Thusyanth Vijayakumar-Mid Point Report 2014-2015

SHH dependent Medulloblastoma: Sox2 loss-of-function and gain-of-function study

Background:

Cancer remains to be the second leading cause of deaths in children, with central nervous system tumours classified as the leading cause of childhood cancer mortality.[1] Moreover, medulloblastoma (MB), a pediatric intracranial tumour, is contemporarily noted to be the most frequent and malignant of all diagnosed brain tumours in childhood.[1] Through recent improvements in cancer therapy treatments, standard risk MB patients were able to achieve an increased 5-year overall average survival rate of 90% as opposed to 50% previously.[2] However, survivors now face the long-term toxic effects of current treatments that often lead to permanent neurological and developmental defects.[3] Despite the state of the art combinatorial treatment methods from surgical resection to radiation therapy and chemotherapy, patients often experience tumour recurrence and tumour metastasis at primary sites.[2] Inter-tumoural heterogeneity is a cardinal factor for the inefficiency of current chemotherapies targeting multiple cancer pathways.[3] Currently, MB is classified into four subgroups based on severity, age of diagnosis, and metastatic potential: WNT signalling pathway dependent, Sonic Hedgehog (Shh) pathway dependent, Group 3, and Group 4.[3]

The Shh dependent MB is one of the most researched subgroup, as its significance in normal cerebellar development hints at the involvement of stem-like cancer cells. According to the cancer stem cell (CSC) hypothesis, a rare population within a heterogeneous cancer noted as CSCs demonstrates stem cell properties such as self-renewal and multilineage differentiation.[4-6] Brain tumour stem cells (BTSCs) identified by a neural stem cell marker CD133 or CD15 make up the subpopulation that can initiate and maintain a primary brain tumour.[5,6] According to the CSC hypothesis, the BTSCs are not targeted by the standard treatment regime; therefore they repopulate and reformulate a secondary tumour.[5,6] Patient relapse arising from tumour recurrence is what contributes to a poorer survival outcome, as additional surgical resection are deemed inefficient in children due to risks of neurological defects, severe toxic side effects and even death.[7]

We discovered that patients exhibiting poorer clinical outcome such as treatment-resistant Shh-dependent MB actually maintain higher Sox2 expression levels. Sox2, a transcription factor required for normal brain development, has been further characterized as a pluripotent and neural stem cell marker that can now be used as a target for Shh-dependent MBs.[8,9] However, to date we do not have congruent research that describes how Sox2 directly interacts with the agonists of the Shh pathway leading to the treatment refractory population of BTSCs in MBs.[8,9]

References:

1.) Gatta G., et al. Survival of European children and young adults with cancer diagnosed 1995–2002. *Eur J Cancer* **45**, 992–1005 (2009)
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4.) Bonnet, D. & Dick, J.E., Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**, 730-7 (1997).
5.) Singh, S.K. et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* **63**, 5821-8 (2003).
6.) Singh, S.K. et al. Identification of human brain tumour initiating cells. *Nature* **432**, 396-401 (2004).
7.) Louis, D.N. et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* **114**, 97-109 (2007).
8.) Sutter R., Cerebellar stem cells act as medulloblastoma-initiating cells in a mouse model and a neural stem cell signature characterizes a subset of human medulloblastomas, *Oncogene* **29**, 1845–1856 (2010)
9.) Ahlfeld, J., Favaro, R., Pagella, P., Kretzschmar, H.A., Nicolis, S., and Schu¨ ller, U. Sox2 requirement in sonic hedgehog-associated medulloblastoma. *Cancer Res.* **73**, 3796–3807 (2013).

Proposal:

I plan to study the role of Sox2 in maintaining stem cell properties in BTSCs and provide a mechanism as to how these Sox2+ cells elude modern clinical therapies. I aim to achieve this goal through two objectives: 1) Create an *in vitro* chemo-radiotherapy treated Shh-dependent MB cell population that mimics the clinical recurrent BTSC profile. Self-renewal assay, flow cytometric analyses, and comparative gene analyses will be used to obtain a differential signature for primary vs. relapse BTSC population. 2) Create an *in vivo* model by using the Sox2 Shh-dependent MB BTSCs identified from objective 1. NODSCID mice will be serially transplanted with the BTSC population obtained from objective 1, and treated with current clinical chemo-radiotherapies to assess tumour-initiating capacity of BTSCs post-treatment. During various time points of the standard therapy we will collect *in vivo* tumour-engrafted mouse models and compare it to existing controlled xenograft mouse models. Immunohistochemistry, stem cell assays, and gene sequencing will be carried out to verify if the BTSC profile obtained through *in vitro* studies is translatable to the *in vivo* model. Ultimately, the differential signature can be used to generate agonists to target the BTSC population that gives rise to relapse, such that the void of surgery in patient relapse can be filled with effective chemotherapeutic treatments.

During the summer (2014):
What we did: **(Preliminary data from Singh Lab)**
- Through literature we have found in various MB patient cohorts, that Sox2 is highly expressed in group 2 (Shh-dependent MB).
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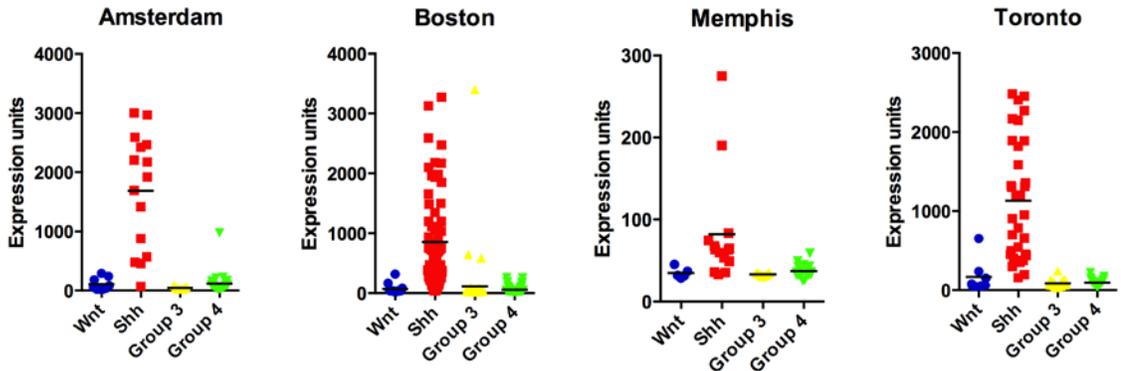
- Through a patient survival analysis we can see that those group 2 MB patients with higher Sox2 expression levels demonstrate lower relapse-free and overall survival percentage.
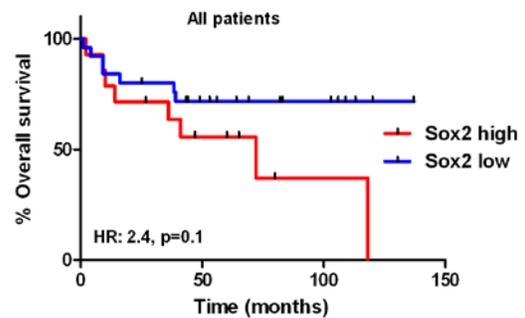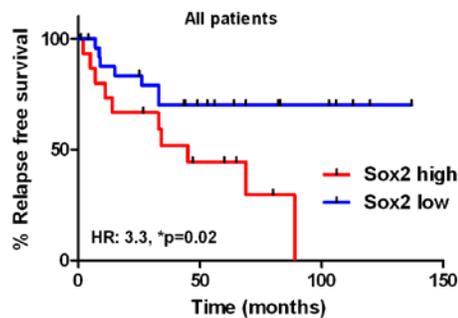
- Of all 4 existing MB subgroups, a smaller group within the SHH(group 2) subgroup that is distinct *via* higher expression of Sox2, display worst patient prognosis.
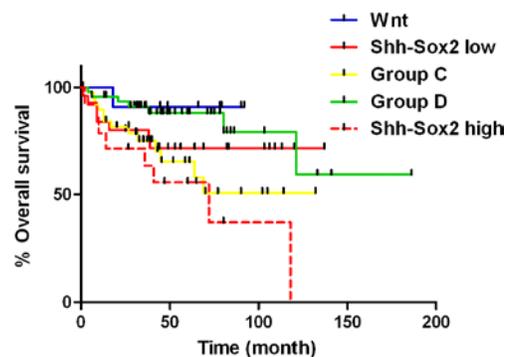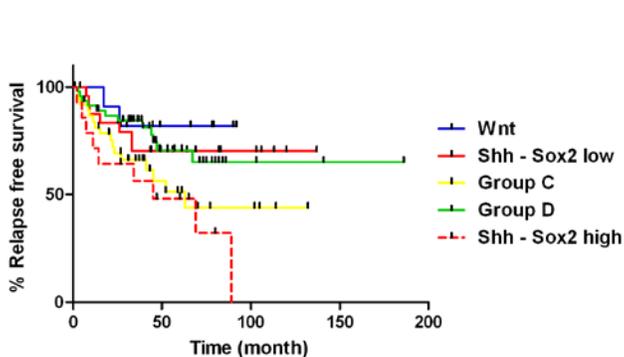
- Thus, the role of Sox2 was selectively chosen for study, to understand its signaling interactions and function(s) within a MB tumour.

Most of the work done was to understand the problem and finding evidence to support it. Training at all levels from molecular to intracranial mouse injections to cell culture techniques were practiced and mastered.

What results we obtained up to date:

- We chose a group 2 cell line, DAOYs and sorted it for CD133+ and CD133-. These cells were also sorted for CD15+ and CD15- using flow cytometry. Those enriched for the markers were assumed to be representative of the BTIC population of the MB tumour samples.
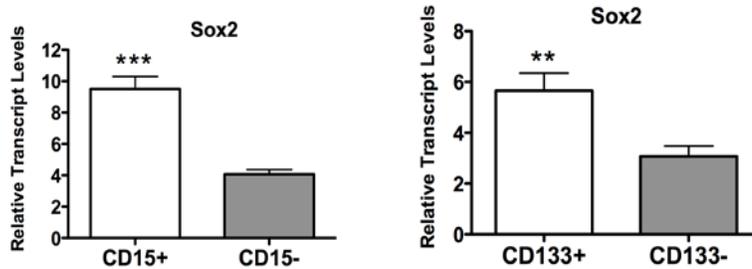
- The BTIC populations of DAOY lines enriched by CD15+ and CD133+ displayed higher Sox2 expression, indicating that Sox2 plays a role in the maintaining and perhaps even initiating the stem-like characteristics of the BTICs. Sox2 could potentially promote the BTICs to acquire the ability to completely reconstitute a complete heterogeneous tumour.

In order to understand the role of Sox2 within the SHH pathway, we needed to evaluate the link between significant factors within the pathway to Sox2. Since we know Gli1 is a down-regulated factor in the Sonic hedgehog pathway, we knocked down and overexpressed Gli1 to examine the effects on Sox2 expression.
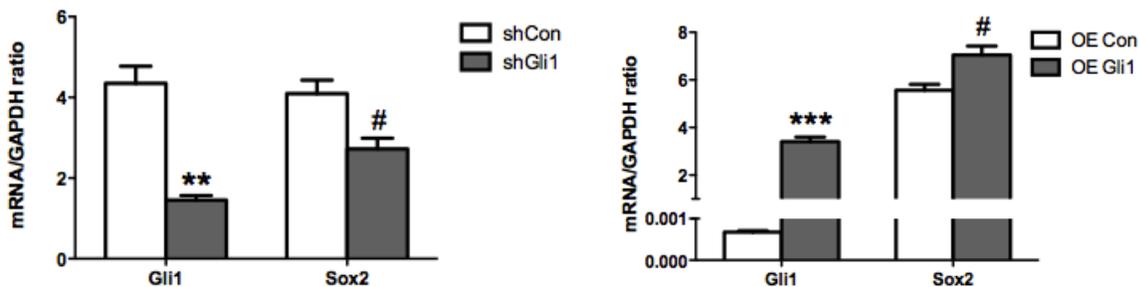
- As demonstrated by our results above, we see that Sox2 expressions fall as Gli1 is knocked down and Sox2 expressions rise as Gli1 is over expressed.

The last set experiments we did near the end of summer and fall term was to optimize knockdowns (KDs) and over-expressions (OEs) of Sox2 in different MB cell lines. Thus, various

vectors were used for transfections and transductions of DAOY cells. Yet the efficiency was not that high.
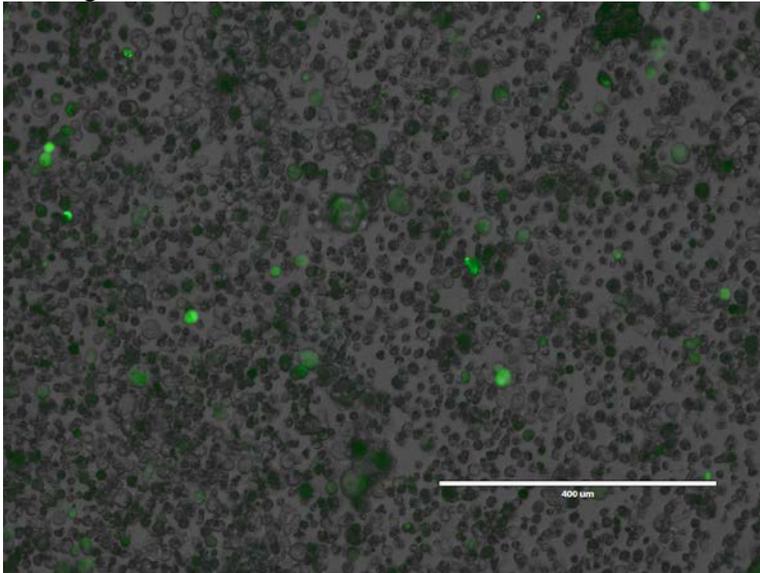
-Transfections that show low efficiency of knockdowns. If GFP marker fluoresces green than it indicates successful transfections or transductions of the vectors. However we do not see much green signals.

In order to enhance the specificity of the KDs and OEs, and increase the efficiency we started to work with a new CRISPR Cas9 technology. The grad students who have mentored me through this stage of the experiments continue to optimize and perfect this technique.

In terms of *in vivo* work, I have tried to optimize a mouse xenograft model that can replicate treatment tumour relapse model. This model has been consistently modified to mimic as much as possible the progression of medulloblastoma tumour in patients with the addition of therapies. The therapies include radiation and chemotherapy (Vincristine, Cisplatin, and Cyclophosphamide).

Mouse model

2 Gy

Sacrifice
7 mice

Chemotherapy

Sacrifice
7 mice

Sacrifice
7 mice

Sacrifice
7 mice

WEEK 1-2

WEEK 3

WEEK 4

TUMOUR RELAPSE!   WEEK 5-6
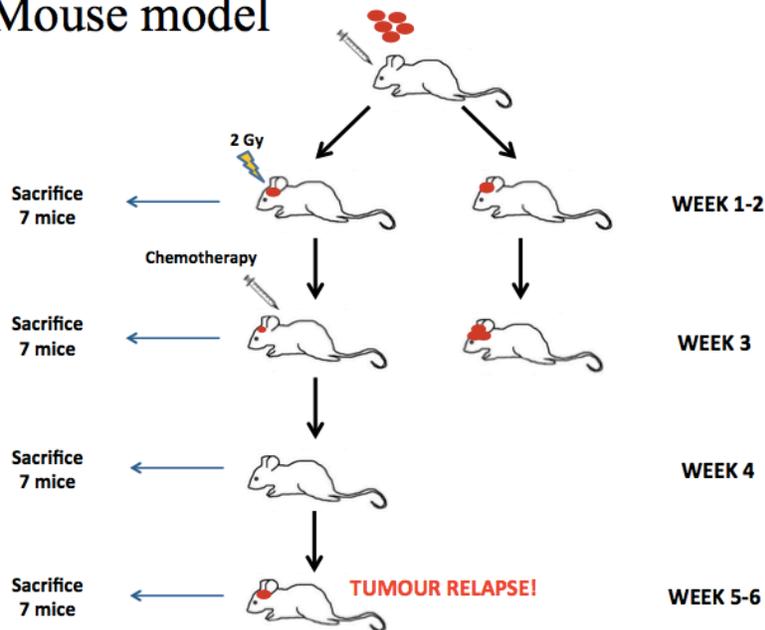
In the following summer I plan to use the animal model displayed above with group 2 MB cells lines such as Daoys to evaluate tumour cells at various time points with the expression of Sox2. There can be a comparison of normal tumour growth and Sox2 expression and how the Sox2 expression varies before, during and after chemo and radiation therapy.

However, first the in vitro experiments of knocking down (and overexpressing) Sox2 and then evaluating the effects on the cells themselves must be done. Thus, westerns and PCR will be done to validate the modulations of the cell lines. Then stem cell assays such as proliferation assays and self-renewal assays will be done to evaluate the functionality of the Sox2 protein and its role in cancer stem cells and treatment evasion.

2015 Summer Plan:

Transfections

Daoys

Another Group 2
cell line

CD133+/- sort

Validation of hypothesis
-transcript and protein
level (Westerns, PCR,
RNA quantification)

Transductions

**Intracranial
injections**

Radiation and
Chemotherapy
treatment
protocol

IHC staining

H & E
staining

Self-renewal
assay

Proliferation
assay