Exploring Brain-Derived Progenitor Cells as a therapeutic delivery system to Glioblastoma

Andrew T. Olin, Western University

Supervisor: Ronald, John A., The University of Western Ontario
: Hebb, Matthew O, The University of Western Ontario

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Neuroscience

© Andrew T. Olin 2023

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Neuroscience and Neurobiology Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/9605

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.
Abstract

Glioblastoma (GBM) is a devastating incurable malignant brain cancer in need of new treatments. We have begun to investigate the feasibility of a primary adult cell type (Brain-Derived Progenitor Cells, BDPCs) as a novel therapeutic delivery system to GBM. Our objective was to track the viability of BDPCs after intratumoral infusion into syngeneic orthotopic rat GBM tumours using non-invasive bioluminescence imaging (BLI). We hypothesize rat BDPCs will survive greater than 1 week following infusion into orthotopic F98 GBM tumors. BDPCs harvested from the cortex of adult Fischer rats were expanded in culture then engineered to co-express firefly Luciferase for BLI as well as the fluorescence protein tdTomato. In vitro assays displayed consistent lentiviral engineering of transgenes as well as statistically significant GBM-homing by BDPCs (p < 0.01). All animals showed in vivo BLI signal until the study’s endpoint, confirming viable BDPCs were still present. Histological examination revealed small numbers of fluorescent BDPCs at the tumours’ invading edges in frozen coronal sections.

Keywords

Glioblastoma (GBM), Neural Stem Cell (NSC), Brain-Derived Progenitor Cell (BDPC), Cell Migration, Lentiviral Engineering, Molecular Imaging, Bioluminescence Imaging (BLI), Fluorescence Microscopy, Cell-Based Therapy (CBT)
Summary for Lay Audience

Glioblastoma (GBM) is an aggressive type of brain cancer that currently has no cure. Despite a multidisciplinary standard of treatment, fewer than 10% of patients survive 5 years post-diagnosis. However, recent research has shown that certain stem cells may be used as a promising therapy for treating GBM. The aim of this work was to investigate the potential of Brain-Derived Progenitor Cells (BDPCs) for delivering therapeutic agents to GBM tumours. BDPCs can be safely obtained from surgical patients, then cultured and engineered in the research laboratory. By engineering BDPCs to emit light under specific conditions, we hypothesized that we would be able to track their viability inside of a rat tumour. The results of this experiment would inform downstream experiments aimed at determining the optimal conditions necessary for achieving BDPCs’ therapeutic delivery to GBM.

The study used a combination of in vitro and in vivo experiments to test the feasibility of BDPCs as therapeutic delivery system for GBM. In vitro experiments involved growing BDPCs in special culture chambers and testing their ability to migrate towards GBM cells. In vivo experiments involved injecting engineered BDPCs into rats’ GBM tumours to see if they would remain viable and for how long.

The results of the study showed that both human and rat BDPCs did significantly migrate towards their respective glioblastoma cells in vitro. Furthermore, BDPCs engineered with molecular imaging transgenes remained viable in our brain cancer model by BLI signal until study endpoint. This manuscript offers promising evidence that BPDC-based therapy could be a valuable tool for treating GBM in the future. By using these specialized cells to deliver targeted therapies, we hope to improve treatment outcomes and ultimately find a cure for this devastating disease.
Acknowledgements

This work was made possible by the efforts of many people, and all the help and support, big and small made a difference along the way. I’d like to start by thanking my academic supervisors Drs. John Ronald and Matthew Hebb. Your instruction in and out of the lab taught me volumes about performing and communicating robust science. Your patience, support, and concern taught me about compassionate leadership and dedication in the face of adversity. I’m honored to contribute to the groundbreaking research in your labs.

This is certainly not without the support of many dedicated scientists, students, and professionals all working towards the collective success of the group; so many of whom were a direct influence on myself and this body of work. Cheryl Johnson, Hu Xu, Andrew Deweyert, Simon Benoit, John Kelly, Amanda Hamilton, Nourhan Shalaby, Xin Yue Wang and many more from the Hebb and Ronald Labs and the CMIG kept me moving forward during this exhilarating period of education. Dr. S. Whitehead and his researchers graciously allowed use of their microscopy suite. Dr. Kim Chadwick generously provided instruction and support for the flow cytometry experiments. I’m grateful for the ACVS staff who housed and cared for our research animals. Susan Simpson and Lara Staeccker of the Neuroscience Graduate Program were crucial in helping me navigate this submission, and my enrollment in the program overall. Your understanding, flexibility, and support is a large reason this work can be shared here. I’m grateful to my advisory committee of Drs. Stephen Pasternak, Cheryle Séguin, Lauren Flynn, and Timothy Scholl for their instruction and feedback in designing and reporting on the experiments in this text, as well as improving my public speaking and presentation skills.

To all the family and friends who helped me get to London, watch our beloved dog Ichi, send care packages, and continually support my journey, I hope to always be able to repay the favor. To my siblings, thank you for being the constant inspiration of how to TCOB in every aspect of my life. To my parents and grandparents, thank you for the opportunities and support that led me here, and the resilience to keep going when knocked down, I needed all of it. To the Lepaks, I’m honored to share this work on behalf of my Aunt Anne, who fought GBM for nearly 6 years, overlapping with my entry to the Neuroscience Program at Western. We were beyond lucky to have this time with you, against all odds.

To Joshua Dierolf, thanks so much for your time, support, and friendship. To Jenny Hodges and our lab mates, thank you for being the latest piece of the puzzle supporting my completion of this work.

To A.C., C.K., and R.B., you’re missed every day, see you on the other side.

A minha mágica Leiriane, valeu pra cuidar de mim, sempre vou cuidar de você.

To the patients who provided samples allowing us to perform these experiments, thank you for your trust and bravery. To the animals used in our studies, thank you for your sacrifice, so that we may learn how to treat and save the patients to come.
# Table of Contents

Abstract .......................................................................................................................... ii
Keywords ......................................................................................................................... ii
Summary for Lay Audience ............................................................................................. iii
Acknowledgements ........................................................................................................ iv
Table of Contents ........................................................................................................... v
List of Figures ................................................................................................................ viii
List of Appendices ......................................................................................................... ix
List of Abbreviations ..................................................................................................... x

Chapter 1 ......................................................................................................................... 1

1 General Introduction .................................................................................................... 1

1.1 Glioblastoma ............................................................................................................. 1

1.2 Standard Treatment of Glioblastoma ....................................................................... 3

1.3 Experimental Treatment of Glioblastoma ............................................................... 4

1.3.1 Cell-Based Treatment of Glioblastoma ............................................................... 5

1.4 BDPCs ....................................................................................................................... 6

1.5 In Vitro Cell Migration Assays ................................................................................ 7

1.6 Preclinical GBM Models .......................................................................................... 9

1.6.1 F98 Fischer Rat GBM Model ............................................................................ 10

1.7 Bioluminescence Imaging (BLI) ........................................................................... 10

1.8 Thesis Overview ....................................................................................................... 11

1.9 References ............................................................................................................... 13

Chapter 2 ....................................................................................................................... 20

2 Evaluating BDPC Viability in a syngeneic orthotopic rat glioma model .................. 20
2.1 Introduction .................................................................................. 20
2.2 Materials and Methods ................................................................. 21
   2.2.1 Cell Culture and Derivations ................................................. 21
   2.2.2 BDPC Engineering ............................................................... 22
   2.2.3 In Vitro BDPC Characterization ........................................... 23
   2.2.4 Syngeneic Fischer Rat BDPC + GBM Model ......................... 25
   2.2.5 In Vivo BLI ........................................................................... 25
   2.2.6 Histology ............................................................................ 26
   2.2.7 Statistics ............................................................................ 26
2.3 Results ......................................................................................... 27
   2.3.1 In Vitro screening shows migratory BDPCs can be stably engineered ...... 27
   2.3.2 Implanted engineered BDPCs remain viable in orthotopic F98 tumours ... 29
   2.3.3 Microscopic examination identifies tdT-positive BDPCs in F98 tumours .. 31
2.4 Discussion .................................................................................... 34
2.5 References ................................................................................... 37

Chapter 3 .......................................................................................... 40

3  Summary and Discussion ............................................................... 40
  3.1 Summary of Findings ................................................................. 40
  3.2 Challenges and Limitations ....................................................... 43
  3.3 Future Directions ..................................................................... 46
  3.4 Summary .................................................................................. 47
  3.5 References ............................................................................. 49

Appendix ......................................................................................... 52

App A In vivo intratumoral BDPC injection study design ..................... 52
App B Animal Research Ethics Approval (2018-025) .......................... 53
App C Animal Research Ethics Approval (2018-026) ........................................ 56
Curriculum Vitae .................................................................................................. 59
List of Figures

<table>
<thead>
<tr>
<th>Figure 1-1</th>
<th>Bioluminescence chemical reaction equation</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2-1</td>
<td>Study design and Lentiviral engineering</td>
<td>27</td>
</tr>
<tr>
<td>Figure 2-2</td>
<td>Multimodal <em>in vitro</em> confirmation of BDPC engineering</td>
<td>28</td>
</tr>
<tr>
<td>Figure 2-3</td>
<td>Transwell migration assays showed increased tropism of hBDPCs and rBDPCs towards glioma cultures</td>
<td>29</td>
</tr>
<tr>
<td>Figure 2-4</td>
<td>Intratumoral BDPC viability shown by longitudinal BLI</td>
<td>30</td>
</tr>
<tr>
<td>Figure 2-5</td>
<td>Brightfield and fluorescence microscopy show characteristics of orthotopic F98 tumours</td>
<td>32</td>
</tr>
<tr>
<td>Figure 2-6</td>
<td>Intratumoral tdTomato-positive BDPCs identified by confocal fluorescence microscopy</td>
<td>33</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix A- *In vivo* intratumoral BDPC injection study design .......................... 52

Appendix B- Animal Research Ethics Approval 2018-025 ........................................ 53

Appendix C- Animal Research Ethics Approval 2018-026 .......................................... 56
List of Abbreviations

GBM: Glioblastoma
CNS: Central nervous system
BBB: Blood-brain barrier
BBTB: Blood-brain-tumour barrier
TMZ: Temozolomide
PFS: Progression-free survival
OS: Overall survival
EGFR: Epidermal growth factor receptor
IDH: Isocitrate dehydrogenase
iNSC: induced Neural stem cell
tdNSC: transdifferentiated neural stem cell
ad-MSC: adipose derived Mesenchymal stem cell
MRI: Magnetic resonance imaging
tdT: tdTomato; red fluorescence protein
FACS: Fluorescence-activated cell sorting
BLI: Bioluminescence imaging
CSCs: Cancer stem cells
PET: Positron Emission tomography
MGMT: O-6-Methylguanine-DNA Methyltransferase
IHC: Immunohistochemistry
ICC: Immunocytochemistry
DAPI: 4',6-Diamidino-2-Phenylindole
IMT: Intratumoural Modulation Therapy
TRAIL: Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand
1 General Introduction

1.1 Glioblastoma

As the leading cause of death in Canada, there remain countless opportunities to improve our understanding of cancer and its treatments. It was estimated that over 150,000 new cancer diagnoses would be made in Canada in 2021. There exists a range in mortality; dependent on the type of cancer, the timing of diagnosis, and access to and availability of treatment options. In the case of brain cancers, there is a great disparity between diagnosed and recovered numbers, as the incidence ranks 18th yet mortality ranks 9th. These cancers are difficult to diagnose and treat, due to several factors that are described below.

Brain cancers are divided, among other ways, by whether they are malignant or non-malignant. Gliomas represent ~30% of all brain cancer cases, with glioblastoma (GBM, a malignant High-Grade Glioma) representing nearly half of all malignant brain cancers (incidence rate ~ 4 per 100,000 people). These cancers arise from glial cells, non-neuronal cell types found in the brain. Within the CNS glial family, there are astrocytes, microglia, ependymal cells, and oligodendrocytes, whose functions include structural support, cellular waste distribution, and modulating signaling events. The classification of gliomas include astrocytomas, oligodendrogliomas, and mixed neuronal-glial tumours, each characterized by distinct differences in cellular morphology, growth rates, and invasion/migration states.

Gliomas are also delineated by grades I through IV; lower-grade gliomas include pilocytic astrocytoma (I) and diffuse astrocytoma, IDH-Mutant (II) while high-grade gliomas include anaplastic oligodendroglioma, IDH-mutant (III) and glioblastoma (IV). Low-grade gliomas carry modest survival times of several years, and some can be cured by surgical resection, which is not possible in HGGs such as GBM. High-grade gliomas suffer a more dismal outcome, and in particular, the WHO Grade IV GBM is considered the most aggressive, with survival times measured in months rather than years.

As previously stated, gliomas comprise ~1/3 of newly diagnosed brain cancers, however, the malignant, high-grade glioblastoma accounts for ~1/2 of all gliomas. These are markedly
more aggressive tumours than lower-grade gliomas, with progression-free survival (PFS) of 8 and overall survival (OS) of 15 months even with treatment. Despite treatment, patients inevitably succumb to the invasive and recurrent GBM\textsuperscript{4}. GBM can be divided between primary and secondary tumours, with the significant majority (>80%) being primary\textsuperscript{38}. Primary have arisen “de novo” presumably from a patient’s own genomic mutations, while secondary have a previous ontology, such as a low-grade glioma that has progressed to glioblastoma\textsuperscript{7}. GBM is marked by nuclear polymorphisms, hypoxia, necrosis, and its ability to invade from one brain region to another, seemingly unchecked\textsuperscript{9}.

The brain is shielded from the rest of the body by the blood-brain barrier (BBB), a network of vasculature-that allows for selective molecular transport as well as immunologic protection\textsuperscript{10}. Of note with GBM is its insidious way of disrupting and modifying the BBB, including creating a Blood-Brain-Tumour Barrier (BBTB)\textsuperscript{11}. This protects the tumour from CNS-immunologic circulating cells and is also used to send signaling molecules to initiate further vascularization\textsuperscript{10}. It also makes treating GBM with conventional systemic chemotherapies even more challenging, as most of the molecules of interest will not cross the normal BBB due to size, polarity, or chemical structure\textsuperscript{12}. Even if they can, as in the lone approved chemotherapeutic temozolomide (TMZ), their distribution within the tumour will be reduced due to this microenvironmental restructuring\textsuperscript{11,19}. The growing tumour will either exert pressure on neighboring areas or overtake said region with infiltrative growth. Both options provide considerable problems to the eloquent nuclei of the brain - as well as their cognitive and life-sustaining functions, making total surgical resection of GBM virtually impossible\textsuperscript{13}.

Generally, these tumours display mutations in gene expression as well as epigenetic markers, such as EGFR, IDH, and MGMT-hypermethylation\textsuperscript{14}. The expression profiles vary from patient to patient, and from core to periphery within a single patient’s GBM\textsuperscript{22,32}. Due to this heterogeneity a lone biopsy may not be fully representative of the molecular makeup of a GBM tumour\textsuperscript{10,38}. While we’ll discuss total treatment below, like other cancers, these mutations can affect treatment outcomes\textsuperscript{14}. This remains a complex issue for all brain cancers, as many are not diagnosed until there is major mobility, sensory or cognitive problem in the patient\textsuperscript{4,9}. This is particularly true in high-grade gliomas, i.e., GBM, whose patients do not have favorable outcomes even with access to treatment.
Cancer stem cells (CSCs) have been linked to initiation and recurrence of GBM and other cancers. They, like non-cancer stem cells, are proliferative and regenerative, as well as not having a terminally differentiated state\textsuperscript{56,63}. Their durability in the face of standard treatment also leads to increased severity upon GBM recurrence. Researchers are now focusing on treatments that can address yet another complication in providing care to GBM patients, but this is an emerging field and requires more study\textsuperscript{56}. Standard treatments will be described, but unfortunately, the prognosis is generally bleak. Fewer than 10\% of patients survive 5 years, and most succumb to a recurrent form of their GBM within 2 years\textsuperscript{16}. This publication aims to share findings of the preclinical development of a novel cellular delivery system to a GBM model, with the overall goal of formulating novel treatments for this fatal brain cancer.

1.2 Standard Treatment of GBM 

This section describes the current standard of treatment for GBM, which yet remains incurable. Overall, glioma treatments depend on the grade, location, and morphology of the tumour\textsuperscript{7}. In the case of high-grade GBM, the treatments are dependent on the location and morphology of the tumour, however, they may include surgical resection to relieve the patient from comorbidities associated with the aggressive tumour growth\textsuperscript{16}. This could include impairment or loss of sensory and motor functions or severe neurological disorders such as epilepsy\textsuperscript{13}.

Unfortunately, the ubiquitous and capable nature of glia means when these cells transform into a neoplasm, they can easily encompass multiple regions of the brain\textsuperscript{13}. This makes resection problematic, as the surgeon must balance removing as much of the tumour without disrupting vital processes. Even when the glioma is compact, rather than diffuse and infiltrative, it may be in a surgically inaccessible area, obscured by numerous eloquent regions. Due to the heterogeneity and complexity of GBM, there is a multidisciplinary approach to treatment, known as the Stupp Protocol\textsuperscript{19}. Together with maximal safe surgical resection, there is radiation therapy delivering 60 Gy over 6 weeks and the lone approved chemotherapeutic, TMZ. TMZ is delivered at 75 mg/m\textsuperscript{2} per day during radiation, followed by adjuvant dosing of 150-200 mg/kg\textsuperscript{2} per day over 6 cycles\textsuperscript{19}.

Median survival is as follows: without treatment = 8 months, surgical resection and radiation = 12 months, and surgical resection with combined radiation + TMZ = 14.6 months\textsuperscript{19,20}. The
prognosis for patients who are not able to undergo full therapy is typically reduced. Of note, these treatments do not change survival dynamics, solely extending the duration of life post-diagnosis. The recurrent nature of glioblastoma is such that >90% of patients succumb to their malignancies within 5 years\textsuperscript{9}. While we do observe an extension of survival dynamics when practicing the Stupp protocol, there remain many challenges to curing patients of malignant glioblastoma\textsuperscript{19}.

Most chemotherapeutic agents cannot cross the blood-brain barrier, and even those that can - such as TMZ, may not be fully delivered to the tumour because it has established a BBTB which can insulate it from such therapeutics due to alterations in permeability\textsuperscript{11}. There is also the cancer stem-cell niche, which is inherently treatment-resistant as well as regenerative\textsuperscript{56,63}. It can initiate GBM recurrence from a small number of cells that remain post-surgical resection.

Recently a novel yet cumbersome cranial stimulation device known as Optune, which delivers tumor treating fields to the brain, has been introduced in the clinic as an added modality to standard treatment\textsuperscript{62}. Further advances in electrode technology and prescribed stimulation parameters have improved on its initial successes\textsuperscript{27}. New technologies to aid in conventional treatments such as gamma knife radiosurgery and fluorescence-guided surgery\textsuperscript{28,29} are also being used in the clinic. While early clinical data is promising, similarly to the Stupp protocol, these are not curative therapies. For decades there have been groups developing alternative and experimental treatments for GBM to circumvent these challenges, which will be partially elucidated in the following section.

1.3 Experimental Treatment of GBM

As GBM remains incurable, many groups have tried to increase the efficacy of GBM treatments. Throughout this text we will cover the more recent successes in preclinical research toward GBM therapies, as well as highlighting the needs for further development. While the traditional chemotherapeutic agent TMZ added months to many GBM patients’ lives, that was introduced to the market ~20 years ago\textsuperscript{20}. Depending on the regulation of the patient’s MGMT gene this may not even benefit ~1/3 to 1/2 of GBM patients\textsuperscript{21}. Another limitation of conventional treatment is the delivery to, and distribution within the tumour, as the alterations in the BBB and BBTB may affect the ability of TMZ to be distributed to the
entire GBM\textsuperscript{22}. While this highlights the need for a dynamic and multidisciplinary approach, ongoing GBM characterization shows this malignancy is ever more complex and resilient than the Stupp Protocol can address\textsuperscript{19,20,32,36,45}.

In attempts to circumvent these factors, numerous techniques have been tested, including novel and repurposed chemotherapeutics, radiation techniques, and implantable therapeutic molecules, with mixed successes\textsuperscript{23-25}. In truly unfortunate cases, the treatment was effective in preclinical animal studies but when translated to human trials did not improve patient outcomes\textsuperscript{26}. As new molecular drivers of the disease are discovered, as well as the biological control of these mechanisms, they are also interrogated as treatment targets\textsuperscript{30-32,35-36}.

Many of the next generation of GBM treatments are biological and cell-based, which gave the inspiration for the trial of our novel cortical-derived progenitor cells, called brain-derived progenitor cells (BDPCs), as a therapeutic delivery system for GBM\textsuperscript{17,33-34}.

1.3.1 Cell-Based Therapy of GBM

Given that many molecular-based treatments cannot cross the BBB, researchers interrogated and repurposed endogenous biological processes to treat GBM\textsuperscript{45,38}. Certain stem/stem-like cells can migrate to areas of injury within the body, including within the brain\textsuperscript{42,43}. This migration is driven partly by the activity of the chemokine receptor 4 (CXCR4) and its ligand SDF-1a, a marker of hypoxia, and has applications in oncology and other CNS disorders\textsuperscript{71,75}. A necessity of any GBM treatment is to be able to affect the entire neoplasm, which again, may encompass many regions of the brain\textsuperscript{4}. To date, researchers have tested different sources and types of cells to deliver anticancer therapies, to discern the most efficacious as well as translatable system\textsuperscript{33,34,44}. Experiments show multipotent cells that can cross the corpus callosum and home to GBM in the contralateral hemisphere, as well as travel from the core of a tumour to track distant “satellite cells”\textsuperscript{33,34}. Zhang showed that NSCs can also track and target the GSC niche, having positive outcomes in their model\textsuperscript{36}. Given that glioblastoma is so heterogeneous amongst patients, being variably infiltrative and migratory, delivering therapeutics that can reach the entire tumour is crucial\textsuperscript{32,33,36,56}. Advances in molecular biology allow for the migratory cells to be used as a delivery system; once engineered, cells can secrete either a genetic or protein product (such as TRAIL) to the ECM\textsuperscript{33,34,44,45}. Neighboring (cancer) cells can then take up the secreted product, potentially
sealing their own fate\textsuperscript{44}. Many cancer cells respond to the activity of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), by initiating downstream caspase-8 or -10 based apoptosis. Non-transformed (non-cancerous) cells do not express the receptor to be affected by TRAIL, which is especially important in treating tumours in a complex environment such as the brain\textsuperscript{39,40}. Early TRAIL attempts fell short in that they could not sustain activity to arrest and shrink the tumour. Soluble TRAIL as it is referred to, was consumed, and degraded at higher rates than what would completely treat a GBM and thus was deemed ineffective\textsuperscript{40,41}. A simpler and more effective delivery system would be necessary to inhibit the growth of fatal glioblastoma. Engineering cells that can persist within the brain and continuously secrete therapeutic proteins, such as TRAIL, is an alternative treatment strategy that is being explored extensively\textsuperscript{34,37,55}.

While there have been many forms of stem cells tested for therapeutic delivery to GBM, it appears that cells of CNS origin will persist in the brain post-transplantation longer than those that originated outside of the BBB, even if they are also stem/stem-like cells, such as adipose-derived mesenchymal stem cells (adMSCs)\textsuperscript{46}. While adMSCs may be relatively abundant and easily accessed compared to NSCs, there are translational issues with using them in the CNS, as fetal and adult MSCs vary in their gene expression profiles\textsuperscript{72,73}. NSCs used in clinical studies may also need alternative cell options, as they have been derived from controversial tissues, immortalized in vitro, and may have an immunogenic profile in some patients\textsuperscript{45,72,75}. A successful alternative to the current adM/NSC offerings will be easily obtained/harvested from a patient or potential matched donor source, not require cellular reprogramming or immortalization to be migratory and viable within the CNS, and be capable of delivering a therapeutic payload to the GBM\textsuperscript{33,37,78}. Next, we will describe the delivery cells from our study (BDPCs)\textsuperscript{17} but in this context, we have an autologous or allogeneic delivery solution without ethical concerns or requiring transdifferentiation\textsuperscript{34,44,46}.

1.4 BDPCs

Our group has recently discovered and started to characterize a cortical cell type obtained during neurosurgery, deemed Brain Derived Progenitor Cells (BDPCs)\textsuperscript{17}. BDPCs can be expanded through many passages, reliably frozen and thawed, and stably engineered to express transgenes of interest using lentivirus. These cells express markers of multiple cell
lineages found in the brain, leading to their name including the term progenitor\textsuperscript{49-51}. They were originally proposed as a therapeutic strategy for Parkinson’s Disease, and still may have applications in various CNS disorders\textsuperscript{17,65}. Given the preclinical success other groups have had using cells even of non-neural origin, we hypothesized BDPCs may be viable when implanted into GBM\textsuperscript{33,36,44}. The various cell types tested by other groups include induced Neural Stem Cells, transdifferentiated Neural Stem Cells, and adipose-derived Mesenchymal Stem Cells (iNSCs, tdNSCs, adMSCs). While capable in GBM models, these cells can require reprogramming, expensive culture reagents like fibroblast growth factor, or come from a non-clinically relevant source; some have succeeded in their preclinical studies but may not have a direct clinical translation\textsuperscript{34,44,46}. Due to their relative ease of collection, expansion, and maintenance compared to several cells tested, we believe BDPCs are a realistic clinical candidate for cell-delivered therapeutics to GBM. BDPCs could be positioned as an autologous or allogeneic cell delivery system and since they can be found in the adult brain do not carry some of the ethical controversies of other cell delivery systems\textsuperscript{52}.

In that our Fischer rat BDPCs have syngeneic and translatable potential not yet seen in other studies we were eager to test them in a preclinical glioma surrogate\textsuperscript{17,58}. Our research group has vast experience engineering niche cell types for molecular imaging, and continues to optimize culture conditions favorable to expanding BDPCs for use as an \textit{in vivo} delivery system within the CNS\textsuperscript{47,66,65}. By using BDPCs as a delivery vehicle for biologic therapies such as TRAIL, we hope to improve the notoriously bleak prognosis of this brain cancer. Should this prove to be effective, there are additional CNS indications that could benefit from targeted cellular delivery\textsuperscript{64,65}.

1.5 \textit{In Vitro} Cell Migration Assays

Cell migration is an endogenous process that is imperative to biological development, immune response, and cancer metastasis\textsuperscript{42,43,49,54}. In the development of a novel cell-based therapeutic delivery system for glioblastoma, we aim to identify and quantify the \textit{in vitro} ability of BDPCs to migrate to human and rodent glioma models. \textit{In vitro} cell migration assays can be used to measure the distance covered during the cells’ movement or the proportion of the initial cell number that was able to migrate within the system\textsuperscript{34}. A wound-healing assay is one basic method for measuring \textit{in vitro} migration. It is performed by
scraping a lesion into a confluent plate of cells, then quantifying the distance covered and elapsed time for the cells to close the artificial wound.

More advanced cell migration assays utilize distinct chambers or regions of a cell culture apparatus so that varying cell types can be probed for their migration (or attraction) profiles. While the current generation of assays are developed using microfluidics or complex detection and imaging methods, the Transwell assay is several decades old and still widely used\textsuperscript{48,68,69}. Although there are multiple ways to perform the assay, depending on the desired measurement, here we’ll highlight the method developed to study BDPC migration toward glioblastoma.

The Transwell apparatus consists of a “hanging basket” chamber that fits into standard culture plates (manufactured to size specifically for the plate type, i.e., 12, 24, 96 well formats). The bottom of the chamber has a porous membrane (the chemistry and pore size of the membrane varies by need) that under our conditions (8\textmu m pore, Polycarbonate membrane) will allow for the BDPCs to migrate from the upper chamber into the awaiting GBM culture. After a period of culture, the chamber membrane is washed and excised from the apparatus, to be prepped for quantification. Briefly, the membrane is stained with DAPI (4’6-Diamidino-2-Phenylindole) and mounted on a glass slide. The number of stained nuclei on the membrane’s lower surface is indicative of active and concerted cell migration, due to the size constraints of the porous membrane not allowing for simple diffusion. To quantify this test in a consistent manner, we used an academic protocol that is run by the open-source software ImageJ (NIH). The images of the membranes covered with stained nuclei are converted into binary (black and white), and processing sequences remove the background debris while digitally circling the nuclei to be automatically counted.

While there are certain limitations of the Transwell assay, in that it is only an endpoint assay, and does not achieve true biological relevance due to its planar nature, it is consistent in manufacture and operation, leading to robust results that can inform downstream experiments. We were able to use the Transwell assay to test rat BDPCs migrating toward the F98 rat glioma cell line and patient-derived human BDPCs toward patient-derived GBM cultures.
1.6 Preclinical GBM Models

Although there is progress being made using human patient derived GBM cell lines, an important component of any disease research is that there exists a model organism where efficacy can be studied prior to human administration. Models can be referred to as syngeneic – meaning of the same species, or xenogeneic (also referred to as a xenograft model), in the example of a human sample implanted into a mouse. While certain GBM-therapeutic studies can be performed by injecting human cells into immunocompromised mice\(^{34}\), it is still warranted to explore the use of syngeneic animal models of glioma, in the hope that they best recapitulate human GBM\(^{53}\).

There are several preclinical glioma models available, in multiple host species such as mouse, rat and cat\(^{53,58}\). The relevant choice can depend on the features of the vivarium (whether the facility can support immunocompromised animals), the type of study (whether mechanistic, therapeutic, etc.) and the type of metrics to be analyzed. The many models can vary in their molecular proximity to human GBM, life expectancy post-inoculation, and treatment response\(^{53}\).

Additionally, the size difference between a mouse and rat brain is significant, so if there is an experimental readout reliant on postmortem brain analysis, it may prove easier to use the larger rat brain. However, if one is testing a human stem cell-based treatment or interrogating growth patterns of a human glioma, it may be necessary to use an immunocompromised mouse, as immunocompromised rat models are not as widely produced. A critique of immunocompromised animal models is that they do not accurately represent disease states; they may show a particular treatment response or differences in patients’ cancers, however, they may mask an endogenous immune response to the neoplasm\(^{23,31,57}\). While certainly more biologically representative than \textit{in vitro} testing methods, immunocompromised glioma models do not substitute for syngeneic models across the board. The work described here involves the viability of a cell delivery system to an orthotopic glioma implant. This can be performed in immunocompromised mice, but given the aforementioned challenges, we opted for a syngeneic rat model of GBM\(^{58}\).
1.6.1 F98 Fischer Rat GBM Model .................................

One of many glioma models, developed in the 1970s by injection of ethyl nitrosourea into pregnant Fischer rats, the syngeneic F98 glioma model is very useful for preclinical studies. It is informative due to similarities with human glioma in the overexpression of analogous proteins, cellular morphology, and invasiveness\(^53\). One important advantage of the F98 over some of the models, such as C6 and RT-2, is that there is no immune interference in the host animal\(^57\). This is crucial to learning the true nature of translational applications as other models may elicit an immune response within the host animal – overshadowing or complicating the results found in trials testing immune therapies\(^31\).

There are advantages of the F98 over other models, such as the larger brain size, allowing for surgical and histological ease over mouse models, for example. Additionally, teratogenicity of the model being 100% means every animal dosed will generate a tumour\(^58\). It is crucial to utilize reliable animal models to recapitulate human findings as best as we can in the laboratory.

1.7 Bioluminescence Imaging (BLI)..............................

Bioluminescence imaging (BLI) is a preclinical molecular imaging technique that provides longitudinal viability measurement for tracking living engineered cells in vitro as well as in vivo\(^54\). The gene controlling this activity confers luminescence in the animal world, such as the light emitted by fireflies, or sea urchins. In the lab environment, the substrate D-luciferin is cleaved by the luciferase enzyme (with the addition of oxygen, Mg\(^{2+}\) and ATP; the latter provided by living cells), with the emission of light being one of the byproducts of this oxidative reaction. The photons of light can be collected using a charge-cooled device camera within a light-tight box, and this is then representative of (living) metabolically active cells.

In recent years, BLI has become common in preclinical cancer research. Our lab and those of our colleagues have used BLI for gliomas, among other cancers, and found it to be very reliable to correlate in vitro to in vivo findings\(^27,47,54,60,61\). BLI is an invaluable tool in the laboratory for preclinical imaging of animal study cohorts because it is non-invasive, relatively inexpensive, and can be conducted by a single operator.
Luciferase + D-Luciferin + O₂ + Mg²⁺ + ATP

→

Luciferase + Oxyluciferin + CO₂ + Mg²⁺ + AMP + \textit{Light}

**Figure 1-1. Bioluminescence chemical reaction equation.**

With necessary cofactors present, the \textit{Luciferase} enzyme catalyzes the substrate D-Luciferin into Oxyluciferin, also emitting a photon of light as a byproduct. These photons are then captured by CCD. Due to O₂ and ATP being required for BLI, it is a readout of viability in preclinical cell tracking experiments.

An advantage to using BLI in animal studies is that it can be conducted longitudinally, without affecting the viability of the subjects. This removes the need to process animals daily for postmortem histology to observe changing cell populations, for example⁶⁷. This can reduce the number of animals necessary for a study, allowing precious lab resources to be spread amongst other experimental techniques. While we utilized BLI to measure the viability of implanted cells, it can also effectively measure cell proliferation and gross migration, both important concepts of oncology research. As there are multiple \textit{luciferases} which are active in the presence of unique and specific substrates, researchers can design complex experiments such as simultaneous spatial tracking of one engineered cell and viability measurement of another⁵⁴,⁵⁹,⁶⁰.

BLI is a relatively simple, inexpensive, and reliable preclinical research method. By increasing sensitivity over fluorescence imaging, and being considerably less invasive than histology, we aimed to detect our engineered and implanted BDPCs accurately and longitudinally in the F98 rat GBM model. To our knowledge this is the first use of an engineered BDPC in a syngeneic F98 tumour. In summary, here we begin to investigate the viability of BDPCs in a syngeneic orthotopic rodent model of glioblastoma using preclinical non-invasive molecular imaging.

1.8 Thesis Overview.............................................................

While therapeutic oncology research has made many advances of late, GBM remains fatal and has not had a significant change in overall survival or length of progression-free survival since the addition of TMZ to the standard of treatment¹⁹. New treatments are desperately needed by this patient community and their families. This work aimed to probe the application of a novel cell type (BDPCs) as a therapeutic delivery system for glioblastoma.
Due to similarities with multipotent cell-therapy candidates, and their CNS origin, we believed BDPCs would be a promising candidate for GBM cell therapy\textsuperscript{34,52,66}.

In the clinic, this therapy would proceed along with the surgical resection plan afforded to the patient by their tumour. Due to the burden of time on GBM patients, BDPCs will ideally be available as a ready-to-administer engineered cell therapy product\textsuperscript{45}. Clinicians may be able to generate stocks of BDPCs from non-brain cancer patients, such as those in a movement disorder clinic\textsuperscript{17}. Should the patient not be able to receive donor cells, their own BDPCs may be isolated and engineered with the oncolytic transgene(s). In this case, the surgeon would collect necessary cortex (for isolation of BDPCs) either during diagnostic biopsy or surgical resection. The BDPCs would then be expanded for therapeutic administration to the tumour’s core or resection cavity as soon as possible. If the patient can accept the donor BDPC product, during biopsy or resection surgery the surgeon will seed the tumour/cavity with therapeutic BDPCs, where they will release effector molecules selected for their tumouricidal capabilities\textsuperscript{34,44}. The patient would then be followed by standard imaging protocols used to track tumour progression and/or recurrence. There have been recent trials using a similar method, however, with an immortalized neural stem cell line that may not be applicable for all GBM patients\textsuperscript{45}. As adult primary progenitor cells, BDPCs offer both allogeneic and autologous options, a vital characteristic which may allow a greater number of patients to receive this treatment.

Prior to engineering said cells with oncolytic products and implanting them into tumour-bearing rodents, we embarked on several experiments to understand their viability in the notoriously inhospitable tumour microenvironment\textsuperscript{10-12,22}. The following sections of the manuscript will show, in detail, the experimental record and our interpretation of the results. The aim of this thesis is to test the hypothesis that based on evidence of their molecular identity and physical origin, BDPCs will remain viable as detected by BLI in a syngeneic orthotopic rodent model of glioblastoma.
1.9 References


60. Williams RJ, Sehl OC, Gevaert JJ, Liu S, Kelly JJ, Foster PJ, Ronald JA. Dual Magnetic Particle Imaging and Akaluc Bioluminescence Imaging for Tracking Cancer Cell


2.1 Introduction .................................................................

Glioblastoma (GBM) is the most common primary malignant brain tumour in adults, and currently has no cure\(^1\). Standard of treatment, which depends on tumour location and morphology, includes surgical resection, temozolomide (TMZ) chemotherapy, and radiation\(^2,23\). Current challenges include drug delivery and uptake, due to the blood-brain barrier and inherent cytoprotective properties of the tumour\(^2\). Despite treatment, the median survival is ~15 months with <10% of patients living 5 years after diagnosis\(^3\). There is an urgent need for more effective treatments for this devastating disease.

A common strategy being explored for next-generation GBM treatment is utilizing cells to locally express and deliver therapeutics\(^4,9\). For instance, cells can be engineered with transgenes to secrete oncolytic proteins or be used in immune-signaling cascades to suppress the tumour’s growth\(^10-13\). A unique characteristic of many of the delivery cells tested in the field is their tumour-homing activity\(^9\). The advantage of exploiting this migratory profile is in the case of when the tumour is either too diffuse or infiltrative to effectively resect, or if it is obscured by eloquent brain regions rendering it inoperable\(^11,14\). Cell types being explored as vehicles include induced and/or immortalized neural stem cells (NSCs)\(^9,11\) as well as mesenchymal stem cells (MSCs)\(^6\), most of which have been derived from extracranial sources. Cell types derived from the brain may have advantages in terms of intracranial survival, thus potentially leading to increased therapeutic indices\(^4-8\).

Our group has previously identified a cell type we termed brain-derived progenitor cells (BDPCs). BDPCs are obtained during adult neurosurgery, and the isolation and outgrowth of BDPCs from the human brain have proven to be robust and reproducible\(^5\). Since the majority of GBM patients undergo neurosurgery, the collection of BDPCs from GBM patients can be done routinely, as we have demonstrated in movement-disorder patients\(^5\). Importantly, compared to multipotent neural cells which are derived from deep in the brain in the hippocampus\(^15\), BDPCs are derived from the outer cortex which has advantages in terms of ease of collection. The BDPCs express molecular markers of multiple CNS cell types\(^5\), and do not require ex-vivo reprogramming like similar cell-therapy-based candidates\(^11\). We can
reliably grow BDPCs obtained from Fischer rat brains, which affords the opportunity to test BDPCs as cellular vehicles in syngeneic GBM Fischer rat models such as the F98 model\textsuperscript{24,25,33}. Pairing a syngeneic orthotopic tumour model and its therapeutic cell system allows for significant ease of use compared to immunocompromised models\textsuperscript{28}.

Here, as a first step towards a novel cell-based therapeutic, we utilized noninvasive bioluminescence imaging (BLI)\textsuperscript{16,17,21,27} to evaluate the longitudinal viability of BDPCs that were intratumourally injected into pre-established orthotopic F98 tumours. BLI involves engineering cells with a luciferase enzyme that catalyzes luciferin substrates to emit photons which are captured by a cooled charge-coupled device\textsuperscript{16,17,27}. The signal generated is relative to the number of engineered cells and their environment\textsuperscript{16,17}. We show that BDPCs remained viable (BLI signal present) up until the tumour endpoint in the F98 model, suggesting they may have potential as a new and relatively persistent cell source for the local delivery of anticancer therapeutics.

2.2 Materials and Methods .......................................................... 2.2.1 Cell Culture and Derivations...............................................  
Patient-derived GBM cells, Fischer F98 rat glioma cells (F98; CRL-2397 ATCC), and human and Fischer rat brain-derived progenitor cells (hBDPCs and rBDPCs, respectively) were all grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10\% heat-inactivated fetal bovine serum (FBS; Life Technologies), 1\% Penicillin/Streptomycin, and 1x Non-Essential Amino Acid mix (Thermo). All cells were grown at 37°C in a 5\% CO\textsubscript{2} incubator. Cells were regularly confirmed to be mycoplasma-negative using the MycoAlert™ Kit on the GloMax® 20/20 Luminometer system (Lonza, Promega).

Patient GBM Cells

Patient-derived GBM cells were collected from informed and consenting patients during neurosurgery by Dr. Matthew Hebb, as previously described\textsuperscript{18,19}. Briefly, the GBM samples were removed and placed into phosphate-buffered saline (PBS) with 0.5\% FBS. Then the tumour tissue was washed in PBS and digested in PBS with 0.25\% Trypsin (Life Technologies) and 75 \(\mu\)g DNase I (Roche) for 20 minutes at 37°C and filtered through a 100 \(\mu\)m cell strainer. The samples were then centrifuged at 1000 x RCF for 10 minutes, the cell
pellet resuspended in complete DMEM, and plated for 30 minutes to allow settling of blood cells. Next, the supernatant was transferred to 2 wells of a 10 µg/mL poly-L-lysine (Trevigen Inc) -coated 24-well plate and incubated at standard conditions. The media was changed 2-3 times per week and 80% confluent cultures were passaged 1:2 onto standard, uncoated tissue culture plates using 0.25% trypsin with 0.53 mM ethylenediaminetetraacetic acid (EDTA; Wisent).

**BDPC Collection**

Human brain-derived progenitor cells (hBDPCs) were obtained during neurosurgical procedures related to movement disorders\(^5\). Small 0.5-cc volume portions of tissue directly beneath the surface of the cortex of both hemispheres were removed and placed at 4°C for no more than 2 hours. The remaining steps for hBDPC preparation mimic that of the GBM cell preparation. The cells are digested, filtered, and centrifuged, then plated to allow separation from blood cells. The remaining supernatant was then transferred to pre-coated 10 µg/mL poly-L-Lysine 24-well plates and incubated at standard conditions. The cultures were similarly passed and maintained following 80% confluent growth. Fischer rat BDPCs (rBDPCs) were isolated from 2x2 mm\(^2\) sections of the outer cortex of freshly sacrificed adult rats and prepared as above.

**2.2.2 BDPC Engineering**

Lentiviral vectors containing the following transgenes driven by the human elongation factor 1α promoter (p-hEF1) were generated: tdTomato (tdT) for fluorescence imaging and flow cytometry; codon-optimized *Firefly luciferase* (Luc2) for bioluminescence imaging (BLI); and rat organic anion transporting polypeptide 1A1 (Oatp1a1). Oatp1a1 is used in the lab as an MRI reporter gene (not performed in this study) but also improves the uptake of D-luciferin for improved BLI sensitivity\(^17\).

Transgenes were separated by P2A and E2A sequences to allow successful coexpression driven by the same promoter. As described by Nystrom et al., 2019\(^17\), the pUltra-Chili-Luc vector (gift from Malcolm Moore; Addgene plasmid #48688) was updated to include p-hEF1 and *firefly Luciferase* 2, separated by the P2A self-cleaving peptide sequence. The E2A-Oatp1a1 sequence was obtained from the LV-PGK-SO (gift from Dr. Kevin Brindle,
University of Cambridge) vector and inserted downstream of Luc2 into the transfer vector. The cloning was performed using the In-Fusion HD Cloning system (Takara Bio USA, Inc). Next the 3rd-generation envelope plasmid pMD2.G and packaging plasmids pRSV-Rev, pMDLg (all gifts from Didier Trono; Addgene plasmids #12259, #12253, and #12251, respectively) were co-transfected with the transfer vector into modified human embryonic kidney cells (HEK 293T) using Lipofectamine 3000 (ThermoFisher Scientific).

Fisher rat BDPCs were then transduced with p-hEF1-tdT(P2A)fLuc2(E2A)Oatp1a1 lentivirus at a multiplicity of infection of 50 along with polybrene (8 μg/mL) for 24 hours. Following transduction, the cells were purified based on tdT-expression using a FACS Aria III cell sorter (BD Biosciences). These cells are referred to as tdT/Luc2/OATP1A1-rBDPCs.

2.2.3 *In Vitro* BDPC Characterization

**Flow Cytometry Analysis**

tdT/Luc2/OATP1A1-rBDPCs were cultured in standard conditions for multiple passages. Cells were collected in PBS, fixed using 4% paraformaldehyde (PFA), and permeabilized using 0.1% Triton-X-100 (Sigma-Aldrich, St. Louis, MO). After washing with PBS, cells were resuspended in PBS + 3% FBS. Flow cytometry to determine the percentage of tdT-positive cells was performed on a Becton Dickinson LSR II SORP flow cytometer running FACSDiva software (BD Biosciences). Side and forward scatter analysis was performed to remove clustered cells, resolving >20k single cells per sample to be analyzed. The cytometry and subsequent data analysis were completed using FlowJo v 9.6.3 (TreeStar, Inc., Ashland OR, USA).

**Immunofluorescence Analysis**

Both naïve and tdt/Luc2/OATP1A1-rBDPCs were cultured on glass coverslips for 72 hours at standard conditions. The coverslips were then fixed with 4% PFA for 10 minutes, permeabilized for 10 minutes with 0.1% Triton-X-100 and blocked for 1 hour with 3% bovine serum albumin (BSA, Santa Cruz Biotechnology). Between steps, the samples were washed three times with tris-buffered saline (TBS), which was also the diluent for permeabilizing and blocking solutions. The primary rabbit anti-*firefly Luciferase* antibody (AbCam, ab21176), diluted 1:300 in TBS, was used for overnight incubation at 4°C followed
by three TBS washes. Far-red Alexa Fluor 647 secondary goat anti-rabbit (Invitrogen, A21244), diluted 1:500 in TBS, was then incubated at room temperature for 1 hour. After washing, the slips were mounted onto glass slides using VECTASHIELD Antifade Mounting Medium (Vector Laboratories, H-1200). Brightfield and fluorescence images (excitation 360 nm for DAPI, 555 nm for tdTomato and 647 nm for Far Red) were taken on an Eclipse Ni microscope run by NIS-Elements AR software (Nikon).

**BLI Analysis**

Five passages after engineering, tdT/Luc2/OATP1A1-rBDPCs were plated in black-bottom 24 well plates at serial concentrations from $5 \times 10^3$ to $2.5 \times 10^5$ cells/well (n=4 per concentration). 24 hours later they were washed 3 times with PBS and incubated with 5 µL of 30 mg/mL D-Luciferin (PerkinElmer #122799). Bioluminescence images of the plates were then collected using an IVIS Lumina XRMS scanner (PerkinElmer). Regions of interest were drawn over the wells and peak radiance (p/s/cm²/sr) in each region was determined using the software LivingImage (Version 4, PerkinElmer). The data of peak radiance per cell number was then plotted and analyzed in GraphPad Prism v8.

**Transwell Migration Assays**

We evaluated the migratory abilities of BDPCs towards GBM cells using well-established Transwell assays with 8 µm-pore 12 mm-diameter polycarbonate membranes (Millipore #PI8P01250)²⁰. The membranes were equilibrated for 12 hours in serum-free DMEM followed by the addition of tdT/Luc2/OATP1A1-rBDPCs (2×10⁴) and incubated for 4 hours before insertion into the wells. Wells contained either media alone (control) or cultured rat F98 cancer cells (2×10⁵) as a potential attractant. The plates were then co-cultured for 24 hours, membranes removed, washed 3 times in PBS, and fixed for 10 minutes with 100% methanol. Membranes were carefully excised from their housings, permeabilized with 0.1% Triton-X-100, and the nuclei of migrated cells were stained with DAPI for 5 minutes each, followed by three PBS washes. The membranes were then mounted in 90% glycerol on glass slides w/ coverslips for microscopic quantification. Similar studies were performed using patient derived GBM (internally called GBM23) and hBDPC (internally called PD6) cells, however, 5×10⁴ of each cell type was used. Fluorescent DAPI images were acquired using an Eclipse Ti microscope (Nikon) and analyzed using ImageJ software. Briefly, membranes
were manually outlined, the background signal was subtracted (grey value =12), and images were transformed to Black + White. Threshold values were then applied (grey values 20-200), followed by Binary and Watershed being applied to detect and quantify individual nuclei >120 pixels\(^2\). The results (n=4-8/sample type) were analyzed using GraphPad Prism8.

2.2.4 Syngeneic Fischer Rat BDPC and GBM Model

All animal procedures were performed in accordance with relevant guidelines and regulations stipulated by an animal use protocol approved by the University Council on Animal Care, Animal Use Subcommittee at Western University (Animal Use Protocol 2018-025). Male Fisher rats (n=4, age, 3-6 months; weight ~200 g; Charles River Laboratories) were used. Rats were anesthetized with 5% isoflurane, maintained at 1% iso (2 L/min O\(_2\)), and placed in a stereotaxic frame for F98 cell implantation into the striatum\(^3\). Their heads were shaved to the skin, and lambda and bregma were identified as cranial landmarks. A burr hole was drilled 2.2 mm lateral to bregma on the right. A 26-gauge micro syringe (Hamilton #80308) containing 4x10\(^4\) F98 cells suspended in 2 \(\mu\)L PBS was advanced to a depth of 6 mm relative to bregma. Cells were then injected at a rate of 1 \(\mu\)L/min. After the injection, the syringe was left in place for 2 minutes to prevent cell reflux. The incision was sutured and 0.1 mL each of 5 mg/mL Metacam and 50 mg/mL Baytril were subcutaneously injected immediately and 24 hours post-operation.

One week later, the skull was reopened and 4x10\(^4\) tdT/Luc2/OATP1A1-rBDPCs in 2 \(\mu\)L PBS were injected in a similar location, although at 5 mm depth relative to bregma, to avoid penetrating the lower edge of the previously seeded tumor. Again, each animal received multiple doses of post-operation analgesics. Animals were monitored for behavioral changes and weighed daily until study endpoint.

2.2.5 In Vivo Bioluminescence Imaging (BLI)

Each animal was imaged at 1, 3-, 6-, 8-, and 10-days post tdT/Luc2/OATPA1-rBDPC injection. Animals received an intraperitoneal injection of 150 mg/kg D-Luciferin (PerkinElmer, #122799), anesthetized as above and imaged every minute for 25-30 minutes using an IVIS Lumina XRMS scanner (PerkinElmer). Regions of interest were drawn over
the brain and peak average radiance (p/s/cm²/sr) was determined using the software LivingImage v4 (PerkinElmer).

2.2.6 Histology

Animals were sacrificed by IP injection of 100 mg/kg Sodium Pentobarbital and intracardial perfusion with saline followed by 4% Paraformaldehyde (PFA). The brains were carefully extracted following decapitation and placed into fresh 4% PFA solution overnight at 4°C. The following day the brains were placed into a 15% Sucrose solution until sunken (~24-36 hours), then placed into a 30% Sucrose solution. When fully sunken in the final cryopreservation solution (~36-48 hours) they were prepared for cryo-sectioning. After embedding in Optimal Cutting Temperature (OCT-) Solution they were sectioned at 10-15 μm thickness through the tumors. Sections were permeabilized using 0.1% Triton-X100, washed 3x with PBS, and stained with Hematoxylin + Eosin (H+E) or DAPI. Once stained, coverslips were applied with 90% glycerol. Images were collected using an Eclipse Ni microscope (Nikon) or an LSM800 confocal microscope (Zeiss).

2.2.7 Statistics

GraphPad Prism v8.3.0 software was used for all statistical analyses. The values of peak radiance versus cell number were plotted as the mean ± standard deviation, and a simple linear regression was used to generate the line of best fit. Statistical significance was established for this and all experiments when p < 0.05. The Unpaired t-test with Welch’s correction was used to analyze the Transwell migration data, due to differences in sample numbers in each group (ranging from n=4 to n=8) and assuming that the groups’ standard deviations were unequal, which was correct. The in vivo BLI data were analyzed by two-way analysis of variance (2-way ANOVA) with Tukey’s multiple comparisons.
2.3 Results ............................................................................................................

2.3.1 *In Vitro* screening shows migratory BDPCs can be stably engineered with lentiviral vectors .................................................................

F98, rBDPC, h-pdxGBM, and hBDPC cell lines were obtained, derived, and maintained in culture. BDPCs were engineered using lentiviral vectors to express tdTomato (tdT) for fluorescence imaging and *Firefly Luciferase* (FLuc) and organic anion transporting polypeptide 1A1 (OATP1A1) for bioluminescence imaging (BLI) (Figure 2-1B). Engineered cultures were sorted to obtain a highly purified population of tdT-positive cells (~95% Figure 2-1C). tdTomato expression was stable at the 9th passage, and all rBDPCs used for later experiments were between passages 5-9 (data not shown).

**Figure 2-1: Study design and Lentiviral engineering.** (A) Overall study design: rBDPCs were engineered, characterized *in vitro*, injected intratumourally into F98 tumour-bearing rats, imaged with longitudinal *in vivo* BLI, and brains were analyzed histologically. (B) Schematic of lentiviral expression cassette for the engineering of rBDPCs. The constitutive human elongation factor 1 alpha promoter (p-hEF1) was used to drive the expression of the fluorescence reporter tdTomato (tdT) and the bioluminescence imaging (BLI) reporter *Firefly Luciferase* 2 (FLuc2). Organic anion transporting polypeptide 1A1 is also coexpressed and helps improve BLI sensitivity via increased D-Luciferin uptake. Each transgene is separated
by a 2A cleavage sequence (P2A and E2A) for efficient co-expression. (C) Analysis of rBDPCs by flow cytometry before and after engineering/sorting. Unsorted cells were ~80% tdT-positive while sorting increased tdT-positivity to ~95%. An inset microscopy image of tdT fluorescence in the sorted cells is shown.

Firefly Luciferase (FLuc) expression and function were assessed in vitro via immunostaining and BLI (Figure 2-2). As expected, naïve F98 glioma or BDPCs did not generate BLI signal either with or without D-Luciferin incubation (Figure 2-2A). In contrast, tdT/Luc2/OATP1A1-rBDPCs did generate BLI signal when incubated with the D-Luciferin (Figure 2-2A). Immunostaining also confirmed FLuc presence (Figure 2-2B). BLI signal significantly positively correlated with cell number (Figure 2-2C, D $r^2=0.9924$; p<0.01).

Figure 2-2: Multimodal in vitro confirmation of BDPC engineering. (A) F98 and Naïve BDPC cultures do not have BLI signal, while engineered BDPCs have BLI signal, only with application of the Luciferase substrate d-Luciferin. (B) tdT-positive BDPCs (i) co-expressed FLuc in their cytoplasm (ii). (C) tdT/Luc2/OATP1A1-rBDPCs were plated in 24-well plates...
(5x10^3 to 2.5x10^5 cells), cultured for 24 hours, and bioluminescence images were taken. (D) Cell number positively correlated with BLI signal (r^2=0.9924, p < 0.01)

Next, we evaluated the migration of both human and rat BDPCs towards patient-derived human or rat GBM cells, respectively, using transwell migration assays (Figure 2-3). Both human and rat BDPCs were found to be migratory towards their respective gliomas. Complete media was used as a negative control. Human BDPCs migrated significantly more (2-fold) to the GBM cells compared to the control (Figure 2-3A,B; p<0.05). Similarly, rat BDPCs migrated significantly more towards F98 cells (Figure 2-3C,D; p<0.05).

![Figure 2-3: Transwell migration assays showed increased tropism of hBDPCs and rBDPCs towards glioma cultures.](image)

(A) DAPI images of hBDPCs after migration towards media alone (top) or patient-derived GBM cultures (bottom). (B) hBDPCs showed significantly increased migration towards patient derived GBM cells compared to media alone (media n=8, cells n=4; p<0.0001). (C) DAPI images of rBDPCs migration towards media alone (top) or rat F98 glioma cultures (bottom). (D) rBDPCs migrated significantly greater towards F98 glioma cells compared to media alone (media n=6, cells n=4; p<0.01)

2.3.2 Implanted engineered BDPCs remain viable in orthotopic glioblastoma model

For our in vivo studies, F98 cells were orthotopically implanted into Fischer rats and one-week later tdT/Luc2/OATPA1-rBDPCs were administered intratumorally. Longitudinal BLI
was performed to assess the viability of the BDPCs (Figure 2-4). Figure 2-4A shows each rat’s BLI signal over time. Animal 1’s signal fluctuated, with a stronger signal early that appeared to stabilize after day 8. Animal 2’s signal fluctuated both up and down, roughly 40% of itself. Animal 3’s signal started lower, increasing to 9X initial values by the end of the study. Animal 4’s signal stayed more consistent, with a steady signal increase, however, the animal had to be removed from the study early due to weight and mobility changes likely associated with the F98 tumour’s progression. BLI signal was not statistically different over time, p-val > 0.05 (Figure 2-4B). Importantly, all animals had BDPC BLI signal at their endpoint, indicating that at least a proportion of the cells remained viable in our syngeneic model.

Figure 2-4: Intratumoral BDPC viability shown by longitudinal BLI. (A) BLI was performed on animals up to 10 days post-tdT/Luc2/OATPA1-rBDPC intratumoural injection. Animal #4 was removed from the study after day 8 due to rapid weight loss and lower activity levels per protocol. (B) Individual plots of daily peak BLI signal of the experimental animals confirm a high degree of variability both within and across animals, although not statistically significant (p-val = 0.3393). However, all animals showed BLI signal at endpoint, confirming that viable rBDPCs were still present. The large variability seen may be partly explained by differences in the extent of surgical wounds, which may absorb BLI light, along with variations in healing or tumor vascularity over time within and between animals.
2.3.3 Microscopic examination identifies tdT-positive BDPCs in established F98 tumours

Postmortem histology was able to highlight characteristics of the F98 glioma, such as cortical infiltration, nuclear pleomorphism and necrosis within the tumour (Figures 2-5 + 2-6). The inset shown in Figure 2-5B is a further magnification of the F98 tumour in Figure 2-5A and shows consistent outgrowth for the model\textsuperscript{18,19}. The red fluorescence overlaid in Figure 2-5C is marked by intense DAPI signal within the tumour (yellow dashed line) and the core of the tumour is visibly necrotic (white dashed line).
Figure 2-5: Brightfield and fluorescence microscopy show characteristics of orthotopic F98 tumours. (A) Brightfield image of rat bearing an F98 tumour receiving intratumoural injection of tdT/Luc2/OATPA1-rBDPCs. Black dotted lines highlight tumour area showing common characteristics of F98 progression. (B) Inset of (A), showing consistent F98 outgrowth. (C) Fluorescence overlay image of cellular (red) and nuclear (blue) channels highlighting the F98 tumour (gold outline), as well as necrotic core (white outline)
There are fluorescence confocal images (Fig 2-6B, C) highlighting multiple tdT-positive + DAPI-positive cells from 2 different animals of the study. Figure 2-6B is the high-magnification confocal inset of the white box in Figure 2-6A which also shows the tumour area highlighted in gold dotted lines. Individual BDPCs are outlined in white dotted lines in Figures 2-6B, C.

Figure 2-6: Intratumoral tdTomato-positive BDPCs identified by confocal fluorescence microscopy. The tumour area in (A) is highlighted by gold dashed lines, while the white dashed box and lines highlight the inset shown in (B) which is red fluorescent and blue fluorescent (DAPI-stained nuclei), the white dashed outlines showing nuclei within outlined tdT-positive cells. (C) The inset of the dotted square in (D) shows confocal high-powered image of the white outlined area in inset (G) showing multiple tdT-positive cells with DAPI-stained nuclei
2.4 Discussion .................................................................

In an attempt to overcome ineffective treatments for glioblastoma, cell-based therapies are being widely explored\textsuperscript{26}. The objective of this study was to evaluate the survival of rat BDPCs in the Fischer rat F98 glioma using BLI.

We show that the BDPCs can be stably engineered to express multiple transgenes including tdTomato, FLuc2, and OATP1A1. Using tdTomato we were able to sort for the engineered BDPCs, allowing for experiments which employed a more homogenous population of trackable cells. The flow cytometry shown here tested cultures that have been passaged 4-8 times after the initial sort (not shown) and were found to contain nearly the same proportions of cells that satisfied the initial criterion of ~95% purity (Figure 2-1C). This consistency is indicative of stability of the construct and the engineering method itself.

Generating the \textit{in vitro} standard curve of BLI signal to cell number (Figure 2-2D) returned a strong ($r^2 = 0.9924$, p-val < 0.01) linear fit, supporting the consistency of the engineering and sorting, as well as the cells being reliable for \textit{in vivo} studies; that lower signal will be indicative of less cells and vice versa\textsuperscript{16, 17}. It is worth noting that the exact correlation of rBDPC BLI signal to cell number is not yet known within an orthotopic brain tumour, as the curve obtained from the culture experiment will not confer accurate \textit{in vivo} quantification of cell number due to differences in signal attenuation in both settings. Should targeted GBM treatment with BDPCs commence, it would then be beneficial to researchers to accurately quantify how many therapeutic cells are viable throughout the study.

Using the Transwell cellular migration assay\textsuperscript{20} we have shown that both the human and rat BDPCs are tumour-homing vectors; both migrate to their corresponding glioma cells \textit{in vitro} (Figure 2-3B,D). When the species are compared to each other, however, there appears to be a gradient of migratory activity. This may have to do with the design of the assay, as slightly different ratios of migrant: attractant cell numbers were used between the rat and human cells. It is noted that in the future we would attempt to utilize non-neoplastic control cells (i.e., naïve glia, neurons and/or fibroblasts) for this assay, which may provide a more robust interpretation of each species’ BDPC migratory profile.
The diffuse and infiltrative nature of gliomas in vivo requires a motile cell delivery system. Should therapeutic cells be administered in a clinic during surgery, they will be tasked with delivering therapeutic cargo to the entire glioma to ensure efficacy. This includes potentially far-reaching deposits of neoplastic cells away from the core of the tumour, as well as in situations where the core of the tumour encompasses multiple nuclei within the brain. Kim, et al., 2006, have shown that by solely delivering therapeutic molecules such as TRAIL intratumourally may have initial efficacy, but distant foci may not be reached by soluble forms of the protein. To overcome this incomplete distribution, migratory cells which are capable of continuously delivering the therapeutic to all locations of the tumour will be used. This has long been a goal of glioma treatment, as systemic administration of chemotherapeutics lags in necessary therapeutic efficacy, as well as has many adverse side effects for the patient.

In that we had a well-engineered and consistently migratory cell type, we commenced introducing the BDPCs to tumours in vivo to determine their viability in an animal model of glioblastoma (Appendix A).

BLI was able to identify engineered BDPCs from the time of implant to study endpoint. The in vivo BLI results did not support the initial hypothesis that peak signal would be measured early after injection and diminish. Although individual animals’ peak signal fluctuated over the experimental period, all the values were within ~ 1 order of magnitude of each other, and we did not observe any significant differences in signal over time across all the animals (Figure 2-4B). Per our F98 model animal use and care protocols (Appendices B, C), the animals reached endpoint prior to observing a significant decrease in the BDPCs’ BLI signal, indicating the BDPCs remained viable until endpoint when the animals were perfused and their brains prepared for histology. Microscopic examination of postmortem brain tissue confirmed consistent F98 growth and identified implanted BDPCs within different areas of the tumours (Figures 2-5, 2-6).

With recent developments in clinically relevant in vivo cell tracking methods, there are higher-resolution readouts available for our future studies. Pairing the OATP1 gene with the NIS reporter allows for quantitative PET measures and anatomically significant MRI results. Given that BLI is a lower-sensitivity preclinical imaging modality than PET-MRI, we would...
expect accurate *in vivo* quantification and spatial resolution of the implanted BDPCs within the F98 GBM model, should we adapt the innovations of Shalaby, Nystrom, et al.\textsuperscript{16,17,34}. One of the ways PET-MRI may return higher resolution data is the NIS/OATP1 system is not reliant on $O_2$ as a cofactor. This oxygen is assumed to be a limiting reagent in the hypoxic necrotic core of the aggressive F98 GBM, potentially diminishing our BLI signal obtained.

In this study BDPCs were engineered for *in vivo* non-invasive molecular imaging and we were able to show that viable cells were detectable for a little over a week within an inhospitable and aggressive orthotopic tumour. Future work will include using clinically relevant reporter genes for imaging modalities such as MRI and PET, and co-engineering BDPCs with both our reporter genes and a therapeutic gene\textsuperscript{27,34,35}. With continued development, BDPCs may be a viable cellular vehicle for delivery of therapeutics targeting glioblastoma\textsuperscript{9,11}. 
2.5 References


Chapter 3 Summary and Discussion

Overview

Since the introduction of TMZ in 2005, survival outcomes for GBM have not advanced\(^1\). Cellular therapies offer new possibilities and hope for patients and their families, as the endogenous properties and activities of the cells tested may confer increased therapeutic biodistribution against the tumour, as well as sparing the patient’s healthy tissue from systemic chemotherapeutic toxicity\(^2\). This study sought to determine the viability of a novel cellular delivery system within a preclinical animal model of glioblastoma.

Our lab has discovered and named a novel cell type, BDPCs, from within the CNS that display characteristics of similar cells used in this field\(^2,3\). To probe and characterize their application as a cellular therapeutic delivery system, we performed several experiments prior to their use in our novel syngeneic preclinical F98 GBM model\(^10\). We verified the stability of lentiviral engineering and their migratory potential using field-standard \textit{in vitro} techniques such as flow cytometry and the Transwell assay\(^4\), respectively. The engineered BDPCs generated \textit{in vivo} BLI signal until the F98 GBM model endpoint, indicative of their viability post-intratumoural injection.

We have begun to show that BDPCs have potential as a cellular therapeutic delivery system against high-grade gliomas. The following will expand on the interpretation of our results, propose a critique of our study design and methods, and suggest further experiments to understand and develop BDPCs as a component of next-generation GBM therapy\(^2,6\).

3.1 Summary of Findings………………………………………………

The cells used here, BDPCs, can be safely obtained during neurosurgery, and based on \textit{in vitro} and \textit{in vivo} studies, can migrate to, and persist within the inhospitable environment of Fisher rat F98 gliomas. As GBM isn’t yet cured, this research is vital to explore all untapped treatment options. The design and results of the study as well as their scientific impact will be summarized in the following sections.

Prior to initiating experiments in our preclinical rodent glioma model (\textbf{Figure 2-1A, Appendices A-C}) we validated the cells using \textit{in vitro} experiments. Lentiviral engineering
was confirmed by flow cytometry, immunocytochemistry (ICC), in vitro BLI, and was found to be consistent throughout numerous passages in culture (Figure 2-1C). While the ICC was able to show immunoreactivity to the *Luciferase*2 transgene, (Figure 2-2B) this is not indicative of the transgene’s functional activity, solely the detection of the encoded protein product. It is crucial to the later *in vivo* work, however, to establish that any signal obtained from BLI would be exclusive to the reactivity of the engineered BDPCs. Naïve BDPCs and F98 cells do not have endogenous *Luciferase* activity, nor do the engineered cells without the application of D-Luciferin substrate. This provides confidence that the signal obtained during the *in vivo* BLI will be specific to the engineered BDPCs’ activity (Figure 2-2A).

Next, serial dilutions of BDPCs were plated to establish a standard curve of cell number to BLI signal. The *in vitro* BLI signal was linear with respect to the cell number plated (\( r^2 = 0.9924 \), Figure 2-2C), also supporting my colleagues’ initial findings showing the stability of lentiviral engineering using multimodal constructs. This linearity is useful in reference to the later *in vivo* BLI experiments, as the values will be different (lower) than here due to tissue-based signal attenuation, but not due to any inconsistency of the transgene expression when introduced to the cells.

While the consistency of the engineering is vital to their performance in our syngeneic orthotopic rat GBM model, it was separately necessary to profile the BDPC’s innate migratory capabilities to GBM. Our assay design used the F98 GBM as the attractant for Fischer rat BDPCs, and a patient-derived GBM culture as the attractant for a BDPC line derived from a patient of the movement disorder clinic. We found that both rodent and human glioblastoma cells promote significantly higher levels of BDPC migration over their media-only control samples (\( p<0.01 \), \( p<0.0001 \), respectively, Figure 2-3). Cells determined by the field as tumour-homing vectors express the CXCR4 receptor, which is active upon detection of its ligand SDF-1α. While we have not yet probed the BDPCs for their CXCR4 expression level, they do maintain similar expression patterns of nestin which confers their progenitor status. Ultimately, understanding this profile in the BDPCs will help us design the most effective therapeutic for the GBM community. From various *in vitro* measures of stability, activity, and migration, we deemed these engineered BDPCs worthy of evaluating in our syngeneic orthotopic F98 GBM model.
There are numerous preclinical GBM models across various host species, which can be problematic for the interpretation of results, particularly in the immunocompromised xenograft model. Some of these models are clinically irrelevant, such as flank-injected tumors, as they do not consider the brain’s complex microenvironment and provide challenges to observing treatment effects, respectively. Our goal was to generate a syngeneic orthotopic tumour and engineered delivery cell model, to best mimic the human GBM condition and its nascent therapy.

Given its history in the field, as well as our group’s experience with this model, we selected the aggressive, human-like Fischer rat F98 glioma, and subsequently isolated BDPCs from healthy adult Fischer rats. Based on the in vitro BLI specificity/activity experiment, our longitudinal in vivo BLI study returned 3 primary results (Figure 2-4). First, the signal generated is likely exclusive to the engineered BDPCs. Second, although there are fluctuations in BLI signal across and within animals, on average, the BLI signal did not change significantly over time. Finally, the BLI signal is still present at the study endpoint. Due to signal attenuation, we cannot correlate the in vivo signal values to the in vitro standard curve shown in Figure 2-2D, so BLI provides a relative measure of cellular viability per animal. Although we observed the in vivo BLI signal present up to the protocol’s endpoint, this is an aggressive brain cancer model and results should be considered in this light. To our knowledge, this is the first study examining the viability of BDPCs implanted into an orthotopic F98 tumour.

Animal #4 was removed from the study 1 day early due to symptoms attributed to the progression of the F98 GBM, confirming its rapid fatality. This raised the question as to whether a less aggressive GBM model would show increased BDPC survival over time, relative to the duration likely required to achieve tumour regression in a human patient. Mathieu and Barth have reported on various GBM animal models, and although the rapid fatality is noted, the Fischer F98 currently remains our preclinical glioma due to its many similarities to human GBM and its lack of immune response in the host animal.

In postmortem histology, we observed consistent tumour size and morphology to our previous F98 model use, thus were largely satisfied with the implant surgeries and the glioma model’s oncogenicity. Both the peripheral brain infiltration and necrotic core of the F98
tumour were clearly visible, and the noninjected contralateral hemisphere was unaffected (Figure 2-5A). The sections were stained with DAPI, and when a tdT-fluorescence-based cell was identified, the 2 channels were overlaid to colocalize the cell body and nucleus (Figure 2-6B,C). This morphological confirmation is vital in glioma histology, as the red autofluorescence of glial cells (including GBM) proved challenging in accurately identifying the red tdT-positive BDPCs without a blue DAPI-stained nucleus. There is also a considerable imbalance of the 2 cell types, which at this point in the F98 model means the BDPCs’ fluorescence may be obscured by the volume of the necrotic core, or tumour mass itself. Although engineered BDPCs contained a luciferase transgene, we confirmed to react with an anti-Luc antibody in vitro (Fig 2-2B), we were unable to use this antibody to visualize BDPCs in IHC sections.

While the total number of implanted BDPCs appears scarce in the histological findings, the in vivo BLI signal obtained is representative of the cells’ presence and viability to study endpoint, confirming our overarching hypothesis that BDPCs have the potential as a cellular therapeutic delivery system to glioblastoma. This work does have areas for improvement and expansion, and the following sections offer our comments on study limitations and next steps.

3.2 Challenges and Limitations…………………………………………………

As with any study, there are limitations to the depth of data obtained from individual experiments. This is accounted for by attempting to design experiments with their appropriate controls as well as a large enough sample size to apply statistical power to the study. We acknowledge that experiments and their artificial environments do not always recapitulate the physiology and biology that we intend to learn about. This is especially important when studying such a dynamic and heterogeneous disease as GBM\textsuperscript{10}. There have been multiple preclinical therapies that showed initial promise but when translated to clinical trials largely failed\textsuperscript{13}. While this work is still far from the clinic, we endeavored to perform a full suite of characterization experiments to best inform the future translation of BDPCs as therapeutic vessels.

As previously mentioned, the Transwell assay, while robust and consistent, is not without its shortcomings. Being that it is an endpoint assay, it requires the completion of the experiment
to obtain info even when establishing conditions of use. The methods described in the literature also range in their setup, requiring troubleshooting by the researcher if their cell type has not been previously assayed\textsuperscript{22,23}. While we did use a media-only control, going forward we would also use fibroblasts, healthy naïve neurons, or both, as additional controls.

Although untested here due to experimental timelines, there is also a modification to the standard Transwell method which aims to increase the assay’s biological relevance. Researchers coat the membrane with a biological matrix such as Matrigel or collagen, which forces the cell to invade and migrate through a tissue-density-type barrier, results of which may be more definitive, or indicative of \textit{in vivo} translation compared to the nude polycarbonate membrane\textsuperscript{24,25}. Other \textit{in vitro} migration assays are also available, but either do not expand on the capabilities and measures of Transwell or require expensive equipment and reagents to perform, which is burdensome on an academic institution\textsuperscript{26,27}.

To develop a novel biologically relevant, longitudinal measurement-capable migration assay, we screened multiple biologic matrices and their physical orientations in parallel with our Transwell work. This was partially inspired by the custom migration assay described by Bago, et al., which we were not able to replicate, and thus sought to innovate a reproducible alternative\textsuperscript{9}. The goal was an assay that, like the Matrigel/invasion Transwell protocol, could better recapitulate \textit{in vivo} migration, without relying solely on endpoint collection. We hoped this would display the kinetics of migration over time, potentially informing the \textit{in vivo} model design. With contributions from many colleagues, notably Joshua Dierolf, we tested many assay configurations but were not able to reliably quantify the migration, and for the sake of resources, moved on with the study.

There are general challenges of using a proliferative brain cancer line and a niche CNS cell such as BDPCs. Their differential outgrowth dynamics require accurate characterization of the F98 to estimate the appropriate number of BDPCs necessary to achieve coverage within the core and around the tumour’s periphery. We were unaware whether we’d be able to resolve the 40k BDPCs in a tumour that by this time would encompass ~1/3 to 1/2 of the animal’s injected hemisphere. We are grateful to the work of Andrea Di Sebastiano, Mitch Cooper, Simon Benoit, Andrew Deweyert, and Hu Xu in the Hebb lab for this background in F98 methodology.
As we commenced in vivo BDPC implantation into orthotopic F98 tumours, there were aspects of the study that were truly novel and experimental. This being the first examination of engineered BDPC’s viability in a syngeneic orthotopic GBM model, we were unaware if the BDPCs would persist in the tumour core, generating BLI signal. We also did not know if the BLI signal obtained would be susceptible to further attenuation by the growing tumour. In hindsight there is a simple in vitro BDPC:F98 cell co-culture BLI experiment that may have informed us of the in vivo BLI signal patterns later observed.

Other variables are the independent requirement of substrate and cofactor distribution in the animal, regardless of the disease being researched. In the case of GBM, this is quite pertinent, as the F98 tumour’s core may exhibit necrosis in just a week’s growth. The hypoxic nature of the F98 model then has the potential to interfere with the results, as O\textsuperscript{2} is a cofactor for the reaction, when it is not available the signal will be diminished\textsuperscript{10,16,19}. While the D-Luciferin substrate can pass the naïve BBB, under the influence of a glioma-BBTB this permeability may not remain favorable or consistent for longitudinal studies\textsuperscript{19,21}.

We aim to be the best stewards of these animals’ care, using as few as possible to complete the study, rather than overbreeding solely to have a large sample size if not necessary. One unfortunate occurrence noted is that in the first cohort of animals that we performed surgeries and subsequent BLI (An#1 from [Figure 2-4A]), our surgical suite did not have the designated clear/transparent sutures and this animal received standard, black-colored sutures. While these sutures are noticeably present in the animal’s head in the photograph (Figure 2-4A), the signal value collected by the CCD was consistent with the others. Although the signal appeared consistent, best practices require the use of the same reagents, supplies, etc. across an entire experiment.

Given the F98’s 100% teratogenicity, we also have a chance to extend the length of our model. Researchers who implant \( \frac{1}{4} \) of the F98 cells as we did here are routinely able to allow their animals to continue \( \sim \)10 days longer than our group is capable of prior to sacrifice\textsuperscript{10}. This extension would drastically improve our ability to determine the long-term viability of BDPCs in an F98 tumour. Whether they would continue to generate BLI signal to a later time point would be valuable information to their application as a therapeutic delivery system for GBM.
After obtaining BLI signal from the BDPCs until the prescribed F98 model’s endpoint, we examined the post-mortem brain tissue using fluorescence microscopy (Figures 2-5, 2-6). There were tdT-positive cells colocalized with DAPI-stained nuclei in multiple animal’s tissue sections, which is expected given that the BLI signal persisted throughout the study. To highlight additional BDPCs, we tested the anti-Luciferase antibody from in vitro ICC (Figure 2-2B). Ideally this would not only circumvent the glial autofluorescence, but to confirm the tdT-positive cells identified also reacted with antibodies specific to the other engineered transgene (Luciferase). While there were brain sections with IHC-based reactivity, presumably highlighting the BDPCs, the images were not consistent enough to include in this report.

3.3 Future Directions…………………………………………………………

While this work spanned the topics of molecular- and cell biology, neuro-oncology, and animal physiology, there are remaining and continuing components of the study that will be discussed here. As mentioned in previous sections, many of our in vitro experiments generated data that qualified the BDPCs to be used in the in vivo studies. The initial results were positive; the engineered BDPCs consistently expressed multimodal preclinical molecular imaging reporters.

The monoculture experiments that lead to Figures 2-2A, 2-2B could be repeated as co-culture experiments (F98 + BDPC) to set the baseline measures for observing therapeutic efficacy if the lentiviral vector can also express an anticancer molecule like TRAIL in BDPCs. Parkins, et al.\textsuperscript{17}, showed that 2 distinct Luciferases and their respective substrates can be used to track the viability and in vivo location of more than one cell type at a time in the same animal or experimental system. A therapeutically engineered BDPC population may confer a decrease in an engineered F98 glioma’s BLI signal if the BDPCs’ intratumoural viability remains comparable to our present study whilst distributing oncolytic transgenes\textsuperscript{9}. This readout may inform us as to the requisite number of therapeutic BDPCs to arrest the progression of GBM.

Barring dual-BLI use there are other non-invasive longitudinal imaging modalities of late, such as the NIS/OATP1B3 PET-MRI reporters described by Shalaby, et al.\textsuperscript{18} These systems also allow for clinical translation, which cannot be said about preclinical BLI. The
combination of highly sensitive, quantifiable PET measures and the increased spatial resolution of MRI results in a more robust readout of the fates of the therapeutic delivery cell (BDPCs) and the neoplasm (F98 GBM) compared to the serial BLI and postmortem microscopic evaluation employed in our current study. The GBM community is eager for increased levels of sensitivity in evaluating much-needed novel therapeutics against this fatal brain cancer.

A common theme in the cell therapy-against-GBM literature is the ability of the (largely) multipotent delivery cells to home to distant tumour foci, not only from within the brain but also from an IV injection. Regardless of the multimodal molecular imaging systems employed, we believe it is still of interest to explore the BDPCs’ in vivo GBM-homing profile. A simple in vivo experiment may confirm or refute our theory that BDPCs are migratory in relation to GBM; seed the GBM tumour in the animals’ striatum as done previously, and rather than injecting the BDPCs directly into the tumour, inject them into the contralateral striatum. Following up with BLI will report on the viability of the BDPCs and may give insights as to whether the cells have migrated to the tumour prior to postmortem histology, for example. In Figure 2-4 the majority of the BLI signal is oriented to the side of the implant (R). In the proposed experiment, I posit that there will be an indicative signal shift from the hemisphere of BDPC injection across the animal’s skull and ultimately displaying the majority of BLI signal on the hemisphere seeded with the F98 tumour. We would then attempt to resolve engineered BDPCs in the contralateral tumour-bearing hemisphere by postmortem microscopic examination of brain sections.

A positive finding on this subject of in vivo GBM-homing capabilities would only reinforce the interest in developing the novel CNS cell type as a therapeutic delivery system to GBM. As there are other yet-incurable gliomas, such as the pediatric DIPG, should this system prove effective in treating GBM, there are other indications worthy of our efforts in developing and applying BDPCs as a therapeutic delivery system.

3.4 Summary

As GBM is yet uncured, patients and their families are in desperate need of novel therapies – as the lone chemotherapeutic, TMZ only benefits ~50% of the patient population. Stem-cell-based delivery of therapeutics to GBM has been successful in preclinical research
models and is now being tested in the clinic. Here we have explored BDPCs as an autologous alternative to the engineered and immortalized cell lines used commonly in this field. Positive findings from this study warrant further investigation as to the application of BDPCs for treatment of glioblastoma.
3.5 Chapter 3 References


Appendix A

Tumour and BDPC Implantation Schematic

Appendix A: Schematic of *in vivo* intratumoral BDPC viability study; (A) F98 cells are implanted in the striatum using stereotaxic surgery (blue dot), (B) 7 days later engineered BDPCs (red dot) are implanted to the same M/L and A/P coordinates, though 1mm dorsal (-5mm vs. -6mm in the D/V axis) in an aim to deposit the cells within the core of the growing F98 tumor (approximate size at day 7 outlined in blue). The animal is then imaged by BLI (C) over 10 days before perfusion and fixation with Saline followed by 4% PFA to prepare brains for cryopreservation in 30% Sucrose and frozen sectioned (10-15µm slices) for histology. Stereotactic coordinate images were obtained from the Rat Atlas^{28}. 
Appendix B

UWO Animal Use Protocol 2018-025

3/22/2018

Changes

Table of Contents

Animal Use Protocol Overview
Funding Source List
Purpose of Animal Use
Hazardous Materials
Animal Movement Between Sites
Animal Groups and Experimental Timeline Overview
Mouse
  Tissue Collection
  Justification for Choice of Species
  the 3Rs: Replace, Reduce, Refine
  Species Strains
  Animal Transfers
  Environmental Enrichment
Animal Housing/Housing and Use Location Information
  Holding beyond 12 hours
  Acclimatization Period & Quarantine
Veterinary Drugs
Experimental Agents Information
SOP List
  Procedures Checklist for Reporting and Training
  Procedures Narrative
  Procedural Consequences & Monitoring
  Endpoint Method Information
  Animal Numbers Requested
Rat
  Tissue Collection
  Justification for Choice of Species
  the 3Rs: Replace, Reduce, Refine
  Species Strains
  Animal Transfers
  Environmental Enrichment
Animal Housing/Housing and Use Location Information
  Holding beyond 12 hours
  Acclimatization Period & Quarantine
Veterinary Drugs
Experimental Agents Information
SOP List
  Procedures Checklist for Reporting and Training
  Procedures Narrative
  Procedural Consequences & Monitoring
  Endpoint Method Information
  Animal Numbers Requested
Personnel List
Protocol Attachments
Amendment Reason

Protocol Introduction
The questions on this page activate specific sections within the AUP form. Note that species selection is part of this introductory page.

Does this AUP involve teaching?
- Yes
- No

Is the animal work on this project shared by another Animal Care Committee?
- Yes
- No

Will you be using hazards?
- Yes
- No

Will live animals be moved outside of their housing facility?
- Yes
- No

Will field studies be conducted?
- Yes
- No

Add/Update/Remove Species Used on this Protocol

<table>
<thead>
<tr>
<th>Species</th>
<th>Agents</th>
<th>Drugs</th>
<th>Restraint</th>
<th>Breeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Rat</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Animal Use Protocol Overview

Animal Use Protocol Title

Assessing the potential of intratumoral modulation therapy for glioblastoma

Application Type. If this is a post-pilot project, please attach the Pilot Report to this section, below.

Full Renewal

Provide Associated Previous Protocol Number

2013-050

Please provide a report detailing the previous AUP's use of animals.

Fisher rats are implanted with F98 glioma cells in the striatum of the brain (40,000 cells per side). During the same surgery, stimulation electrodes are also implanted. After 4-7 days of tumor growth, one side is stimulated 24/7 by different frequencies (between 100 Hz and 140 kHz) and 2.1V with different polarities and electrode designs in order to test how this affects tumor growth on the stimulated side in comparison to the non-stimulated side. Animals are sacrificed 15 days after cell implantation and 6 days of stimulation or when reaching euthanasia criteria, whichever comes first.

Generally, we euthanized animals 15 days after cell implantation, without them showing major symptoms of the brain tumor. Only one rat (out of 92) had to be euthanized one day earlier because it had reached euthanasia criteria. A total of three rats died under anesthesia during the implantation surgery for unknown reasons. They were all from the same litter. Finally, one rat died at the first tumor resection attempt under anesthesia. This was due to the challenging technique of tumor resection in the small rat brain. Techniques have since been refined, so that we didn't have any further mortalities. The results of this study so far has been submitted for publication to scientific reports, and will be hopefully published shortly.

Additional animal groups were added, where human glioblastoma cells are injected into immunosuppressed rats. Here the tumors often don't take at all, or take much slower.
Using non-scientific language, please describe the project's purpose, expected benefit, and a brief summary of your work with the animal model(s).
*Please be aware that in the event of communications with Western Media Relations and the PI is not available, this summary will be sent to Western Media Relations.*

There is currently no cure for brain tumors (glioblastoma), and affected patients have a survival time on average of 8 months after first diagnosis. We have evidence from cultured human glioblastoma cells, plus there is evidence from transcranial stimulation by electrodes glued onto the head, that electrical stimulation can slow down, if not completely stop, cancer cell growth. In this project, we use a well established rat brain cancer model in order to test whether stimulation with an intratumoral electrode (same as the ones that are currently used for deep brain stimulation of Parkinson patients) 24/7 can stop brain tumor growth. Rats will be injected with rat or human glioma cells into the brain on both sides. In one side we will implant an electrode into the developing tumor and stimulate 24/7. After different survival times, animals will be sacrificed and tumor size will be assessed. In a separate group, the tumor will grow for 4-7 days and a tumor resection will be performed to see if the electrical stimulation can impede the regrowth of tumor. If successful, this treatment could be readily be turned into an experimental treatment in the clinic.

GLOSSARY OF TERMS - Identify each individual scientific term and abbreviation using CAPITAL LETTERS, and then briefly define each term to be referenced in any section of this protocol.

* e.g. ALLELE - The genetic variant of a gene responsible for the different traits of certain characteristics and genetic diseases.

  - Glioblastoma - the most common form of brain cancer
  - Transcranial - through the skull (the patient is wearing a bath cap with electrodes in it)
  - Intratumoral - inside the tumor
  - HFS- high frequency stimulation (usually frequencies of 100-300 Hz
  - AEF - alternating electrical fields. Electrical fields which alternate in polarity at a very high frequency of 100-300 kHz
  - Monopolar - an electrode that only contains one lead. A reference electrode is placed somewhere under the skin. Large stimulated areas are the result
  - Bipolar - an electrode with at least two leads (plus and minus pole). Small stimulated areas mainly close/between the two leads are the result

Here is the link to CCAC's Policy on Scientific Merit and Ethical Review of Animal-based Research:


Has the work outlined in this AUP received favourable scientific peer review?

Yes ☐ No ☐

Do you wish to provide a funding peer review assessment, which may be considered in lieu of internal scientific peer review? If ‘YES’, please attach the funding assessment.

Yes ☐ No ☐

If this is a RESEARCH AUP, please provide a list of one to three publications relevant to the work outlined in this AUP.

If this is a research AUP, attach an OUTLINE for scientific merit reviewers that provides sufficient information that another scientist working in the same field of study could effectively review this AUP's scientific merit, below. Pls may utilize whichever format best describes its scientific merit, e.g. background, rationale, hypothesis, objectives, experimental procedures

Using only key words, specify the animal models and procedures described within this AUP.

  - Anaesthesia (gas), stereotaxic surgery, Special agents (F98 cells), human glioma, chronic implantation of cannula/electrodes, chronic electrical stimulation in the awake animal, daily monitoring, euthanasia, tumor resection
Appendix C

UWO Animal Use Protocol 2018-026

3/22/2019

Table of Contents

Animal Use Protocol Overview
Funding Source List
Purpose of Animal Use
Hazardous Materials
Animal Movement Between Sites
Animal Groups and Experimental Timelines Overview
Mice
- Tissue Collection
- Justification for Choice of Species
  - the 3Rs: Replace, Reduce, Refine
- Species Strains
- Animal Transfer
- Environmental Enrichment
- Animal Housing, Housing and Use Location Information
- Holding beyond 12 hours
- Acclimatization Period & Quarantine
- Veterinary Drugs
- Experimental Agents Information
- SOP List
- Procedures Checklist for Reporting and Training
- Procedural Narrative
- Procedural Consequences & Monitoring
- Endpoint Method Information
- Animal Numbers Requested
- Rat
- Tissue Collection
- Justification for Choice of Species
  - the 3Rs: Replace, Reduce, Refine
- Species Strains
- Animal Transfer
- Environmental Enrichment
- Animal Housing, Housing and Use Location Information
- Holding beyond 12 hours
- Acclimatization Period & Quarantine
- Veterinary Drugs
- Experimental Agents Information
- SOP List
- Procedures Checklist for Reporting and Training
- Procedural Narrative
- Procedural Consequences & Monitoring
- Endpoint Method Information
- Animal Numbers Requested
- Reptilian List
- Protocol Appendices
- Amendment Reasons

Protocol Introduction
The questions on this page activate specific sections within the AUP form. Note that species selection is part of this introductory page.

Does this AUP involve teaching?

- Yes  
- No

Is the animal work on this project shared by another Animal Care Committee?

- Yes  
- No

Will you be using hazards?

- Yes  
- No

Will live animals be moved outside of their housing facility?

- Yes  
- No

Will field studies be conducted?

- Yes  
- No

**Add/Update/Remove Species Used on this Protocol**

<table>
<thead>
<tr>
<th>Species</th>
<th>Agents</th>
<th>Drugs</th>
<th>Restraint</th>
<th>Breeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Rat</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

**Animal Use Protocol Overview**

**Animal Use Protocol Title**

Using human brain cells for cell-based therapy

**Application Type. If this is a post-pilot project, please attach the Pilot Report to this section, below.**

- Full Renewal

**Provide Associated Previous Protocol Number**

2014-001

**Please provide a report detailing the previous AUP's use of animals**

We have done some initial studies in the last AUP period, demonstrating that we can track the fate of implanted cells in the mouse brain for up to 14 days, using different techniques, such as Bioluminescence imaging, as well as cells transfected with iron particles and MRI. There was no mortality or morbidity in these studies, but very inconsistent survival of the implanted cells, as far as we could track them. We now use different protocols for transfecting the cells, and seem to have far better survival and tractability.

Using non-scientific language, please describe the project's purpose, expected benefit, and a brief summary of your work with the animal model(s).

*Please be aware that in the event of communications with Western Media Relations and the PI is not available, this summary will be sent to Western Media Relations.*

My collaborator Matthew Hebble harvests small quantities of human brain tissue during neurosurgery in brain tumor patients and during deep brain stimulation electrode placement in Parkinson Disease patients. When kept in cell culture, these human brain cells grow and divide and start to carry markers for oligodendrocytes, which normally provide the myelin sheets in the
brain. They also release neurotrophic factors that are essential for promoting neuronal cell survival. We want to explore their potential to be re-introduced into the brain of rats or mice to repair cell damage in MS patients or to promote cell survival in neurodegenerative diseases. As a first step, we want to inject these cells into brains of immunodeficient mice and see whether they integrate, form myelin sheaths, and/or release neurotrophic factors. We also want to use rats and inject rat cortical reprogrammed cells back into the rat. Finally, we want to inject human patient-dervied cortical cells also into immunodeficient rats.

GLOSSARY OF TERMS - Identify each individual scientific term and abbreviation using CAPITAL LETTERS, and then briefly define each term to be referenced in any section of this protocol.

e.g. ALLELE - The genetic variant of a gene responsible for the different traits of certain characteristics and genetic diseases.

- myelin sheath: a fatty insulation sheet around the neuronal cell processes. Like cable insulation it is important for proper function.
- oligodendrocytes: Glia (non-neuronal) cells in the brain that form the myelin sheaths
- neurotrophic factors: small molecules that promote survival and growth of neurons
- demyelination: loss of myelin sheaths due to disease

Here is the link to CCAC’s Policy on Scientific Merit and Ethical Review of Animal-based Research:


Has the work outlined in this AUP received favourable scientific peer review?

Yes ☐ No ☐

Do you wish to provide a funding peer review assessment, which may be considered in lieu of internal scientific peer review? If YES, please attach the funding assessment.

Yes ☐ No ☐

If this is a RESEARCH AUP, please provide a list of one to three publications relevant to the work outlined in this AUP.

If this is a research AUP, attach an OUTLINE for scientific merit reviewers that provides sufficient information that another scientist working in the same field of study could effectively review this AUP’s scientific merit, below. PIs may utilize whichever format best describes its scientific merit, e.g. background, rationale, hypothesis, objectives, experimental procedures

Using only key words, specify the animal models and procedures described within this AUP.

Fundamental Research, stereotaxic surgery under gas anesthesia, microinjection of cultured human glioblastoma cells into brain, injections of post OP analgesia, animal survival for different time periods, in vivo imaging under anesthesia, euthanasia through transcardiac perfusion.
Curriculum Vitae

Andrew Olin

**Education:** University of Western Ontario

- Master’s Candidate, Neuroscience Graduate Program
  UW-Madison
- B.S. Neurobiology 5/2017
  Madison College
- A.A.S Biotechnology Lab Technician 5/2014

**Research and Teaching Experience:**

*Graduate Research Assistant*

Hebb/Ronald Labs, UWO 9/2018 -

Working in collaboration with Clinical Neurological Sciences Department of University Hospital (Dr. Hebb) and Robarts Research Institute (Dr. Ronald) to advance novel therapeutics towards glioblastoma. Laboratory techniques include primary mammalian cell culture, cloning and genetic engineering, flow cytometry, stereotaxic rodent brain surgery, bioluminescent imaging, and immunohistochemistry. The thesis project entails characterizing the viability of a novel cell type within orthotopic brain tumour models using multiple preclinical imaging technologies.

*Associate Research Specialist*

Burger Lab, UW-Madison 6/2017-8/2018

I worked on two projects related to Parkinson’s disease and Alzheimer’s disease. We examined the phosphorylation states of particular proteins involved in synaptic plasticity, as to whether these states confer healthy or damaged behavior in cells and in animals. These experiments included behavioral techniques, biochemical analysis of cell and tissue extracts, and electrophysiology. I also trained new students in the lab and assisted in other projects using novel gene editing techniques to control the expression of these proteins. In addition, I was responsible for equipment maintenance, reagent inventory and ordering, and general lab managing tasks.

*Undergraduate Research Assistant, Teaching Assistant*

Burger Lab, Neurobiology and Behavior Course, UW-Madison 6/2016-5/2017

As an advanced undergraduate, I joined Dr. Burger’s lab to work on a project to study the molecular mechanism of environmental enrichment on learning and memory in aging. Environmental enrichment (EE) has been shown to enhance quality of life and cognitive ability in rats. Working with Giuseppe Cortese, a postdoc in the lab, we were able to show
that EE enhances learning and memory and synaptic plasticity via activation of the p70 ribosomal S6 kinase. I was able to assist in experiments, data analysis and interpretation, as well as manuscript development ultimately leading to publication in Neurobiology of Aging.

With Drs. Burger and Ziskind-Conhaim as mentors, I was able to develop content for, and assist in the teaching of an upper-level laboratory course on techniques in neurobiology. Conceptual and hands on principles of electrophysiology were taught using crawfish, through intracellular recordings and pharmacological effects on receptors.

**Recognition and Awards:** American Society for Neural Therapy and Repair (ASNTR) Annual Conference Travel Award 4/2017 Dean’s List with Honors, Madison College 5/2014

**Publications:**


**Poster Presentations:**


-presented at multiple conferences
Neuroscience Research Day 2/2020
Clinical Neurological Sciences Research Day (virtual) 5/2020

Olin, A., Cortese, G., and Burger, C. Optimization of the Radial Arm Water Maze to Distinguish Learning and Memory Performance in Aged Rats

Olin, K., Olin A., and Burger, C. The Synaptic Scaffolding Protein Homer1c is Necessary for Successful Learning and Memory and for Induction of Group 1 Metabotropic Glutamate Receptor-Mediated Long-Term Depression.

-presented at multiple conferences
Alzheimer’s Disease and Related Disorders Research Day 6/2017
American Society for Neural Therapy and Repair Annual Conference 4/2017
Olin, A., Drone, D. Utilization of a periplasmic signal sequence to overcome host cell toxicity of a recombinant nuclease.

Mentored Research Poster Session, UW-Madison 5/2015

Donovan S., Olin, A., Thao, T.W., Thiessen, A., Tubon, T.C. Differentiation of iPS IMR90-4 Cells into Neural Precursors using E6 Media

Wisconsin Stem Cell Symposium: From Stem Cells to Blood 4/2014