RESEARCH PLAN

A. SPECIFIC AIMS. The long-term goal of these pilot studies is to prevent glioblastoma (GBM) recurrence, and brain metastases, by removing the senescent microenvironment that supports it. In the current studies, we are using GBM as a model and proof of principle. GBM is the most common and aggressive brain tumor. It kills most people within 2 years. Despite surgery, radiation, and temozolomide chemotherapy, most GBMs recur within 6 months. While the incidence of GBM dramatically increases after age 60, and continues to increase with age, little research has focused on the age-dependent microenvironmental factors that promote growth and recurrence. Instead, the dogma is that the age-GBM association is due to accumulation of mutations within a cell of origin. *Our central hypothesis is that the aging brain and senescent microenvironment caused by radiation promote tumorigenesis and recurrence.* While this idea is new and potentially risky, there is evidence that bolsters our premise that non-cell autonomous mechanisms in the microenvironment may play a role in brain tumors – and as relevant to the Longer Life Foundation reviewer critique (see below) – applicable to tens of thousands of patients who develop brain metastases from other cancers: (1) the senescent microenvironment promotes cancers in other organs[1,2] and we believe these lessons apply to the brain; (2) advanced age decreases immune system efficacy in clearing brain tumors[3], which leaves open the possibility that other aged non-tumor cells play a role as well; and (3) there is one report that eliminating radiation-induced senescent cells may increase survival in a mouse glioma model[4]. We will test our hypothesis in the following mechanistic and pre-clinical Aims (**Fig 1**), which will provide data necessary to purse deeper mechanistic and clinical studies:

Aim 1. Determine whether senescent cells increase cell proliferation and DNA damage. Using cell lines, we will test whether senescent cells promote hallmarks of cancer.

Aim 2. Determine whether senolytic therapy can prevent tumor recurrence. By repurposing already in-human pharmacologic agents to remove senescent cells, as well as genetically modified mice, we will test whether the senescent microenvironment drives brain tumorigenesis and recurrence.

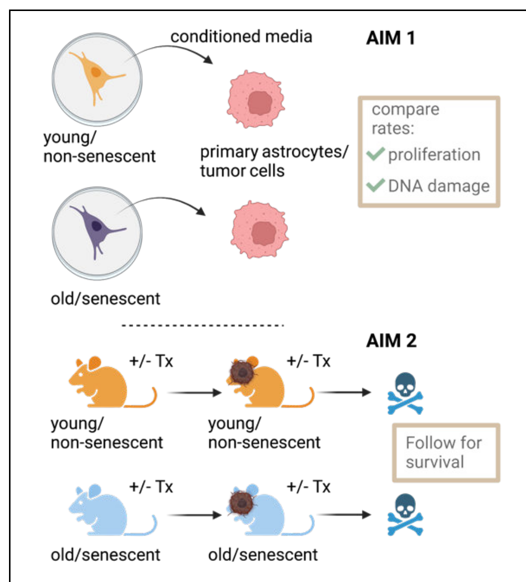

Figure 1. Schema of Aims. Aim 1 uses cell models to test the hypothesis that senescent cells promote key characteristics of cellular transformation: increased proliferation and mutagenic stress. **Aim 2** uses agents approved for humans as well as genetically modified mice to test whether ablation of senescent cells prolongs survival of mice with brain tumors.

This work is significant because it will add to our understanding of how aging, which the World Health Organization finally recognizes as a disease, increases the risk of GBM pathogenesis and recurrence in a non-cell autonomous manner. This work is conceptually innovative because it will suggest senolytic therapy is a promising way to prevent primary GBM and its recurrence after treatment. The principles will be applicable to metastatic tumors to the brain, which affect a much larger population and are a major cause of morbidity and mortality. We are well positioned to quickly translate positive findings to a clinical trial.

B. SCOPE OF WORK AND RELEVANCE TO THE MISSION OF THE FOUNDATION

Each of the Aims of this proposal will generate preliminary data that we will leverage for NIH R01 support to clarify the mechanisms by which aging causes GBM recurrence and enables metastatic tumor cells to thrive in the brain. More specific to the goals of the Longer Life Foundation, the data we will obtain in this proposal will support a phase I clinical trial of senolytic therapy to prevent GBM recurrence, with

support from one of the following sources: Bristol Meyers Squibb, the Barnes Foundation/Institute of Clinical and Translational Sciences, the Siteman Investment Program, or NIH.

Clinical applicability and path to translation: Dasatinib, which is an agent with senolytic properties, is already FDA-approved for a completely different indication. Quercertin is a nutritional supplement. As such, the findings from this study can be quickly translated to patients. Dr. Chheda has experience with operating clinical trials: he holds two FDA investigational new drug licenses and is PI of 4 investigator-initiated interventional trials, and the national PI of one consortium trial. At the end of one year of the funding period, we envision a phase I clinical trial to find the maximal tolerated dose of the combination of dasatinib and quercertin in patients with either primary or metastatic brain tumors. We envision we can complete enrollment in less than 1 year, and we would follow this with a phase II trial.

Applicability to broad audiences or populations: One reviewer of our LOI noted that GBM patients offer a "[V]ery limited population that would add value to insurance medicine at this time." We would like to emphasize that we are elucidating the mechanism by which the aging brain microenvironment supports cancer, using GBM as a model. The findings of this work will be applicable to brain metastases, which are a major cause of death and morbidity in patients with breast or lung cancer, which afflict tens of thousands of patients a year. A question that might arise is why we do not do these studies using brain metastasis models rather than primary GBM models. First, our lab has well established expertise to perform these experiments, and we have experience with the nuance of these tumor models. Second, the path to clinical translation is quicker in the GBM population, as the readouts for efficacy will be faster than in metastatic disease. Once this path is established, application to other indications in broader populations will be faster.

Enhancement of length or quality of life: Recurrent disease is what kills patients with GBM. By preventing or delaying recurrence, we will have a major impact on their length and quality of life. Assuming the same brain microenvironment principles apply to tumors that metastasize to the brain, we believe we can significantly extend the length and improve the quality of a vast number of lives.

Elucidation of mechanism of disease, even if patient population is not large: After successful completion of our Aims, we will have identified which non-tumor senescent brain cell populations can support cancer cells. This will form the basis of further studies identifying the mechanism by which these aging and senescent cells perform these pro-tumor functions.

Application of new technologies to questions related to The Longer Life Foundation's Mission: We are applying a new technology to study aging and senescence, and cancers of the nervous system. The INK-ATTAC mouse enables inducible elimination of $p16^{Ink4a}$-positive senescent cells upon administration of a drug, AP20187[5]. The use of this model to study ageing and brain cancer is technically innovative.

C. BACKGROUND AND SIGNIFICANCE

Glioblastoma (GBM). Most patients with glioblastoma who have been treated with existing standard therapies have recurrence within 6 months and almost all die within 2 years. Standard therapy for GBM is surgery, followed by radiation and the alkylating chemotherapy, temozolomide, which was approved twenty years ago. Targeted therapies have had limited success in GBM: no single targeted or combination therapy has been successful in increasing the time to recurrence or overall survival.

A major gap in knowledge is how the normal brain microenvironment supports tumor pathogenesis. While most patients with glioblastoma (GBM) are in their 60s, almost all preclinical studies over the past 30 years have used very young mice (6-8 weeks old) that do not model GBM as a disease of aging. Second, there has been little exploration of how radiation-induced

senescence promotes tumor recurrence. Both of these issues are also applicable to understanding and preventing brain metastases from other primary tumors, as well as their recurrence after radiation, which is a standard treatment for breast and lung brain metastases.

Senescence. Cellular senescence is an irreversible state of cell cycle arrest. Senescence cells accumulate with ageing. It has been established that the senescent microenvironment can promote cancer[1,2], although this has not been yet shown in the brain. At least partially, senescent cells affect their surrounding milieu through secretion of factors, collectively called the senescence associated secretory phenotype (SASP). In addition to age, the senescent cell state can be acquired through various stresses, including radiation.[1,2,4,6] There is one report that eliminating radiation-induced senescent cells may increase survival in a mouse glioma model[4]. In this proposal, we use aged mice, and also radiation, to study the impact of the senescent state on brain tumors.

Senolytics. Various agents, termed senolytics, can kill senescent cells. These include dasatinib, quercertin, ABT263, ABT737, FOXO4-DRI, and bafilomycin A[7]. In this proposal, we use a combination of dasatinib and quercertin, because they have both been used in humans and the dosing by which they can reach the brain and target resident brain senescent cells in mice has been established[8-10]. Dasatinib is FDA-approved for Philadelphia chromosome-positive (Ph+) chronic myeloid leukemia (CML) in chronic phase. Quercetin, is a nutritional supplement. Given these treatments are safe in humans, there is potential for rapid clinical translation of this work.

INK-ATTAC mouse. We will use genetically modified mice carrying the INK-ATTAC transgene, which enables inducible elimination of $p16^{Ink4a}$-positive senescent cells upon administration of a drug, AP20187[5]. This mouse was generated on the BubR1 progeria mouse background, a model for a genetic disorder which causes premature and rapid aging.

Significance. Our work is *significant* because it may lead to development of the first aging- and senescence-targeting therapies to treat patients with cancer.

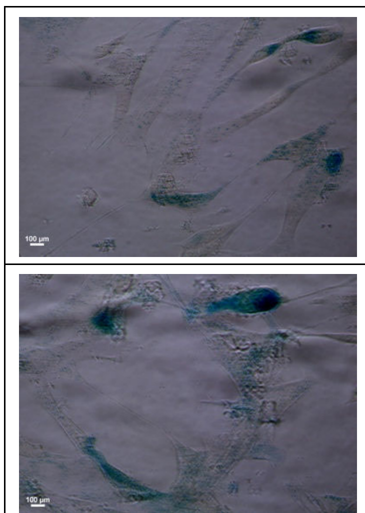

Figure 2. Isolation of murine astrocytes and β-galactosidase staining for senescence. 8 week old mouse brains were harvested and processed to isolate primary astrocytes. They were then treated with 0Gy (top) or 4Gy (bottom) of radiation and 1 week later were stained with β-galactosidase. Note the 4Gy condition has more senescent cells. Scale bar, 100uM.

Innovation. These studies are *conceptually innovative* because of focusing on developing therapies that target brain tumor cells directly, they focus attention on the microenvironment that supports these tumors. In addition, despite decades of research, there has been almost no investigation into why brain tumor incidence increases with age. Instead, the dogma is that the age-GBM association is due to accumulation of mutations within a cell of origin. This work is *technically innovative* because it uses the INK-ATTAC mouse, which has not been used for studies of brain tumors.

D. PRELIMINARY DATA

Models of glioma. We have published expertise using both xenograft[11,12] and syngeneic[13-15] mouse glioma models. We have generated immortalized, non-transformed astrocytes[15] for studies in Aim 1.

Isolation of specific primary cell types from mouse brains. PhD candidate/Visiting Scholar Amanda deAndrade Costa has trained in neuroscience laboratories and has expertise isolating primary brain cells from mice. Mouse brains are mechanically and enzymatically dissociated, myelin is removed, and subjected to a Ficoll gradient. Depending on the cell type, cultures are treated with factors (e.g. 5ng/ml GM-CSF for microglia). We are also optimizing β-galactosidase staining (**Fig 2**).

E. RESEARCH DESIGN AND METHODS

Aim 1. Determine whether senescent cells increase cell proliferation and DNA damage.
1.A. We will isolate brains from 2 month old and 2 year old mice. Then separately, we will prepare organotypic brain slices or isolate neurons, astrocytes, microglia and oligodendroglia cells. Using β-galactosidase staining, we will confirm older brains and cells have a greater percentage of senescent cells. We will culture sections or cells, and apply the conditioned media to murine glioma cell lines (e.g., CT2A and GL261) or immortalized astrocytes. We will assess relative cell proliferation of these target cells by CellTiter Glo assay (Promega). We will also assess rates of DNA damage, as a preliminary surrogate for mutagenesis, using comet assay and immunofluorescence for g-H2AX. Negative controls will include fresh, non-conditioned media. Positive controls for DNA damage will be 2Gy radiation. We expect that proliferation rate and rate of DNA damage will be increased in the target cells when they are conditioned with media from the aged brains or cells. This will suggest that a secreted factor from aged cells promotes two key hallmarks of cancer.

1.B. We will isolate cells from 2 month old mice and induce senescence using radiation. We will optimize our protocol and confirm senescence using β-galactosidase staining. We will then use conditioned media in a similar fashion as **1.A.** using the same target cells. We expect that conditioned media from senescent cells, compared to control cells, will induce greater proliferation and DNA damage in target cells. This will suggest that a secreted factor from senescent cells promotes phenotypes associated with cancer.

Statistical considerations. Each experiment will be performed using pooled brain isolations from 2 mice, and using these, all studies will be performed with three biological replicates (brain isolations four different times). We are limited to mouse numbers and replicates given that each aged mouse is over four hundred dollars. We will quantify percentage of β-galactosidase positive cells, g-H2AX foci, as well as relative luminescence values from CellTiter Glo with Fisher's exact test for each cell type (neurons, astrocytes, etc.). Each cell type will be considered separately. We will perform studies using male and female mice, not mixing sexes. If we find differences present between old and young in one sex and not the other, we will pursue this interesting observation in future studies.

Expected Outcomes, Potential Pitfalls, and Alternatives. These are standard isolations and assays for our laboratory, and we do not anticipate technical difficulties. We expect that brain cells and slices from older mice will promote more proliferation and DNA damage of target cells. We also expect that radiation-treated cells will do the same. If we find that there is no increase in proliferation in already transformed cells (CT2A and GL261), but there is increase in immortalized cells, this will suggest that the senescent microenvironment may preferentially play a role in the initiation of tumors as opposed to further accelerating cells that are already tumorigenic. If we find that immortalized cells are more susceptible to DNA-damage from senescent cell conditioned media, we will interpret this as an important causal step enroute to transformation. If, as expected, that senescent cells increase relative cell number of target cells, we will validate with assessment of cell cycle using flow cytometry. If, as expected, we see an increase in DNA damage in target cells, we will follow with deep sequencing of cells to test whether they develop a greater mutational burden compared to target cells treated with young cell media.

Aim 2. Determine whether senolytic therapy can prevent tumor recurrence. Using genetic models and repurposing already safe in-human pharmacologic agents to remove senescent cells, we will test whether they contribute to tumorigenesis and tumor recurrence.

2.A. A major challenge in neuro-oncology is GBM recurrence after radiation, which is the standard of care. Radiation induces senescence in the brain[4]. Toward advancing clinical translation, we will test whether pharmacologic ablation of senescent cells improves outcomes after radiation. We will irradiate the right hemisphere of 20 month old C57BL6/J mice with 2Gy/radiation or 0Gy x 5 days and wait one month. We will treat 50% of the mice in each group with dasatinib 5mg/kg and quercertin 10mg/kg by oral gavage, three consecutive days/week, every two weeks for 8

weeks. The control treatment will be the vehicle, 60% phosal/10% ethanol/30% PEG-400. Then, we will implant 40K syngeneic murine GL261 glioma cells transduced with luciferase into the right hemisphere. No therapy will be given after the tumor cells are implanted; therefore, we do not expect any confounding of a direct anti-tumor cell effect of the senolytics. We expect two findings from this experiment: (1) irradiated mice treated with senolytics will live longer than those treated with vehicle (model of senolytics in the tumor recurrent setting); and (2) the 0Gy control mice treated with senolytic will live longer than the 0Gy control mice treated with vehicle (model for prevention of tumors in aged patients treated with senolytics). Either of these outcomes will make a unique impact on the field and will be strong data to leverage further funding and interest.

2.B. To test the hypothesis that radiation-induced senescence in the tumor microenvironment favors recurrence, we will use the INK-ATTAC mouse. This mouse enables inducible elimination of $p16^{Ink4a}$-positive senescent cells upon administration of a drug, AP20187[5]. We will either deliver 2Gy/day x 5 days or 0Gy control to the brains of 8 week old mice. We are using this age mice as we will not have time to breed and age them out in the funding period. However, this work will be relevant to the recurrence model. At 1, 2, and 4wk post radiation, we will confirm induction of senescence by harvesting brains and performing immunohistochemistry for β-galactosidase. After confirming the best timepoint that demonstrates induction of senescence in the brain, we will deliver radiation to another set of mice. At the defined time point, each group will be treated with AP20187 or control, to delete senescent cells or leave them intact. One week later, we will implant syngeneic glioma cells, as in **2A** and follow them for survival. We expect that mice in which senescent cells are eliminated will live longer.

Statistical considerations. In Aim 2A, fifteen 20 month old mice, and fifteen 2 month old mice will be used for each condition; there will be 4 conditions: with and without radiation and with and without senolytic treatment. Since these are pilot studies, we have no preliminary data to perform a power calculation, however, we believe this size of cohorts is financially feasible (each 20 month old mouse is $412) and will demonstrate evidence of an effect worth pursuing when further funding becomes available. In Aim 2B, 20 mice will be used per group. This is based on the reasonable availability of mice after breeding in the funding period. For survival analysis, we will use the Kaplan-Meier (KM) method and log-rank test to assess differences between/across groups and will estimate hazard ratio (HR) from the Cox model. Based on our previous data using the same syngeneic glioma model[13], to achieve 80% power, we used 16 animals per group.

Expected Outcomes, Potential Pitfalls, and Alternatives. We are facile with intracranial tumor injections and delivery of hemi-brain radiation with the Washington University SAARP facility, and as such, we do not anticipate technical difficulties. In terms of treatment of the INK-ATTAC mice, our neighboring lab uses these mice regularly, and drug administration of AP20187 is straightforward and achievement of senolysis is validated. We expect that aged mice have shorter survival, and that senolytic therapy will significantly increase survival, even to the extent of survival of the young, untreated mice. An implicit limitation in these studies is the use of only a total of 30 aged mice, and one syngeneic mouse model. This is due to cost. However, the purpose of these studies is proof of principle and to provide pilot data for further mechanistic and clinical translation studies. If we do not find a difference in the aged mice versus the young mice, treated with 0Gy of radiation, we will consider using slower growing and less aggressive brain tumor models to see an effect. We will also test whether a genetically modified mouse model that develops brain tumors that we have currently breeding in the lab has a difference in tumor pathogenesis in old versus young mice. In this model, we induce spontaneous tumors in neural stem cells in which PTEN is surrounded by LoxP sites, by administering avian virus carrying Cre recombinase and the PDGFRα transgene[16]. We will continue to age these mice and either initiate tumors in young or old mice to further test the hypothesis. If we find that the dasatinib and quercertin combination does not increase survival, but the AP20187 treatment does, we will test whether other senolytic compounds, such as ABT263, ABT737, FOXO4-DRI, or bafilomycin A have an effect. Another option to consider is that the dasatinib and quercertin did not perfuse the brain at a high enough concentration. This is less likely given the published literature; however, we could also directly deliver these compounds to the brain using an Alzet osmotic pump. Regardless, future studies

will use mass spectroscopy to measure the concentration of senolyic agent that reaches the brain parenchyma.

Summary. Upon completion of these studies, we will have (1) preliminary data that a secreted factor from senescent cells can promote several hallmarks of cellular transformation and (2) a signal that senolytic therapy may be worth pursuing in mechanistic and translational studies to prevent tumor recurrence, and brain metastases in patients with systemic cancers. Such findings will be impactful for the field of neuro-oncology, where the main focus has been on developing better tumor cell-targeted agents. It will also suggest that there may be contexts where we should consider reducing radiation in order to prevent microenvironmental promotion of tumor recurrence. These findings will be relevant to patients with primary brain tumors, as well as the tens of thousands of patients each year who develop brain metastases. Collectively, these findings will improve the length and quality of lives of patients with cancer.

TIMELINE

Aims	Q1	Q2	Q3	Q4
Aim 1. Determine whether senescent cells increase cell proliferation and DNA damage.				
Aim 1A. Conditioned media from old or young mouse brain cells	X			
Aim 1B. Conditioned media from irradiate or non-irradiated cells.		X		
Aim 2. Determine whether senolytic therapy can prevent tumor recurrence.				
Aim 2A. Dasatinib and quercertin in old and young mice, in vivo.		X	X	
Aim 2B. INK-ATTAC mouse studies.			X	X

PLANS FOR FUTURE FUNDING

We will leverage these pilot studies to deepen mechanistic understanding of how the ageing brain and treatment-induced senescent brain cells support and promote primary and metastatic brain tumors. We will also leverage the studies in Aim 2 to directly impact patient lives.

Therefore, we will pursue future funding along both mechanism and translational tracks.

June 2023: R01 Application to National Institute of Neurological Disorders and Stroke (NINDS), Clinical Neuroimmunology and Brain Tumor (CNBT) study section. Aim 1: Determine the mechanism by which senescent brain microenvironment promotes primary and metastatic tumor pathogenesis. Aim 2: Test whether standard of care and/or immune checkpoint blockade can be enhanced by senolytic therapies. Aim 3: Develop a mouse model that enables ablation of specific brain cells across space and time.

July 2023: LOI submission to Bristol Myers Squibb for dasatinib supply for an investigator initiated phase I trial of dasatinib combined with quercertin in patients with glioblastoma or astrocytoma.

September 2023:
A. Siteman Investment Program. Aim 1: Perform a phase I investigator initiated trial of dasatinib combined with quercertin in patients with glioblastoma or astrocytoma. Primary endpoint: toxicity. Aim 2: Brain imaging correlates of senolytic therapy.

B. The Foundation for Barnes-Jewish Hospital and the Institute for Clinical and Translational Sciences application. Perform a phase I investigator initiated trial of dasatinib combined with quercertin in patients with glioblastoma or astrocytoma.

C. American Brain Tumor Association Discovery Grant. Aim 1. Combination of oncolytic virus therapy and senolytic therapy to target the senescent immune system in mouse models of glioblastoma. Aim 2. Identification of senescent associated secretory phenotype (SASP) factors that support radiation resistance.

October 2023: R01 With Clinical Trial Application to National Cancer Institute (NCI), Developmental Therapeutics study section. Aim 1: Perform a phase I investigator initiated trial of dasatinib combined with quercertin in patients with glioblastoma or astrocytoma. Primary endpoint: toxicity. Aim 2: Brain imaging correlates of senolytic therapy. Aim 3. Immune correlatives of senolytic therapy in primary and metastatic brain tumor patients.

May 2024: Department of Defense IDEA award. Aim 1: Senolytic therapy in murine primary and metastatic brain tumor models. Aim 2: A new genetically modified mouse model to selectively ablate senescent brain cells over time and space.

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