is vital for *Sox2* expression (22). Studies done on hippocampal development proposed that Sox2 itself targets *Shh* as an agonist as well, bringing the causal relationship around full loop (23). This means that *Sox2* would be strongly upregulated in an auto-feedback loop involving the SHH pathway. There is no surprise then that the tumor cells, with highly activated SHH pathway due to *ptch1* LOH, would have a robust GFP signal that is absent in other cell lines.

DISCUSSION

The results of this study show the effect of growth factor withdrawal on the *Sox2* expression of three representative stem cell populations: WT P7 for the wild-type cerebellar stem cell population, 3-week Ptch1;p53 for the aberrant stem cell population, and Ptch1;p53 tumor for the cancer stem cell population.

The original question of whether or not aberrant stem cells retain differentiation capacity while tumor stem cells resist them, has yielded surprising discoveries on the nature of *Sox2* expression and possible implications for the stemness of each tested population.

First of all, the experiments revealed that the potential tumor initiating stem cells, rather than exhibiting an on/off *Sox2* expression, displayed a fluctuation of *Sox2* expressing steady states. The most robustly GFP fluorescent population not present in the other two cell lines demonstrated a possible interplay between *Sox2* and the SHH pathway in which *Ptch1* LOH ramps up the SHH pathway signaling. *Sox2* expression is increased by upregulated Gli, the downstream effector of the SHH pathway, and Sox2 feeds back into the SHH pathway by upregulating *Shh* expression. This potential auto-feedback loop explains the highly proliferative nature of SHH tumor stem cells and may partially explain the reason for their malignant nature when compared to the yet pre-malignant Ptch1;p53 stem cells. The auto-feedback loop also explains why the tumor stem cells seem relatively resistant to differentiation cues upon growth factor withdrawal. The clinical implication of this finding is that upon LOH,

Ptch1;p53 tumor stem cells may act as robust cerebellar tumors that have the ability to retain their stem-like state to confer metastasis.

The study also confirmed the aberrant stem cell population's ability to retain differentiation capacity, as made evident by the large drop in *Sox2* expressing cells upon removal of growth factors. Compared to the wild-type stem cell population which seemed to have completely lost *Sox2* expressing cells under (-) growth factor condition, the aberrant stem cell population could be seen settling to a lower *Sox2* expressing steady state. This relative resistance to differentiation cues could also be explained by *Ptch1* heterozyogisty, which would upregulate SHH pathway that could potentially feed into the *Sox2* auto-feedback loop. Compared to the tumor stem cells with LOH though, this yet haplo-insufficient population would have a relatively weaker upregulation of the SHH pathway and therefore weaker resistance to differentiation cues.

Put together, the growth factor withdrawal experiments implicate Sox2 as a reliable marker for determining the stemness of different cerebellar stem cell populations. Additional characterization studies of Sox2 will help establish it further as a prospective marker for isolating tumor initiating stem cells, and in so doing, it could solidify this aberrant stem cell population as the true cell origins of SHH medulloblastoma. However, in order to ascertain the relevance of the experimental outcome, it is important to discuss some of the issues with the data attained.

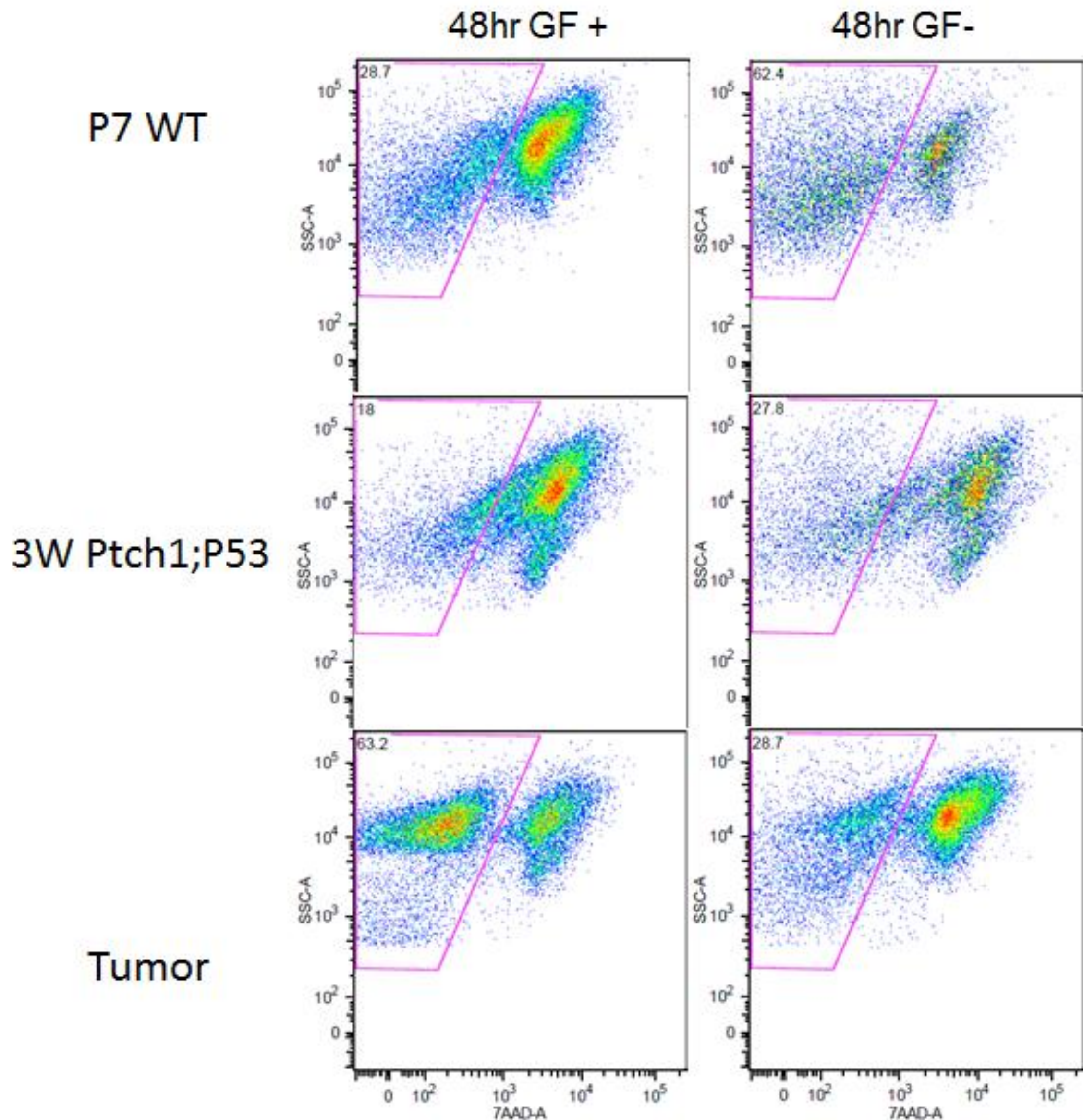

Figure 9. 48 hour growth factor withdrawal FACS analysis of live cell population. Side-by-side comparison of P7 WT, 3W Ptch1;p53, and Ptch1;p53 Tumor under GF(+) and (-) conditions. The plot shows the subset of FSC vs SSC cells that were stained with a with live-dead stain. The gating indicates a conservative live cell gate. The numbers shown on the top left corner for each graph is the percentage of total FSC vs SSC subset that that are live cells according to the gating.

One potential problem with interpreting the experimental data stems from the fact that the observed cells had a high cell death ratio (Figure 9). What's perplexing about this was that there was an extremely high cell death rate for all cultures regardless of growth factor conditions. Such a global increase in cell death implies a problem with the cell preparation. It is entirely possible that the mechanical trituration was too harsh, causing cells to rupture. The issue with this large dead cell population is that it could potentially skew the experimental data. Because it is uncertain what the exact cause of cell death is, it's impossible to say whether the cell death was indiscriminate or targeting a particular group within the culture.

To minimize cell death, some modifications were made to subsequent experiments. Namely, cells were triturated more gently and growth factor removal experiments were performed by allowing a 24hr post-passaging recovery for cells before splitting them into (+) & (-) growth factor media. Also, cells were passaged more regularly to keep them healthier and avoid overgrowth.

An encouraging piece of data from the live cell analysis is the fact that Ptch1;p53 tumor shows a predicted cell death pattern between (+) and (-) growth factor conditions (63.2% live cells and 28.7% live cells, respectively; Figure 9). A recent publication has proved that Sox2 directly upregulates Survivin, an inhibitor of the mitochondria-dependent apoptotic pathway in NSCs (24). Without a compensatory mechanism, decreased *Sox2* expression would therefore lead to cell apoptosis. Because growth factors seem to be required for proper

maintenance of *Sox2* expression, it makes logical sense that the Ptch1;p53 tumors without growth factors have a drastic drop in cell survival due to decreased Survivin.

Another problem that has the potential to skew the experimental data lies in the number of events analyzed per sample on the LSR. For example, the number of cells analyzed for its GFP fluorescence in the 3-week Ptch1;p53 (+) growth factor condition was a mere 3,500 (Figure 4). The scientific standard for FACS requires ~20,000 cells to be analyzed for drawing a meaningful conclusion. Thus, with a small sample size, it is difficult to tell if the fluorescence pattern observed is a true representation of the actual *Sox2* expression profile. Similarly, it is impossible to make statistical significance calculations due to the fact that only one experiment was performed per sample. Altogether,the scarcity of experiments gives less confidence to the data. Therefore, careful planning in subsequent experiments to ensure an adequate number of biological replicates (n=3) as well as sufficient cell numbers for each sample would allow the derivation of a meaningful experimental outcome with more confidence.

Lastly, the experiment could be set up in the future to allow for more prolonged growth factor removal time points. The decision to experiment with the 48 hour time point was largely due to two factors. First, as shown above, there was significant cell death. The study would have required a very large cell population just to be able to have enough cells for an adequate sample size. But even if this was done, the fraction of dead cells would hinder interpreting the

experimental results. Secondly, the kinetics of the *Sox2* transcriptional response is yet largely unknown. This means that the changes observed in GFP fluorescence could be occurring very quickly and therefore require a shorter rather than longer time point of observation. Such reasoning is complicated by the fact that GFP stability is also unknown. If the GFP is very stable, one could expect a slower *Sox2* transcriptional response while a shorter half-life GFP would result in quicker *Sox2* transcriptional action in response to growth factor withdrawal. Because of this transcriptional uncertainty, it is worth looking into different ways of preserving cell survival for longer withdrawal time points. Previous growth factor withdrawal studies were done with substrates (poly-L-lysine coated wells) that cells could adhere to and differentiate. By facilitating differentiation under (-) growth factor conditions, it might be possible to increase the survival of cells that might otherwise be unable to differentiate and die as a consequence

Future Direction

The limitation of using bulk cultures for doing growth factor removal studies is that there is a heterogeneous mixture of cell types within the culture. As seen with the Ptch1;p53 tumor stem cells, *Sox2* expression seems to exhibit varying steady states. Yet without isolating a homogenous group from the heterogeneous mixture it is impossible to tell whether the cells are locked into expressing a certain steady state level of GFP. In other words, it cannot be said

whether the aberrant stem cells and tumor stem cells have a stochastic *Sox2* expression level that will fluctuate at any given time or if highly *Sox2* expressing cells will always remain robustly *Sox2* expressing. In light of results indicating a triphasic *Sox2* expression level, it will be interesting to see if the isolated robustly GFP fluorescent population can regenerate the intermediate and low GFP fluorescent populations.

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