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Brain Tumour Foundation of Canada Medical Student Fellowship Final Report

IMPACT STATEMENT

The Brain Tumour Foundation of Canada summer studentship provided me with the unique opportunity of conducting cutting-edge brain tumour research under the mentorship of Dr. Sheila Singh, while also having the privilege of interacting with patients and their loved ones through events supported by the McMaster Children's Hospital. These memorable experiences served as a strong motivator for my summer research since I was able to reflect on the importance and value of my work whenever there was an unexpected hurdle to overcome. In terms of my exposure to pediatric oncology, my summer research term allowed me to appreciate the difficulties associated with conducting research in pediatric brain tumours as these tumours are rare in the general public, which may limit the number of primary samples that can be collected during the course of a 2-month research term. To further complicate the work we do, not all samples are proliferative, consisting of health cells, which may limit the number of experiments and replicates that can be performed on a given sample. Nevertheless, I was able to develop several new practical research skills in molecular biology. These include: molecular cloning, immunoprecipitation, and chromatin immunoprecipitation. On a more conceptual and theoretical front, I developed greater insight into the dynamic nature of signaling pathways and their role in cancer. For example, the Wnt pathway has long been considered as a promoter of tumorigenesis and indicator of poor prognosis. However, my summer research term has indicated that this notion is truly context-dependent and is not the case in all types of cancer. Therefore, the idea of a constantly evolving pathway with different roles and functions depending on the stage of tumour progression and type of tumour may be considered by some to further complicate our understanding of cancer, but such ideas may also trigger novel avenues for targeted therapy if these pathways can be leveraged at a point during benign tumour growth.

The opportunity to work with Dr. Singh in the clinic and operating room and then to "cross the bridge" by her side to the research laboratory and ask the pertinent questions that were triggered from our clinical encounters has been a priceless and truly motivating experience. Words cannot express the gratitude that I have for programs such as the Brain Tumour Foundation of Canada summer studentship in making such memorable and impactful experiences a reality. Lastly, my summer research has further solidified my desire to merge my research interest in neuro-oncology with a clinical career in neurosurgery focused on surgical neuro-oncology such that I may translate the observations made in the clinic and operating room based on a patient's clinical features and tumour location/size to the laboratory where I may investigate the biology of these tumours and apply these findings to those same patients seen in clinic or the operating room.

INTRODUCTION

Brain tumours represent the leading cause of childhood cancer mortality, due to their highly aggressive and often incurable nature.[1] This proposal aimed to address novel therapeutic opportunities to improve the overall survival and quality of life of children with medulloblastoma (MB) by targeting what we believe to be at the root of the problem: brain tumour-initiating cells (BTICs). These tumour-initiating cells (TICs) (also known as cancer stem cells) represent a relatively small fraction of tumour cells that have the ability to initiate and maintain tumour growth, unlike all other cells of the bulk tumour mass.[2,3]

MB is the most frequent malignant pediatric brain tumour. Multiple integrated genomics platforms have reproducibly shown MB to be categorized into four subgroups, each distinct in terms of prognosis and predicted therapeutic response.[1,4-7] MB subgroups 1 and 2 are characterized by exclusive aberrant activation of the canonical Wnt and Shh signaling pathways, respectively. Activation of the Wnt signaling pathway characterizes a distinct molecular subgroup associated with a favorable prognosis. In contrast, molecular subgroups 2, 3, and 4 are associated with intermediate-poor outcome, with subgroup 3 and 4 tumours being exclusively characterized by metastatic disease and treatment recurrence.[1]

The Wnt protein family regulates cellular interactions during embryogenesis and is essential for normal neural development.[8] Although deregulated Wnt signaling has been implicated in the genesis of a variety of human cancers,[9-11] activated Wnt signaling through beta-catenin (β-catenin) in melanoma and pancreatic cancer has recently been shown to promote the conversion of neoplastic lesions into ostensibly more benign tumours.[12,13] This intriguing observation invites the hypothesis that Wnt signaling can be leveraged clinically to prevent aggressive tumours at the expense of potentially acquiring more benign

and treatable tumours.[13] We aim to utilize our BTIC model system to experimentally apply this rationale to MB patients with poor outcome and treatment-refractory metastatic tumours

Hypothesis: **Activation of the canonical Wnt signaling pathway in human MB subgroup 3 and 4 BTICs will reduce stem cell self-renewal, tumour initiation, and overall disease burden.**

Objective 1: To characterize the *in vitro* effects of activated Wnt signaling in human Daoy and Med8a MB cell line BTICs (subgroup 3) on *in vitro* stem cell properties of self-renewal, proliferation, and differentiation.

Objective 2: To characterize the *in vivo* effects of activated Wnt signaling in human Daoy and Med8a MB cell line BTICs (subgroup 3) on survival and tumour size.

METHODS

Cell culture

Human brain tumour samples were obtained from consenting patients, as approved by the Hamilton Health Sciences/McMaster Health Sciences Research Ethics Board. Briefly, samples were dissociated in artificial cerebrospinal fluid containing 0.2 Wunisch unit/mL Liberase Blendzyme 3 (Roche) filtered through 70 μm cell strainer. Tumour cells were resuspended in tumour sphere medium consisting of a chemically defined serum-free neural stem cell (NSC) medium, and plated in an ultra-low attachment plate (Corning). The components of our complete NSC media have been previously described.[2,3] Daoy (American Type Culture Collection) and Med8a (kind gift from Dr. James Rutka) MB cell lines were also cultured in tumour sphere medium conditions.

Wnt3a-expressing (L-Wnt3a) and control (L-control) mouse L-Cell fibroblasts were obtained from Dr. Bradley Doble as a kind gift. Both, L-Wnt3a and L-control cells were cultured adherently in EMEM supplemented with 10% FBS at a density of $1x10^6$ cells in a 10 cm plate. In order to select for Wnt3a-expressing cells, L-Wnt3a cells were treated with 0.4 mg/mL of G-418. L-Wnt3a cells were not treated with G-418 after intial selection and propagation.

Quantitative real-time-polymerase chain reaction

Total RNA was isolated using the Qiagen RNeasy Micro kit (Qiagen) and reverse transcribed using Invitrogen's Superscript III First Strand Synthesis kit (Invitrogen). Quantitative PCR was performed using the Chroma4 (Bio-Rad) with iQSYBR Green qPCR kit (Quanta VWR). Data were presented as the ratio of the gene of interest to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as control. The program Primer3 (NCBI, Primer-BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design primer sequences.

Western blotting

In order to assess the effect of GSK3 inhibition on cytoplasmic β-catenin accumulation, cells were lysed using a hypotonic lysis buffer and the cytosolic proteins were separated by 10% Bis-Tris SDS-PAGE and blot transferred onto a PVDF membrane. Western blots were probed with a monoclonal rabbit-anti-human β-catenin antibody (1:800,000) and mouse-anti-human GAPDH antibody (1:10,000). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit and antii-mouse IgG (H&L), respectively. The bands were visualized using an Immobilon Western kit (Millipore).

Viral production and transduction

An inducible form of the lentiviral vector EF1α-β-catenin(ΔGSK3)//SV40-PuroR was given as a kind gift from Dr. Bradly Doble. This vector contains an activated form of β-catenin through quad mutations of sites phosphorylated by glycogen synthase kinase-3 (GSK-3) (S33A, S37A, T41A, S45A).[14] The inducible construct was under doxycycline-controlled transcriptional activation, which enabled inducible Wnt activation that may be reversibly turned on or off based on the presence of the tetracycline analogue doxycycline (Tet-On system).[15] In the canonical Wnt pathway, the ultimate outcome of ligand-initiated signal transduction is the activation of genes regulated by TCF/lymphoid enhancer factor (LEF) transcription factors.[16,17] Therefore, our TCF-reporter construct driving GFP (7xTCF-eGFP//SV40-mCherry)[14] was co-transduced with our inducible construct and used to assay TCF activity in MB BTICs in the presence and absence of doxocycline. Presence of activated Wnt signaling was confirmed with GFP

expression from the TCF reporter qRT-PCR for *Axin2*, a well-characterized tissue-independent downstream target gene in the canonical Wnt pathway.

We developed replication-incompetent lentiviruses by co-transfection of the expression vectors and ViraPower packaging mix (Invitrogen). Viral supernatant was harvested 48 hours after transfection, filtered through a 0.45-mm cellulose acetate filter, and precipitated by PEGit (System Biosciences). The viral pellet was resuspended in 0.5 ml of phosphate-buffered saline and stored at 80^{o}C. Daoy and Med8a cells were transduced with lentiviral vectors and treated with the respective antibiotics after 48 hours of transduction to develop stable cell lines.

LiCl Treatment

Daoy and Med8a MB cells were plated at densities of $1x10^6$ cells/well in eight wells of two 6-well ultra-low attachment plates each. CHIR0021 and DMSO vehicle control treatment concentrations included: 0 mM, 10 mM, 20 mM, and 40 mM. Cells were cultured in complete NSC media at 37^{O}C for 48 hours. Treatment with CHIR0021 and DMSO vehicle control were repeated 3 times after which the cells were plated to assess self-renewal capacity, proliferation, and differentiation potential.

Wnt3a treatment

Conditioned media was collected from L-Wnt3a and L-control cells after 4 days of culture. Media were filtered using a 0.2 μm filter from both cell types and stored at 4^{O}C. After 48 hours of culture in native media for Daoy and Med8a cell lines, media was removed and the cells were split using trypsin. Cells were counted using trypan blue exclusion dye with an automated cell counter. $1x10^6$ Daoy cells per plate were then cultured adherently in L-Wnt3a and L-control conditioned media. Daoy cells remained in these culture conditions for 48 hours, after which the cells were split with trypsin, counted, and plated for self-renewal assay, proliferation assay, or used to perform real-time quantitative PCR.

Neurosphere size assay to determine BTIC proliferative potential

Proliferative potential of MB stem cells was determined according to neurosphere size. 200 single cells were plated in a 96-microwell plate in 0.2 mL tumour sphere medium. After 5 days in culture, the size (diameter) of resulting secondary neurospheres was determined using light microscopy and Metamorph 7.1 (Molecular Devices).

Cell proliferation assay

Triplicates of single cells were plated in a 96-well plate (100μL/well) at a density of 1,000 cells/well in complete tumour sphere media and incubated for three days. Alamar Blue (Invitrogen), a Resazurin-based fluorescent indicator of cell metabolism, was added (20μL) to each well approximately 18 hours prior to the readout time point. Fluorescence was measured using a FLUOstar Omega Fluorescence 556 Microplate reader (BMG LABTECH) at excitation and emission wavelengths of 535nm and 600nm respectively, according to recommendations given by the manufacturer. Proliferation was calculated as fold increase in viable cells over day 0.

Secondary sphere formation assay

After primary sphere formation was noted, spheres were dissociated to single cells and replated in tumour sphere medium as previously described.[2,3] To quantify stem cell frequency, the secondary and tertiary sphere formation rate was calculated from the number of spheres formed by 2000 dissociated single cells.

Differentiation assay

Cells were differentiated in NSC media supplemented with 20% fetal bovine serum (FBS) which was replaced every other day. Differentiation was carried out for 7 days after which, differentiated cells were trypsinized and harvested for subsequent gene expression analyses.

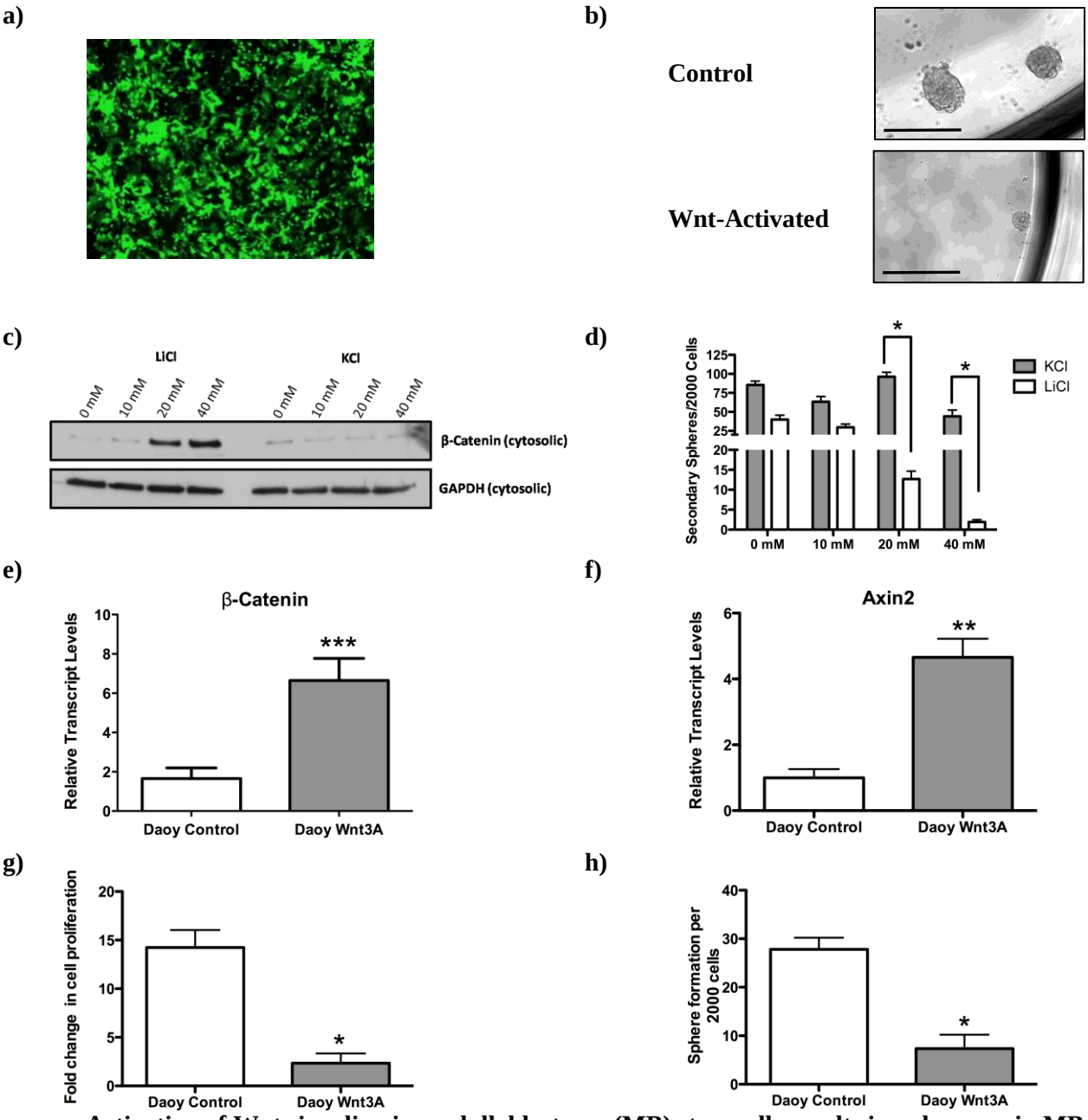
***In vivo* MB stem cell injections & xenograft generation**

$1.0x10^6$ Daoy MB EβP and GFP cells were each injected intracranially into 10 pairs of mice aged 4 weeks as previously described.[3] Briefly, BTIC samples were injected into the right frontal lobe of NOD-CB17-SCID mouse brains according to Research Ethics Board-approved protocols (n=20). Mice were injected with biological replicates consisting of 10^6 single-cell suspensions.

Statistical analysis

For all *in vitro* studies, biological replicates from at least three samples were compiled for each experiment in order to achieve statistical power; unique samples were not pooled before analyses. Data have been represented as mean±s.e.m. Student's *t*-test analyses were performed using the Prism 4.03 software package (GraphPad Software). The independent Student's *t*-test was used to compare continuous variables between two groups. The level of statistical significance was set at 0.05 for all tests.

REPRESENTATIVE RESULTS



Activation of Wnt signaling in medulloblastoma (MB) stem cells results in a decrease in MB stem cell properties. Data presented in the above figures is only representative of the results accumulated throughout the summer term. The data above is from the Daoy (Group 3) MB cell line which was cultured in stem cell conditions to select fro brain tumour-initiating cells (BTICs). This work was also repeated in Med8a (Group 3) MB stem cells with similar findings in a decrease in MB stem cell properties following Wnt activation. (a) Cells treated with both vectors (Wnt-activating and Wnt-reporter) fluoresce green. (b) We then cultured these cells in neural stem cell conditions to select for BTICs and found the tumour

spheres generated from the Wnt-activated cells to be much smaller than our control cells. This finding indicates a decrease in self-renewal and proliferative capacity of Wnt-activated MB stem cells. We then treated tumour spheres with a small molecule GSK3 inhibitor, LiCL, which also activates Wnt signaling. (c) Cytosolic protein levels of β-catenin increased with an increase in concentration of LiCl as indicated by western blotting in comparison to control cells treated with KCl. Given that β-catenin is a downstream target gene for Wnt signaling, we were able to conclude that we had sufficiently activated the pathway in a dose-response fashion. (d) Using the LiCl and KCl treated cells, we assessed secondary sphere formation and showed a decrease in self-renewal capacity in response to increasing doses of LiCl (P=0.0055 at 20mM and P=0.0074 at 40 mM). We then activated Wnt signaling at the receptor level by culturing MB stem cells in Wnt3a ligand conditioned media. Analysis of transcript levels of Wnt signaling target genes (e) *β-catenin* and (f) *Axin2* by qRT-PCR shows an elevated expression in Wnt3A conditioned cells compared to control (P<0.0001 and P=0.0002, respectively). (d) Wnt-activated MB stem cells were also less proliferative when compared to control cells as analyzed by Alamar blue assay (P=0.0286) and (e) displayed a marked reduction in self-renewal capacity as indicated by secondary sphere formation (P=0.0321). *P<0.05, **P<0.005, ***P<0.0001

DISCUSSION

The Wnt family of secreted glycoproteins regulates cellular interactions during embryogenesis and is essential for normal neurogenesis[8]. Deregulated Wnt signaling has been implicated in the genesis of a variety of human cancers, including breast, colon, and MB[9-11]. It has been suggested that cancer cells take over Wnt-mediated stem and progenitor cell self-renewal as part of the mechanism leading to carcinogenesis[18]. However, in cases of melanoma and pancreatic cancer, activation of canonical Wnt signaling, which occurs through the protein, beta-catenin (β-catenin), has recently been associated with the conversion of neoplastic lesions into ostensibly more benign tumours[19,20]. Furthermore, in melanoma, activated Wnt/β-catenin has been shown to synergize with the small-molecule inhibitor, PLX4720, to decrease tumour growth *in vivo* and increase apoptosis *in vitro* of $BRAF^{V60E}$ mutation-driven melanoma[19]. Such intriguing observations invited our hypothesis that Wnt signaling can be leveraged clinically to prevent aggressive tumours at the expense of potentially acquiring more benign and treatable tumours[20]. We have shown through a number of techniques (viral genome integration of Wnt-activating construct, small molecule inhibition of Wnt inhibitors to activate the pathway, and direct activation of the pathway through ligand-receptor interactions) that activated Wnt signaling attenuates the stem cell properties of MB BTICs, thereby making these cells much more response to conventional chemotherapy and radiotherapy.

Our results provide preliminary hope for novel strategies towards the treatment of MB. Proposed experiments for the remainder of my school year include repeating the *in vitro* experiments in surgically-resected primary patient samples and to translate my *in vitro* research findings into an *in vivo* mouse model by performing intracranial injections of Wnt-activated and control MB stem cells. The outcome of this experiment is expected to yield data on the role of activated Wnt signaling in improving survival and reducing MB tumour burden. Our long-term research interests will be to characterize MB BTICs generated from recent Group 3 MB mouse models[21,22]. In collaboration with Dr. Robert Wechsler-Reya, we will co-transduce our inducible Wnt-activating and reporter construct with the characteristic Group 3 Myc-driven construct[21]. In treating mice with doxycycline, we will assess our capacity to reduce tumour growth and prolong survival in mice at different dosages of activated Wnt signaling. We will further study the effects of treating Group 3 MB mouse models with small-molecule GSK3 inhibitors to asses the clinical utility and off-target effects of activating Wnt signaling in a pre-clinical model of Group 3 MB. These experiments will most definitely provide us with a comprehensive understanding of the protective nature of the Wnt pathway within the context of MB. Our hope is to demonstrate novel context-specific roles for Wnt signaling and more importantly, identify novel strategies for treating Group 3 and 4 poor-outcome pediatric medulloblastoma.

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