

Plexin-B2 Mediates Angiogenin-regulated Stemness of
Glioma Stem Cells and is a Therapeutic Target to Prevent
GBM Recurrence

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Abstract

Glioblastoma (GBM) is a fatal disease. The standard of care of GBM has not changed over decades. Tumor recurrence is nearly universal and the recurred GBM lacks any effective therapeutics. Glioma stem cells (GSCs) constantly promote tumor heterogeneity, adaptability, and reconstitution after therapeutic interventions, and are the main culprit behind treatment failure. Characterization of the pathways that maintain the stemness properties of GSCs, including self-renewal ability, differentiation potential, and tumor-initiating capacity, will help to make inroads into the discovery of new targetable molecular determinants, all with the aim to eliminate, diminish, or mobilize GSCs to overcome therapeutic resistance.

Angiogenin (ANG) is a secreted ribonuclease known to regulate angiogenesis, hematopoiesis, and neurogenesis. Plexin-B2 (PLXNB2) is a transmembrane protein originally identified from GBM by differential screening between high grade and low grade astrocytoma, and was later on characterized as the functional receptor for ANG. Expression of both *ANG* and *PLXNB2* have been shown to be inversely correlated with overall survival of GBM patients. ANG and PLXNB2 have also been reported to promote GBM progression by enhancing perivascular invasion of GBM cells. However, their roles in GSC biology remain unknown. This thesis investigates the regulatory function and mechanism of ANG-PLXNB2 in GSCs with a particular emphasis on self-renewal, tumorigenicity, therapeutic response, and disease recurrence.

Here, we report that ANG enhances the stemness of patients-derived GSCs by promoting small RNA biogenesis, in particular, the production of tRNA-derived stress-induced small RNAs (tiRNAs), primarily the bioactive 5'tiRNA-Gly-GCC. PLXNB2 mediates this function of ANG in GSCs. PLXNB2 inhibition, either by gene knockout or by specific monoclonal antibodies (mAbs), reduces self-renewal, induces differentiation, and diminishes tumor-initiation capacity of GSCs. Combinatorial treatment of

temozolomide (TMZ) and PLXNB2 mAbs delayed the recurrence of GSC tumors in an orthotopic xenograft animal model and prolonged the survival of tumor-bearing mice. Post-mortem pharmacodynamical examination showed that PLXNB2 mAb therapy resulted in a decrease in tumor angiogenesis and in tumor cell proliferation, an increase in tumor cell apoptosis, and most significantly, a reduction of the tiRNA-Gly-GCC level in the tumor tissues, indicating an on-target effect of PLXNB2 mAbs that prevented the function of ANG from producing bioactive small RNAs.

Dedication

This work is dedicated to my loving parents, Cuilan Zhao and Zhipeng Yuan

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List of Abbreviations

ABE	Angiogenin Binding Element
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ANG	Angiogenin
ANGBP	Angiogenin Binding Protein
ATR	Ataxia Telangiectasia mutated and Rad3-related
BBB	Blood-brain Barrier
BDNF	Brain-derived Neurotrophic Factor
BEV	Bevacizumab
BER	Base Excision Repair
BLI	Bioluminescence
BMP	Bone Morphogenic Protein
CGP	Cerebellar Granule Cell Precursor
CML	Chronic Myelogenous Leukemia
CNS	Central Nervous System
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CSF-1	Colony stimulating factor 1
CTCF	Corrected Total Cell Fluorescence
DAPI	4',6-diamidino-2-phenylindole
DCX	Docetaxel
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eIF2 α	Eukaryotic Initiation Factor 2 α
ELDA	Extreme Limiting Dilution Analysis
ER	Endoplasmic Reticulum
EV	Extracellular Vesicle
FBL	Fibrillarin
FBS	Fetal Bovine Serum,
FDA	U.S. Food and Drug Administration
FGF	Fibroblast Growth Factor
Fluc	Firefly luciferase
GBM	Glioblastoma
GFAP	Glial Fibrillary Acidic Protein
GLUT3	Glucose Transporter, Type 3
GMPs	Granulocyte-Monocyte Progenitors
GSC	Glioma Stem Cells
GSI	Gamma(γ)-secretase Inhibitor
hESC	Human Embryonic Stem Cell
HIF2 α	Hypoxia-Inducible Factor 2 α
HSPC	Hematopoietic Stem and Progenitor Cell
IDH1	Isocitrate Dehydrogenase Gene 1
Ig	Immunoglobulin
IRES	Internal Ribosomal Entry Sequence
i.p.	Intraperitoneal
KO	Knockout
LATS2	Large Tumor Suppressor Kinase 2
LT-HSC	Long-term Hematopoietic Stem Cell

mAb	Monoclonal Antibody
mRNA	Messenger RNA
MGMT	O ⁶ -methylguanine-DNA methyltransferase
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging
NF1	Neurofibromatosis Type 1
NICD	Notch Intracellular Domains
NLS	Nuclear Localization Sequence
NOS-SCID	Nonobese Diabetic Severe Combined Immunodeficiency
NPC	Neural Progenitor Cell
NSC	Neural Stem Cell
NT	Nucleotide
O ⁶ -MG	O ⁶ -methylguanine
pAb	polyclonal antibody
PABP	Poly(A)-binding Protein
PARP-1	Poly (ADP-ribose) Polymerase-1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PDGFRA	Platelet-Derived Growth Factor Receptor A
PKM	Pyruvate Kinase Isozyme
PLXNB2	Plexin-B2
RNase	Ribonuclease
PNET	Primitive Neuroectodermal Tumor
RNH1	Ribonuclease/Angiogenin Inhibitor 1
RNP	Ribonucleoprotein
RPM	Reads Per Million Reads
<i>RRS1</i>	Regulator of Ribosome Synthesis 1
RTK	Receptor Tyrosine Kinase
RTK Inhibitor	Receptor Tyrosine Kinase Inhibitor
rRNA	ribosomal RNA
SEMA	Semaphorin
SG	Stress Granule
snoRNA	Small Nucleolar RNA
snRNA	Small Nuclear RNA
SVZ	Subventricular Zone
TAM	Tumor-associated Macrophage
TCGA	The Cancer Genome Atlas Research Network
tiRNA	tRNA-derived stress-induced small RNA
TKI	Tyrosine Kinase Inhibitor
TMZ	Temozolomide
TNF- α	Tumor necrosis factor α
tRF	tRNA-derived fragment
tRNA	transfer RNA
UCE	Upstream Core Element
URI	Unconventional Prefoldin RPB5 Interactor
VEGF	Vascular Endothelial Growth Factor
V-SVZ	Ventricular-subventricular Zone
WHO	World Health Organization
WT	Wild Type
5'-UTR	5'-Untranslated Region

Chapter 1: Introduction

1.1. Therapeutic challenges in GBM: overcoming therapeutic resistance

Malignant brain tumors are among the most feared types of cancer with a dismal prognosis and devastating repercussions on quality of life. Gliomas, accounting for 80% of all malignant brain tumors, are histologically diagnosed and assigned a specific grade according to the four-tiered World Health Organization (WHO) classification system (Louis et al., 2007). The WHO grade I-IV indicates increasing degrees of undifferentiation, anaplasia, and aggressiveness. Grade I gliomas are often well-demarcated slow-growing tumors associated with favorable prognoses. Grade II gliomas also grow slowly but often invasively, thus impeding complete surgical resection. Grade III gliomas are fast-growing and are characterized by diffusive brain parenchyma infiltration, histopathologic feature of anaplasia, and an inherent tendency to malignant progression. WHO grade IV is reserved for GBM, the most lethal type of human gliomas. GBM is a disease of clinical and morphological heterogeneity that distinguish from grade III gliomas by markedly increased cellular pleomorphism and the presence of glomeruloid microvascular proliferation and necrotic foci that are often surrounded by pseudopalisading cancer cells.

In this section, epidemiology and therapeutic challenges of GBM is discussed, with emphasis on unmet medical needs, the importance to identify new therapeutic targets, and tumor heterogeneity underlying the resilience of GBM.

1.1.1. Epidemiology of GBM

GBM is the most common and highly malignant tumor that accounts for 48.6% of all malignant tumors in the central nervous system (CNS). The average annual incidence of GBM per 100,000 people is approximately 3.23 in the United States and 3.5 worldwide (Ohgaki and Kleihues, 2005; Ostrom et al., 2020). Prevalence studies (The Central Brain Tumor Registry of the United States Statistical Reports) revealed a steady

increase of incidence rates over the last decades and projected 12,970 new cases in 2021.

GBM are more common in males than females (1.6:1) and in white than black, American Indian, and Asian populations (2:1). Yet, the causes and underlying biological basis of these disparities remain elusive. GBM is less common in children and young adults. As age advances, the incidence of GBM pronouncedly increases with a median age of 64 at diagnosis (Weller et al., 2015).

To date, ionizing radiations is the only definite factor that is recognized as a causative agent for GBM development (Ostrom and Barnholtz-Sloan, 2011; Ron et al., 1988; Sadetzki et al., 2005). The radiation exposure upon diagnostic scans does not suffice as a risk factor in that the risk is both dose and volume dependent (Bondy et al., 2008; Minniti et al., 2005). Despite extensive studies regarding the use of mobile phones and pesticide exposure, no definitive association with gliomagenesis has been reported (2010; Omuro and DeAngelis, 2013; Ostrom et al., 2014). In addition, several rare hereditary syndromes are associated with an increased risk of GBM ($\leq 1\%$), including neurofibromatosis type 1 and 2, Turcot syndrome and Li-Fraumeni syndrome (Ohgaki et al., 2010). Furthermore, GBM are inversely associated with the presence of allergy or atopic disorders that often lead to increased immunoglobulin (Ig) E levels, such as asthma, eczema, and hay fever (Louis et al., 2007). Of note, lifestyle changes, including cigarette smoking, alcohol consumption, or drug use, cannot help people to avert GBM. In short, there are no prevention measures and early detection for this disease.

1.1.2. GBM remains a universally lethal disease with a rapid clinical course

The clinical suspicion of a brain tumor is commonly based on the development of neurological symptoms, such as a unilateral headache with progressive severity and the focal onset of seizures in a previously healthy individual. Gait imbalance, hemiparesis, or

aphasia are also clinical manifestations, reflecting the mass, location, as well as spread dynamics of the intracranial tumors (Omuro and DeAngelis, 2013). Brain magnetic resonance imaging (MRI) is a frequently used diagnostic modality, however, the conclusive diagnosis of a GBM can only be made by pathology. On top of high-grade glioma characteristics (e.g. anaplasia, high mitotic rates, and invasiveness), the definitive histopathological signatures for GBM lesions are the presence of glomeruloid microvascular proliferation and necrotic foci that are often surrounded by pseudopalisading neoplastic cells (Louis et al., 2007).

GBM is one of the most aggressive neoplasms with a median survival of merely 3 months without treatment. The current standard of care for new diagnosed GBMs encompasses maximally safe surgical debulking followed by fractionated radiotherapy with concomitant adjuvant chemotherapy with DNA alkylating agent, TMZ, and followed by up to six cycles of TMZ alone (Stupp et al., 2005). Yet, the intrinsic capacity of GBM tumor cells to infiltrate normal brain parenchyma renders radical surgical excision virtually impossible. Despite the above-mentioned care regimen, all GBM patients inevitably experience tumor relapse and disease progression after 7-10 months. There is no standard of care for recurrent GBMs that are resistant to radio- and cytotoxic chemotherapy. To date, available salvage treatment options include anti-angiogenic therapy (bevacizumab), TMZ rechallenge, other alkylating agents (carmustine and lomustine), and the application of low-intensity alternating electric fields by TTFIELDS (Novocure) (Fisher and Adamson, 2021; Omuro and DeAngelis, 2013). Yet, their efficacy and therapeutic outcomes are limited.

Bevacizumab is the only FDA-authorized mAb for GBM treatment. Mechanistically, bevacizumab abrogates tumor vessel growth by blocking vascular endothelial growth factor (VEGF)/VEGFR signaling, an attempt for “tumor starvation”. Although the use of bevacizumab benefited highly symptomatic patients with brain

edema and even transitorily enhanced drug delivery, it failed to prolong overall survival, caused severe side effect due to impaired VEGF functions (e.g. arterial hypertension, hemorrhages, and congestive heart failure), and is associated with emergence of resistance and rapid disease progression (Omuro and DeAngelis, 2013; Tamimi and Juweid, 2017).

GBM remains a universally lethal disease with a rapid clinical course. Once bevacizumab and TMZ rechallenge fail recurrent GBM, no therapeutic options are available and remaining survival drops to 3-6 months. Despite all the scientific and clinical efforts, GBM patients have one of the grimmest prognoses with a median survival of merely 14.6 months and 5-year survival of 7.2% (Ostrom et al., 2020). The failure to prevent tumor recurrence and progression is the major cause of GBM patient demise. Over the past decades, treatment outcomes have merely improved, and new therapeutic approaches are desperately needed.

1.1.3. Tumor heterogeneity underlies therapeutic resistance

Tumor heterogeneity is a hallmark of GBM and a major obstacle to successful treatment. Studies that unveil this complexity by multi-omics characterization of GBMs across and within tumors provide new insights to dissect intra- and inter-tumoral heterogeneity of GBM and may open an avenue to tackle this extremely resilient disease.

1.1.3.1. Intertumoral heterogeneity of GBM

GBM is one of the most molecularly profiled human malignancies by the efforts of The Cancer Genome Atlas Research Network (TCGA) and others. Interpatient GBMs have been classified into three subtypes based on their distinct genomic, epigenetic, transcriptomic, and proteomic profiles, and termed as proneural, classic and

mesenchymal for their characteristics reminiscent of distinct neural cell types (Brennan et al., 2013; Verhaak et al., 2010; Wang et al., 2017). Proneural tumors harbor aberrant platelet-derived growth factor receptor A (*PDGFRA*) amplifications and frequent mutations in tumor suppressor *TP53* and isocitrate dehydrogenase gene, *IDH1*. The classical subtype of GBMs typically display preponderant epidermal growth factor receptor (*EGFR*) amplifications and homozygous deletion of *CDKN2A*. Mesenchymal subtype of GBM is defined by neurofibromatosis type 1 (*NF1*) deletion, *PTEN* mutation, and elevated expression of genes in tumor necrosis factor and nuclear factor- κ B pathways. These landmark analyses also revealed divergent clinical associations of different GBM subtypes. Patients harboring proneural GBM tended to have more favorable prognoses but had no survival benefits from aggressive treatment. Similar treatment regime, on the contrary, significantly delayed the mortality of classical and mesenchymal GBMs that are often related to poor survival (Bhat et al., 2013; Verhaak et al., 2010). Anti-angiogenic therapy with bevacizumab prolonged the progression-free survival of both mesenchymal and proneural GBMs with the standard-of-care therapy. However, the overall survival benefit was restricted to patients who bear proneural *IDH1* wild-type tumors (Masui et al., 2016; Sandmann et al., 2015).

To date, GBM patients have been treated with the same protocol for decades despite advances in molecular classification and clinical correlation analysis. Intertumoral heterogeneity render different treatment efficacies across GBM patients, highlighting a compelling need to further explore molecular landscape of each subtype and to identify cell(s) of origin(s) for establishing more effective therapeutic regimens. The differing phenotypes may be attributed to the cellular origins of GBM. However, though neural stem cells, CNS progenitor populations, and mature astrocytes have been proposed, the bona fide cells of origin for GBM remains undetermined.

1.1.3.2. GBM is a disease of intratumoral heterogeneity

Extensive intratumoral heterogeneity has long been pathologically documented for GBM. GBM was originally named as “glioblastoma multiforme”, an attempt to stress the strikingly diverse cellular composition within a tumor. Understanding of the complexity remained obscure until technology breakthrough in single cell-sequencing analyses that captured another layer of intratumoral heterogeneity - at the molecular level. A spectrum of subtypes can co-exist within the same GBM tumor, neoplastic region, or a “hybrid” single cell (Cloughesy et al., 2014; Osuka and Van Meir, 2017). In-depth analyses further revealed that cells from the same tumor can harbor different genetic alterations, display divergent epigenetic profiles, and exhibit distinct functional characteristics in the perspective of proliferation, stemness potentials, and responses to therapeutics (Patel et al., 2014; Sottoriva et al., 2013). Intercellular heterogeneity confounds treatment strategies and lays the foundations for disease recurrence.

Intratumoral heterogeneity is a dynamic ecosystem. From clonal evolution viewpoint, intratumoral heterogeneity represents the evolution history of a malignancy from the tumor-founding cell(s), termed cancer stem cells (CSCs). Divergent genetic makeups gradually accumulate in early lineage multipotent cells that subsequently give rise to a variety of more differentiated subclones under the selective pressures of tumor microenvironment and therapeutic forces (Osuka and Van Meir, 2017). Hypoxia, immune response, niche factors for preserving stemness, and bioactive molecules secreted by tumoral or stromal cells all actively influence the cellular and molecular phenotype (Li et al., 2009; Pietras et al., 2014; Zhou et al., 2015). Additionally, subtype transitions have been suggested in longitudinal tumor analyses that proneural primary tumors tended to shift toward mesenchymal phenotypes upon tumor relapse (Phillips et al., 2006). Furthermore, mesenchymal signatures could be induced in murine neural stem cells and human proneural GSCs through activating master transcriptional regulators, STAT3,

C/EBP β , and TAZ (Bhat et al., 2011; Carro et al., 2010). Finally, cytokines (TGF- β and TNF- α) and NF- κ B signaling have been found to regulate transcriptome plasticity in GSCs, enabling subtype transition from a more sensitive proneural state to a more resistant mesenchymal state (Bhat et al., 2013; Segerman et al., 2016).

Intercellular heterogeneity of GBM poses a serious challenge to effective treatment. In-depth understanding of GSCs, a potential GBM-founding cell displaying a striking degree of plasticity may provide an unprecedented insight to target GBM.

1.2. GSCs: driving GBM resilience

Intratumoral heterogeneity and plasticity make GBM a highly adaptable and resilient disease, for which a cure has not been achieved. Atop the apex of GBM hierarchy, GSCs encompass a spectrum of multipotent microstates that constantly adapt to the changing microenvironmental cues and therapeutic insults, resulting in tumor heterogeneity, adaptability, and ultimate therapy resistance (Prager et al., 2020).

1.2.1. GSCs

Two decades ago, several laboratories reported the successful isolation of GSCs or previously designated “human brain tumor initiating cells” (Galli et al., 2004; Singh et al., 2004). GSCs are defined as a rare cell fraction capable of reproducing heterogeneous parental gliomas. GSCs co-opt developmental programs and are endowed with essential functional characteristics of CSCs: high oncogenic potential, unlimited self-renewal ability, and multilineage differentiation capacities (Lathia et al., 2015).

GSCs have been shown to be a reason of GBM resistance to surgical, pharmacological, and radiation intervention. For instance, pulse-chase experiments demonstrated that GSCs sustain long-term GBM tumor growth through repopulating

highly proliferative cells after chemotherapy (Chen et al., 2012a). Molecular studies revealed that GSCs confer tumors resistance to therapies via diverse mechanisms, including overcoming stringent cell survival and proliferation checkpoints (Bao et al., 2006; Kim et al., 2015b; Venere et al., 2014; Wang et al., 2010) and promoting a more resistant subtype - mesenchymal differentiation by NF κ B activation (Bhat et al., 2013). Moreover, GSCs are constantly changing and actively evolving in response to the hostile GBM microenvironment to survive, to preserve stemness, and to fuel progressive tumor growth (Gimple et al., 2019). For example, in the poorly vascularized necrotic tumor regions, GSCs utilize hypoxia-inducible factors, HIF-2 α , to equip themselves survival advantages and promote self-renewal (Li et al., 2009). It has also been reported that under the ischemic tumor environment, GSCs quickly adapt to a reduced oxygen consumption state by elevating SHMT2 expression that rewires the metabolic state through suppressing pyruvate kinase activity, thereby shifting metabolism away from the TCA cycle, and thus supporting survival (Kim et al., 2015a). Upon glucose restriction, GSCs preferentially upregulate high affinity neuronal glucose transporter type 3 (GLUT3) that allows GSCs to outcompete neighbor cells for glucose uptake to thrive and even enrich despite of limited nutrient availability (Cosset et al., 2017; Flavahan et al., 2013). Upon glutamine deprivation, GSCs utilize glutamine synthetase to autonomously replenish glutamine, support *de novo* purine biosynthesis, and thereby sustain biomass production (Tardito et al., 2015).

1.2.2. Cellular origin, methods of identification, and functional validation of GSCs

While successful isolation of GSCs has been achieved two decades ago, controversy remains regarding the origin of the cells. In early 2000s, several teams reported that genetic alterations make neural stem cells (NSCs) (Alcantara Llaguno et

al., 2009; Holland et al., 2000; Zhu et al., 2005) and differentiated astrocytes (Bachoo et al., 2002) permissive for gliomagenesis. Recent years, accumulated evidence from transcriptome and high-throughput sequencing analyses favors the notion that GBM originates from subventricular zone (SVZ)-derived NSCs. Pollen et al. reported that transcriptional profiles of primary human GBM cells have profound similarity to those of immature SVZ glial cells (Pollen et al., 2015). Deep sequencing studies revealed that 42.3% of the patients bear shared *TERT* mutation between GBM lesions and the matched normal SVZ tissues (Lee et al., 2018b). More than 80% of the IDH-wild-type patients share somatic mutations between tumors and matched disease-free SVZ tissues. Most importantly, *in vivo* experiments that utilized genome-edited mice further pointed to the role of transformed NSCs in gliomagenesis that induction of driver mutation in the SVZ-NSCs give rise to murine GBMs (Lee et al., 2018b). Though applicability of murine etiology in modeling human disease needs to be further determined, findings to-date support the notion that transformed SVZ-NSCs that retain multipotency and fuel tumor heterogeneity are the likely cellular origin of GSCs. Yet, we cannot exclude the possibility that genetic perturbed differentiated GBM cells can undergo developmental reprogram and re-gain stem cell capacities (Gimple et al., 2019).

GSCs underlie glioma initiation, progression, and therapy evasion. Therefore, identification and enrichment of GSCs become essential in order to study their functional characteristics and target them for therapeutic development. CD133, a five-transmembrane cell surface glycoprotein encoded by the *PROM1* gene, is a marker for human normal NSCs (Uchida et al., 2000) and is enriched in brain tumor cells that have increased ability of self-renewal, proliferation, and multilineage differentiation (Singh et al., 2003). A hundred CD133+ cells were capable of re-propagating tumors with characteristics similar to the original tumors of the patient in NOS-SCID (Nonobese diabetic-severe combined immunodeficiency) mouse brains, whereas 10,000 CD133-

cells failed to do so (Singh et al., 2004). While CD133 protein expression (not *PROM1* mRNA) remains the most widely used GSC markers to date, it should be noted that some CD133- cells that represent different growth pattern and subtype profiles also fulfill stem cell criteria (Beier et al., 2007). Meanwhile, more cell surface antigens have been proposed as putative GSC markers, such as CD44 (Anido et al., 2010), L1CAM, and CD15 (Gimple et al., 2019). CD44 is enriched in GSCs that undergo mesenchymal transitions (Bhat et al., 2013; Brown et al., 2017). CD44+ mesenchymal GSCs show enhanced invasiveness and radio-resistance than CD133+ GSCs. CD133+ GSCs have been shown to co-segregate with L1CAM+ cells (Bao et al., 2008). L1CAM expression is essential to retain the survival and proliferation ability of CD133+ cells. Along *in vitro* cell passaging, CD133 expression has been found to decrease in CD15+ cells (Mao et al., 2009). CD133- CD15+ cells retain GSC characteristics and thus CD15 is often employed together with CD133 to avoid isolation omission (Son et al., 2009). Yet, utility of CD15 as a GSC marker still faces challenges in that some cells show abilities to interconvert between a CD15+ and a CD15- state. These CD15- cells give rise to heterogeneous tumors *in vivo* that contain CD15+ cells (Kenney-Herbert et al., 2015). Besides cell surface structural proteins, molecular regulators that maintain self-renewal of normal stem cells may also serve as potential GSC markers, such as SOX2 (Hemmati et al., 2003), NANOG (Ben-Porath et al., 2008), OCT4 (Schoenhals et al., 2009), MUSASHI-1, and ID1 (Anido et al., 2010). Despite great effort devoted, no marker or signature set can definitively or comprehensively mark GSCs. This observation may echo the nature of GSCs that they constantly change to create adaptable molecular profiles in response to temporal and environmental cues.

Functional validation of GSCs employs the defining criteria for CSCs: sustained self-renewal, differentiation potentials, and tumor-initiating capacity (Lathia et al., 2015). Among them, self-renewal ability can be evaluated by an *in vitro* sphere formation assay,

and differentiation potentials can be examined in another *in vitro* assay through growth factor withdrawal or serum induction (Guichet et al., 2013; Park et al., 2017; Pollard et al., 2009). The tumor-initiating capacity needs to be assessed *in vivo*, most often in an orthotopic xenograft model in immunodeficient mice (Lathia et al., 2015).

Sphere formation assay evaluates if a cell (at low seeding density) can re-propagate as the compact spheroid in an undifferentiated format. While this method is widely used to examine the self-renewing of potential CSCs, caveats with this approach are inevitable, which include 1) some GSCs cannot propagate as organized tumorspheres, such as TMZ-resistant giant cell GBM GSCs, MGG18 (**Figure 1.6.**), 2) quiescent GSC populations are left out, which by no means possess less tumor-initiating capacity. Therefore, the gold standard of GSCs would be the ability to generate heterogeneous GBM recapitulating the pathological characteristics of the primary GBM in the orthotopic region of the brain, the SVZ, of immunodeficient mice. Sphere formation, *in vitro* differentiation, and orthotopic tumor initiation assays were all employed in this work.

1.2.3. GSCs: mechanisms of resilience

GSCs are a major cause of GBM intratumoral heterogeneity, profound adaptive capacity, and devastating therapy resistance.

1.2.3.1. GSC intrinsic regulatory factors

GSCs are maintained and regulated by intrinsic and extrinsic factors. Intrinsically, replication checkpoint alterations, including *TP53*, *PTEN*, *CDKN2A*, *NF1*, *ATRX*, *RB1*, and *TERT* (Brennan et al., 2013; Negrini et al., 2010), are most founded mutations in GBM. GSCs that bear these mutations take advantages of damage recognition and DNA repair machineries, such as Chk1, Chk2, ATR, ATM, and PARP1 (Bao et al., 2006; Peng

et al., 2016), to enhance genetic instability tolerance, promote cell survival, and foster GBM plasticity. Pharmacological inhibition of DNA repair pathways has been pursued vigorously in cancer therapy under the rationale that cytotoxic agents and DNA repair inhibitors can work together to promote cell killing (Helleday et al., 2008). In the context of GSCs, inhibition of the critical DNA repair enzyme, poly (ADP-ribose) polymerase-1 (PARP-1) potentiates GSC sensitivity to TMZ (up to 3-fold reduction of TMZ IC₅₀) (Tentori et al., 2014). Another *in vitro* work also revealed that the maximum radiosensitization can be achieved if both ATR and PARP pathways are simultaneously targeted (Ahmed et al., 2015). Of note, there is a significantly greater magnitude of chemosensitization effect of ATR and PARP inhibitors on GSCs than on bulk GBM population. Ever since PARP inhibitors (PARPi) were approved for treating patients with advanced breast, ovarian, pancreatic, and prostate cancers, a keen interest arose in investigating their efficacies in treating brain cancers (Gupta et al., 2018; Rouleau et al., 2010). However, gliomas displayed more varied responses to PARPi treatment in preclinical studies and thus the answers remain elusive. More trials in combination with radiation or cytotoxic agents are continuously being tested (Gupta et al., 2018).

Epigenetic regulation plays critical roles in maintaining GSC stemness. For instance, EZH2, a catalytic component of the PRC2 complex, has been shown to retain GSC self-renewal potentials by directly targeting the promoter of *MYC*, a vital regulator of self-renewal (Suvà et al., 2009). Chromatin remodeling protein HELLS also maintains GSC stemness through co-binding with MYC/E2F3 to the promoter regions of MYC/E2F3 target genes (Zhang et al., 2019a). In GSCs, loss of polycomb-mediated repression results in widespread activation of non-physiologic transcription regulators that aberrantly activate WNT signaling and maintains GSC stemness (Rheinbay et al., 2013). Further, in the presence of differentiation cues, GSCs manifest a significantly higher non-CpG methylation at certain promoters as compared with their normal

counterparts, NSCs. Epigenetic modulation renders transcriptionally repressive states and thereby retains GSC primitiveness (Zhou et al., 2018). In response to sustained receptor tyrosine kinase (RTK) inhibitor treatment, H3K27 demethylase KDM6A/B mediates chromatin remodeling to reprogram GSCs back to a Notch-high primitive cell state for adaptation and drug tolerance (Liau et al., 2017). In short, GSCs are characterized by permissive epigenetic landscapes that promote signaling promiscuity, cell stemness, and resistance acquisition. Epigenetic inhibitors that seek to remodel GSC epigenetic profiles hold promises for better treatment. Application of epi-drugs on GBM are currently under clinical trials (Ghasemi, 2020; Safa et al., 2015; Valor and Hervás-Corpión, 2020).

As aforementioned, GSCs excel at metabolic flexibility (Harland et al., 2021; Prager et al., 2020). Different from the differentiated GBM cells that predominantly rely on pyruvate kinase isozyme (PKM) 2 for aerobic glycolysis, GSCs increase their mitochondrial utilization by expressing the PKM isozyme 1 that facilitates oxidative phosphorylation (Vlashi et al., 2011). Duraj et al. profiled bioenergetic regulation of different GSCs using different metabolic inhibitors (metformin, dichloroacetate, sodium oxamate, and diazo-5-oxo-L-norleucine) and found that GSCs are metabolically heterogeneous and highly reprogrammable: GSCs can exhibit highly glycolytic, oxidative, or co-existing metabolic phenotypes under basal or treated conditions (Duraj et al., 2021). Another work that analyzed bioenergetic behaviors of isogenic GSCs further revealed that both aerobic glycolysis and oxidative phosphorylation serve to provide energy in clonally derived GSCs (Shibao et al., 2018). The metabolic choices are highly influenced by GBM microenvironment, such as hypoxia, and are flexible to switch in response to metabolic stresses or energy pathway therapies. Metabolic heterogeneity of GSCs stresses the changelings in developing effective metabolic

treatment regimens in GBM and underscores the importance of targeting GSC stemness.

1.2.3.2. GSC extrinsic regulatory factors and aberrant signaling pathways

The extrinsic regulatory factors include but are not limited to niche factors, microenvironment, immune system, and aberrant signaling pathways. These extrinsic factors play important roles in promoting stemness and adaptive resistance of GSCs.

In response to hypoxic environments (Man et al., 2018; Qiang et al., 2012), aberrant perivascular nitric oxide signaling (Charles et al., 2010), and radiation insults (Wang et al., 2010), GSCs activate and/or amplify Notch signaling to reinforce GSC stemness. GSCs also activate Notch pathway through a close engagement of tumor microvasculature (Calabrese et al., 2007). Endothelial cells supply GSCs ligands for Notch activation and thereby retain the self-renewing state of GSCs.

Tumor necrosis factor α (TNF- α) signals through NF- κ B to promote mesenchymal differentiation in GSCs resulting in enrichment of CD44^{high} subpopulation that have increased radio-resistance (Bhat et al., 2013). Similarly, perivascular niche factor osteopontin signals through CD44-HIF-2 α axis to enhance GSC stem cell properties and radiation resistance (Pietras et al., 2014).

Moreover, GSCs cooperate with differentiated counterparts and immune cells to protect themselves from differentiation. GBM cell-secreted brain-derived neurotrophic factors (BDNF) activate receptor NTRK2-mediated signaling in GSCs to promote self-renew (Wang et al., 2018b). Tumor-associated macrophages (TAMs), M2, employ secretory pleiotrophin to turn on receptor PTPRZ1-mediated paracrine signaling to maintain GSC stemness (Shi et al., 2017).

WNT signaling has crucial roles in stem cell regulation and is often aberrantly activated in GSCs (Lee et al., 2016a). It has been reported that WNT activators, such as

PLAGL2 (Zheng et al., 2010), FOXM1 (Zhang et al., 2011), Frizzled4 (Jin et al., 2011), and permissive epigenetic landscapes (Rheinbay et al., 2013) trigger WNT pathway activation in GSCs to preserve stemness.

GSCs also frequently hijack a number of growth factor signaling pathways to promote self-renewal and survival. For examples, PDGFR β and IL-6 receptor mediated signaling pathways maintain GSCs through the activation of STAT3 (Kim et al., 2012; Wang et al., 2009), autocrine TGF- β signaling preserves the stemness of GSCs via upregulation of self-renewal genes including SOX2 and SOX4 (Ikushima et al., 2009).

1.2.4. Unmet medical needs in current GBM therapies

Over the past decade vigorous efforts have been made to understand genetic, epigenetic, metabolic, and microenvironmental underpinnings of GSCs under the rationale that GSCs dynamically reconstitute tumors for heterogeneity and adaptation and are the main cause of therapeutic failure. For this reason, a large number of preclinical studies have been conducted in attempt to identify novel stemness regulators and related blockage therapies.

As described above, stemness of GSCs can be maintained by Notch pathway and thus Notch blockage has been explored as a strategy to decrease GSC stemness. As Notch activation requires γ -secretase-mediated release of the Notch intracellular domains (NICDs) that turns on Notch-dependent transcription (Bray, 2006), γ -secretase inhibitors (GSIs) have been examined for their effects on GSC stemness and the results showed that Notch inhibition pronouncedly suppressed GSC survival, impaired tumor engraftment, and enhanced GSC radio-sensitization (Fan et al., 2006; Wang et al., 2010). In clinical trials (phase 0/I and II), RO4929097 (GSI) was found to achieve a sufficient tumor penetration in the brain, significantly decreased NICD expression in

tumor cells, and most importantly specifically reduced CD133+ cell population. However, it failed trials treating 1) newly diagnosed GBM in combination with TMZ and radiotherapy (Xu et al., 2016) and 2) recurrent GBM as a single treatment agent (Peereboom et al., 2021). One of the reasons for the failure could be that recurrent tumors arose with a Notch-independent profile, highlighting the complexity of therapeutic evasion primed by tumor adaptability. Concomitant targeting of multiple pathways, such as GSIs plus angiogenesis inhibitors, might be a necessity for a better GBM treatment regime.

Aberrant activation of RTK pathways also promotes GSC stemness including PDGFR signaling. Preclinical analyses reveal that genetic or pharmacological inhibition of PDGFR strongly suppressed STAT3 activation in GSCs and attenuated cell survival, self-renewal, and tumorigenicity (Cenciarelli et al., 2016; Kim et al., 2012). Imatinib mesylate (Gleevec), a small molecule inhibitor of PDGFR and other tyrosine kinases approved for treating multiple types of leukemia (Heldin, 2013), has been shown to enhance the efficacy of radiotherapy for xenografts in murine models (Oertel et al., 2006). Phase II evaluation of imatinib, however, gave disappointing results. While imatinib was successfully delivered to GBM lesions, it showed no measurable efficacy against either newly diagnosed or recurrent GBM in combination with radiotherapy or hydroxyurea (Raymond et al., 2008; Razis et al., 2009; Reardon et al., 2009; Sautter et al., 2020). Imatinib, unexpectedly, has been found to promote GSC motility via augmentation of p130Cas and FAK signaling (Frolov et al., 2016) and elicit sustained ERK activations in GBM cells (Dong et al., 2011). These unforeseen adverse effects might account for trial failure and tyrosine kinase inhibitors (TKIs) application should be assessed with great caution. Moreover, a number of small oral TKIs that target PDGFR together with VEGFR, such as sorafenib, nintedanib and crenolanib were demonstrated to show no clinical impacts on GBM treatment (Di Nunno et al., 2020). Selective

inhibitors targeting EGFR (e.g., dacomitinib and erlotinib), neurotrophin receptor kinase (e.g., larotrectinib), and ERBB-2 (e.g., lapatinib) also failed to bring survival benefits on adult GBM patients (Di Nunno et al., 2021). Effective therapeutics of GSCs using TKIs remain to be a clinical challenge and await further investigation.

GBM microenvironment is characterized by a chaotic mass of poorly functioning blood vessels that impedes oxygen delivery for vigorous tumor metabolism and gives rise to perivascular, necrotic, and hypoxic niches (Gimple et al., 2019; Tang et al., 2021; Wirsching et al., 2021). These niches maintain GSC stemness, facilitate their adaptation to harsh tumor microenvironment, and promote more resilient phenotypes of GSCs. Intensive immune cell infiltration is another feature of GBM tumors that actively drives GSC evolution to evade immunosurveillance and to acquire a more resistance phenotype. Therefore, GSC niches have emerged as novel targets for GSC elimination. Anti-angiogenic therapy has been investigated since early 2000s, with bevacizumab being the only tumor vasculature modulating agent authorized for GBM treatment to date (Wirsching et al., 2021). While administration of bevacizumab renders transitory benefits for tumor-vascular “normalization”, the use of bevacizumab often causes severe side effects and leads to "rebound" progression. Bevacizumab did not prolong the overall survival of patients with newly diagnosed or recurrent GBM under either TMZ or lomustine treatment regimens. Moreover, anti-angiogenic inhibition has been shown to elevate the expression of CXCR4, a vessel-cooption mediator, in GSCs (Pham et al., 2015). While plerixafor, a CXCR4 inhibitor, has been demonstrated to effectively slow down post-bevacizumab tumor progression on murine models, bevacizumab plus plerixafor treatment showed limited efficacy in clinical trials with high grade gliomas (Lee et al., 2018a). Moreover, dysfunctional GBM vasculature fosters the formation of necrotic-hypoxic niches in the perivascular region that may further promote GSC stemness and GBM progression and recurrence. Hypoxic stress activates HIF-2 α

signaling that has been shown to promote mesenchymal transition of GSCs that are often associated with poor survival and radio-resistance (Seidel et al., 2010). HIF-2 α inhibitor (PT-2358) and drugs that target epigenetic regulators (EZH2 inhibitor, tazemetostat; BMI-1 inhibitor, PTC596) to prevent hypoxia-induced mesenchymal transitions have been demonstrated to have anti-tumor benefits in pre-clinical settings (Wirsching et al., 2021). Nevertheless, none of them have reached to clinic yet due to limited blood-brain-barrier penetration or low therapeutic efficacy. Immune cell infiltration has been recurrently documented for patients undergone anti-angiogenic therapies (Tang et al., 2021). Tumor-associated macrophages (TAM) are the most abundant inflammatory cells infiltrating GBM lesions and have been demonstrated to engage in close interaction with GSCs. TAM-GSC crosstalk maintains GSC stemness (pleiotrophin-PTPRZ1 paracrine signaling) (Shi et al., 2017), suppresses immune surveillance (polarization of TAMs into immunosuppressive M2) (Prosniak et al., 2013), and promotes disease progression (production of proinflammatory cytokines) (Tang et al., 2021). Colony stimulating factor-1 (CSF-1) has an essential role in regulating TAM survival and CSF-1 receptor blockade has been shown to effectively deplete TAMs in tumors. Several animal studies have revealed that long-term suppression of TAM-GSC crosstalk by treating with CSF-1 receptor inhibitors significantly regressed tumors, slowed down progression (Pyonteck et al., 2013), and delayed tumor recurrence after radiotherapy (Stafford et al., 2016). Yet, a clinical trial that assessed the efficacy of pexidartinib (a CSF-1 receptor inhibitor) in combination with the standard of care of GBM reported minimal clinical benefits for newly diagnosed GBM (Colman et al., 2018).

Rather than a static population, GSCs exist as a spectrum of multipotent states and are constantly changing in phenotypes and genotypes as the consequence of evolution adaption. Differentiation therapy, an attempt to constrain tumors with restricted transcriptional and metabolic options, emerged as a promising strategy to combat GSC-

mediated adaptive resistance. In addition to aforementioned blockade of stemness maintenance, differentiation therapy aims to direct GSCs into more confined energy valleys, ideally targetable states by existing treatment regimes. Bone morphogenic proteins (BMPs) that instruct astroglial differentiation of neural stem and progenitor cells have been demonstrated to have pro-differentiating capacities for GSCs. BMP4-induced differentiation dramatically impaired the ability of engrafted GSCs to establish intracerebral tumors (Piccirillo et al., 2006). BMP7-mediated GSC differentiation negatively modulated the expression of O⁶-methylguanine-DNA methyltransferase (MGMT) and drug efflux transporters and synergized with TMZ to attenuate tumorigenicity (Tso et al., 2015). BMP2, similarly, elicited a strong differentiation and subsequent combination treatment with TMZ down-modulated the HIF-1 α -dependent MGMT expression and significantly sensitized GSCs to chemotherapy (Persano et al., 2012). Nevertheless, some major concerns of BMP differentiation therapy have been noticed. BMP-differentiated cells were found highly susceptible to cell-cycle re-entry and de-differentiation. Chromatin accessibility studies further revealed that BMP signaling failed to epigenetically silence SOX genes that largely undermine GSC differentiation commitment (Carén et al., 2015). Moreover, BMP activation has been shown to direct a subset of GSCs toward a more quiescent cell state that is intrinsically resistant to radio- and chemo-therapies (Sachdeva et al., 2019). For instance, BMP4 triggered G1 cell cycle arrest in EGFR^{low} GSCs that protected them from TMZ-mediated cell death (Ciechomska et al., 2020). All these findings pinpoint the genetic, epigenetic, and transcriptional complexity underlying BMP-mediated differentiation and related treatments must be evaluated with great caution. An accumulating number of studies suggest that steering differentiation direction into neurogenic programs renders GSCs a more stable state of differentiation: a neuronal fate that resists cell cycle re-entering. Neuronal differentiation of GSCs has been proved to elicit massive cell death, abrogate

tumorigenicity, and significantly prolong the survival of tumor-bearing mice (Guichet et al., 2013; Park et al., 2017; Su et al., 2014). It has been reported that neuronal reprogramming of GSCs could be achieved by simply combining three approved drugs, fasudil, tranilast, and TMZ, termed as FTT cocktail (Gao et al., 2019). FTT cocktail-mediated differentiation therapy potentially attenuated GBM cell malignancy and potentiated cytotoxic effects of TMZ in the murine model. Directing GSCs toward a neuronal cell fate may be a new avenue to improve therapeutic efficacy in GBM. The long-term effects of neuronal reprogramming (such as, if induced neuronal cells makes connections with established neural circuits that interferes normal brain functions) need further investigation.

Notwithstanding intense translational research and clinical trials over the past decades, TMZ remains to be the most effective adjuvant chemotherapy that significantly improved patient survival and quality of life when administrated concomitantly with radiotherapy (Stupp et al., 2005). Yet, GBM is notorious for its therapeutic resistance and TMZ resistance is the main reason contributing to treatment failure. TMZ is an oral DNA alkylating agent that elicits mismatch repair, induces cell cycle arrest at G2/M, and eventually triggers cell death. MGMT that directly repairs TMZ-induced cytotoxic O⁶-methylguanine (O⁶-MG) lesions is the primary contributor to GBM chemoresistance and is highly expressed in many types of GSCs (Mrugala and Chamberlain, 2008; Pistollato et al., 2010). Elevated MGMT levels in GSCs were resulted from the hypomethylation of *MGMT* gene promoter, aberrant pathway activation (JNK, MEK/ERK, and WNT/ β -catenin), and increased HIF-1 α signaling within a hypoxic tumor environment (Jiapaer et al., 2018; Persano et al., 2012). Thus, compounds that down-modulates MGMT expression or activity via employment of irreversible pseudosubstrates for MGMT (O⁶-benzylguanine), methylation of MGMT promoter (through GSK3 β inhibitors), and

suppression of MGMT transcription (through interferon- β) have been intensively assessed in clinical studies to overcome or to reduce resistance (Jiapaer et al., 2018; Nakada et al., 2012). However, no significant survival benefit has been observed in phase II clinical trials for O⁶-benzylguanine (Quinn et al., 2009) and interferon- β (Wakabayashi et al., 2018), and the efficacy of GSK3 β inhibitors in TMZ sensitization remains to be investigated.

TMZ resistance can also result from mechanisms independent of MGMT. Inactivation of mismatch repair (MMR) leads to resistance because TMZ relies on an intact MMR system to induce cytotoxic effects (Atkins et al., 2015). MMR genes are highly susceptible to TMZ-induced mutations and their inactivation mutations were predominantly found in recurrent GBMs. Base excision repair (BER) activity also modulates TMZ-induced cytotoxicity because BER functions by rapidly fixing alkylating agent-induced N3 and N7 methylation damages (Erasmus et al., 2016). GSCs are intrinsically chemo-resistant largely due to the enhanced DNA repair capacity. PARP is currently one of the most investigated BER enzymes and PARP inhibitors were found to strongly sensitize GSCs to TMZ treatment under an *in vitro* setting (Tentori et al., 2014). More clinical trials for PARP inhibitors in combination with radiation or cytotoxic agents are underway (Gupta et al., 2018).

Despite innovations in neurosurgical techniques, advances in radiotherapy, and intense translational research, GBM remains a fatal disease and the standard of care has not changed over the past decades. GBM recurrence is universal and renders a desperate disease state absence of any effective therapeutics that eventually causes patient demise. GSCs impose serious challenges on GBM treatment by promoting tumor heterogeneity, adaptability, and reconstitution after therapeutic interventions (Prager et al., 2020). GSCs, the main culprit behind tumor recurrence and treatment failure, are

sustained and powered by the principal characteristic of CSCs, stemness. Aforementioned preclinical and clinical studies have made fruitful progress in contextualizing GSC stemness regulation, yet the efficacy of related treatments is yet to be established. Collectively, mining GSC stemness/differentiation pathways is an essential step to make inroads into the discovery of next targetable stemness regulators to eliminate GSCs, all with the aim to overcome therapeutic resistance and defeat GBM.

1.3. The biology of ANG

ANG is a 14 kDa vertebrate-specific secreted ribonuclease (RNase) that was originally identified as a tumor angiogenic factor for its potent ability to promote blood vessel formation (Fett et al., 1985; Marx, 1985). ANG, also known as RNASE5, belongs to the pancreatic RNase superfamily and has 35% amino acid identity with RNASE A, the prototypic member of this family. ANG shares the same promoter and 5'-untranslated region (5'-UTR) sequence with RNASE4 that also has roles in angiogenesis, neuroprotection, and tumorigenesis; however, functional associations of the two have not been fully determined (Hofsteenge et al., 1998; Li et al., 2013; Sheng and Xu, 2016). Crystallization analyses revealed that the pyrimidine base-binding center in ANG is occluded by the side chain of Gln-117, and the resultant structure makes ANG retain merely 10^{-5} to 10^{-6} -fold of catalytic activity of RNASE A (Russo et al., 1996). Nevertheless, the seemingly minuscule ribonucleolytic activity is essentially critical for ANG to enable diverse physiologic and pathologic functions, including angiogenesis (Shapiro et al., 1986), neurogenesis and neuroprotection (Subramanian and Feng, 2007), hematopoietic regeneration (Goncalves et al., 2016; Silberstein et al., 2016), innate immune reactions (Hooper et al., 2003), and nearly all steps along tumorigenesis (Li et al., 2020; Li and Hu, 2010; Sheng and Xu, 2016; Tsuji et al., 2005). In addition to the catalytic center (Shapiro et al., 1986), ANG contains two other functional domains: a

typical receptor binding region (Hallahan et al., 1991; Hu et al., 1991) and a classic nuclear localization sequence (NLS) (Moroianu and Riordan, 1994). Via a receptor-interacting loop region (from Lys-60 to Asn-68), ANG binds to the cell surface receptor, now identified as PLXNB2, and undergoes receptor-mediated endocytosis (Yu et al., 2017).

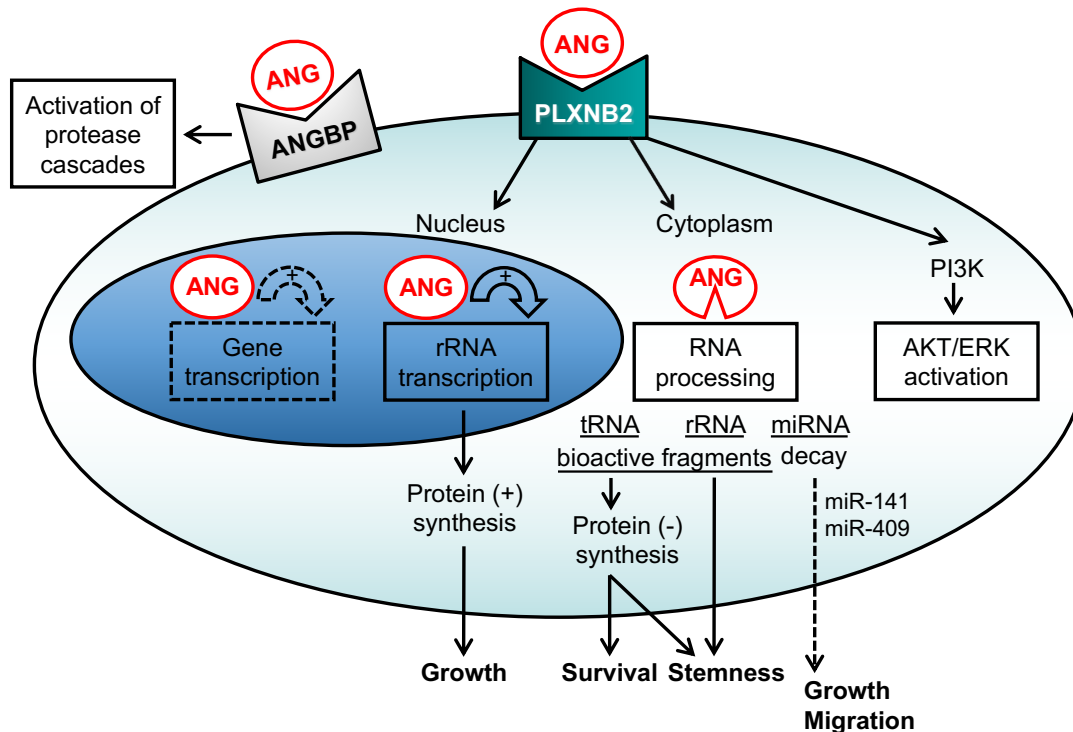


Figure 1.1. Schematic representation of the mechanism of action of ANG.

ANG is a secreted RNase with cell-type- and context-specific roles in growth, survival, stemness, migration, and beyond. (a) ANG undergoes receptor PLXNB2-mediated endocytosis. Under growth conditions or in more differentiated cells, ANG accumulates in the nucleoli where it regulates rRNA transcription, processing, and maturation, thereby promoting growth. Nuclear ANG may act as a transcriptional factor or chromatin remodeling activator to modulate gene transcription. Under stress or in primitive cell types, cytoplasmic ANG cleaves tRNA to produce tiRNAs or 5S rRNA to produce 3'-end fragments of 5S rRNA. These bioactive small RNAs function in promoting survival or stemness. Cytoplasmic ANG may also regulate gene expression via the endonucleolytic decay of the specific microRNAs. (b) ANG can activate signal transduction pathways, including PI3K/AKT and ERK1/2 pathways, in certain cell types. (c) Extracellular ANG interacts with cell-surface actin on endothelial cells, by which they dissociate as a complex called ANG binding protein (ANGBP) that activates protease cascades, such as plasminogen activator/plasmin serine protease system and the matrix metalloproteinase system. By this, ANG stimulates ECM degradation and thereby promotes migration and invasion.

The NLS domain mediates ANG nuclear import. Nuclear ANG accumulates in the nucleoli where ANG regulates ribosomal RNA (rRNA) biogenesis and thus promotes growth (Moroianu and Riordan, 1994; Tsuji et al., 2005; Xu et al., 2003). Another major RNA processing event is related to transfer RNA (tRNA) metabolism, presumably involves cytoplasmic ANG. In response to stress and or in primitive circumstances, cytoplasmic ANG is proposed to cleave a subset of tRNA to produce tiRNA (Fu et al., 2009) that halts ribosome assembly and suppresses cap-dependent protein translation (Ivanov et al., 2011; Yamasaki et al., 2009) so as to conserve energy for cell survival and for preservation of stemness (Goncalves et al., 2016). In addition to the two major RNA processing events (**Figure 1.1.**), ANG also mediates the metabolism of 5S ribosomal RNA (Li et al., 2020) and microRNA (Thomas et al., 2018; Weng et al., 2022; Weng et al., 2012), and is involved in gene transcriptional regulation (Ang et al., 2013), signaling pathways activation, and extracellular matrix degradation (Sheng and Xu, 2016).

1.3.1. Nuclear ANG regulates rRNA biogenesis

Ribosome biogenesis lies at the core of cell growth with a rate-limiting step, rRNA transcription (Li and Hu, 2010; Mélése and Xue, 1995). It is now clear that, under growth conditions, ANG accumulates in the nucleolus where it 1) remodels chromatin to activate the rDNA loci, 2) binds at upstream core element (UCE) region to enhance the promoter occupancy of RNA polymerase I pre-initiation complex (Sheng et al., 2014b), and most importantly, 3) directly binds at angiogenin binding element (ABE) on the promoter of ribosomal DNA to stimulate 47S pre-rRNA transcription (Xu et al., 2003). It has been proposed that the complex 47S pre-rRNA processing and maturation events contains an initial cleavage reaction at the A₀ site resulting in removal of 5'-external transcription spacer of 416 nucleotides, which greatly facilitates the overall processing

speed (Maden, 1990). However, the endoribonuclease(s) responsible for the A₀ cleavage has not been identified after many decades of research. The work of Monti et al. suggested that ANG, very likely, served as an endonuclease that made the cleavage and enhanced subsequent processing events (Monti et al., 2009).

ANG nuclear accumulation and ANG-stimulated rRNA biogenesis have been demonstrated in proliferating endothelial cells (Hu et al., 2000; Xu et al., 2002), cancer cells (Tsuji et al., 2005), and motor neurons (Wu et al., 2007), and are of pivotal importance for the process of angiogenesis, tumorigenesis, and motor neuron physiology (Li and Hu, 2010; Sheng and Xu, 2016). While other potent angiogenic factors, such as VEGF and FGF, signal through tyrosine kinase receptors to activate protein synthesis through the mTOR-S6K-S6P pathway, ANG controls rRNA biogenesis tempo in the nucleus and is a permissive factor for other angiogenetic actions to induce protein synthesis. It has been experimentally demonstrated that inhibition of ANG nuclear import strongly suppressed proliferation of endothelial cells *in vitro* and angiogenesis *in vivo* despite the presence of other angiogenic molecules (Kishimoto et al., 2005). Besides angiogenic activity, ANG plays an important role in regulating cancer cell proliferation. Cancer cells relies on continuous protein synthesis to satisfy their elevated metabolic demands (Hanahan and Weinberg, 2000). For this purpose, cancer cells hijack normally well-controlled ribosome biogenesis machinery to constitutively import ANG into the nucleus where rRNA is constantly transcribed, and thereby ANG sustains the unrestricted growth of cancer cells (Tsuji et al., 2005). In essence, nuclear ANG drives tumor malignancy via dual avenues: sustaining unlimited cell proliferation and promoting tumor angiogenesis. Neomycin and its derivative neamine were found to effectively halt ANG nuclear import. It has been demonstrated that ANG nuclear translocation blockade by either neomycin or neamine potently inhibited cancer cell proliferation and tumor angiogenesis (Hirukawa et al., 2005; Hu, 1998; Ibaragi et al.,

2009a; Ibaragi et al., 2009b; Yoshioka et al., 2006). Undoubtedly, neamine was shown to inhibit the angiogenetic activities induced by other angiogenic factors (Kishimoto et al., 2005). Moreover, strong ANG nuclear accumulation has been documented for motor neurons of both prenatal and adult spinal cords (Wu et al., 2007). In the process of neurite outgrowth and pathfinding, motor neurons show robust demands for ribosomal biogenesis that relies on an intact ribosomal and translational machinery (Kishikawa et al., 2008). The ANG mutations S28N and P112L identified in ALS patients were found to be "loss of function" mutations owing to, at least partially, their deficiencies in ANG nuclear translocation (Wu et al., 2007).

Collectively, rRNA transcription is an essential step for ribosome biogenesis and a major determinant for growth control. Under growth conditions or in differentiated cancer cells, ANG undergoes nuclear translocation to promote rRNA biogenesis and thereby enables physiological and pathological functions of ANG.

1.3.2. Cytoplasmic ANG regulates tRNA metabolism

In addition to nuclear accumulation, ANG is also found in cytoplasmic compartments where it exerts distinct biological effects. In response to stress, ANG is accumulated in the cytoplasm where it cleaves tRNA at the anticodon loop to produce a specific type of tRNA-derived fragments (tRFs), tiRNAs (Fu et al., 2009; Yamasaki et al., 2009). ANG-mediated tiRNA biogenesis is a precisely regulated process. The extent of cleavage is determined by the abundance of cytoplasmic ANG and tRNA, the level of the ANG inhibitor, ribonuclease/angiogenin inhibitor 1 (RNH1), the rate of global protein synthesis, and the type and the intensity of the stress (Saikia et al., 2012). In addition, the specificity of cleavage for tRNA species is largely affected by tRNA modifications (Blanco et al., 2014; Chen et al., 2019b; Schaefer et al., 2010; Wang et al., 2018a; Zhang et al., 2018). For instance, NSUN2, an RNA methyltransferase, mediates

sequence- and structure-specific m⁵C modifications on the tRNA. NSUN2-mediated methylation protects selected tRNA species from ANG-mediated cleavage, whereas *NSUN2* loss induces aberrant tRNA hypo-methylation that over-augments cellular stress response and leads to cell death (Blanco et al., 2014).

In response to environmental insults, mammalian cells undergo stress-induced translational reprogramming that downregulates global protein synthesis while permits damage repair events for adaptation and survival. This process is often driven by the phosphorylation of the translation initiation factor, eukaryotic initiation factor 2 α (eIF2 α) (Holcik and Sonenberg, 2005), and is accompanied by the sequestration of untranslated mRNAs into special cytoplasmic foci, stress granules (SGs) (Emara et al., 2010). SGs were found to direct various incoming mRNA transcripts to sites of translation, storage, re-initiation or decay - a process of translational reprogramming (Yamasaki and Anderson, 2008). SG formation is a major component of cellular stress response, and its assembly can be triggered by phospho-eIF2 α . The work of Emara et al. reported that ANG-induced 5' tiRNAs, but not 3'-tiRNAs, promoted SG assembly and translational repression. This process was demonstrated to be independent of the classic eIF2 α phosphorylation stress response program. Of note, the 5'-monophosphate modifications on 5' tiRNAs were proved to be essentially important for SG assembly. This may partially explain the distinct functional effects of the two tRNA fragments produced by ANG (Emara et al., 2010). Ivanov et al., further revealed that the presence of a 5'-oligo-G motif (at least four guanines) was an indispensable prerequisite for tiRNAs to induce translational repression (Ivanov et al., 2011). This terminal oligo-guanine conferred 5'-tiRNAs capacities to 1) displace eIF4G/eIF4A from uncapped mRNAs and partially from capped mRNAs and 2) displace eIF4F from m⁷G capped mRNAs. Thus, 5'-tiRNA suppresses cap-dependent protein translation but has no effect on internal ribosomal entry sequence (IRES)-mediated protein translation. By this way, IRES-mediated protein

translation is preferentially enriched by ANG through the 5'-oligo-G motif containing 5' tiRNA under stress conditions (Ivanov et al., 2011). Among all human tRNAs, this motif was only found in tRNA^{Ala} and tRNA^{Cys} (H. sapiens tRNA alignment: <http://lowelab.ucsc.edu/GtRNAdb/Hsapi19/Hsapi19-align.html>). It has been long observed that IRES-mediated translation is activated or augmented for survival, adaptation, or cell-fate decision purpose under physiological, stress, or pathological conditions (Komar and Hatzoglou, 2011). Therefore, ANG-induced 5' tiRNAs activates an alternative stress response pathway that suppresses the "energy-expensive" global protein translation and permits IRES-mediated translation of genes associated with survival and adaptation (Ivanov et al., 2011). It is of great interest to elucidate the precise molecular roles of different tiRNA species as well as the regulatory machineries enabling their circumstance-specificity.

Loss-of-function mutations in *ANG* have been documented in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD) (Prehn and Jirström, 2020; van Es et al., 2011). These disorders are characterized by increased endoplasmic reticulum (ER) stress and cell death due to accumulation of misfolded protein aggregates in the cell (Soto and Pritzkow, 2018). Reduced RNase activity is the foremost functional abnormality of these *ANG* variants found in ALS and PD (Bradshaw et al., 2017; Gagliardi et al., 2019; Wu et al., 2007). In response to hypoxic stress or nutrition starvation, *ANG* was found to exert survival-promoting functions on motor neurons (Kieran et al., 2008; Sebastia et al., 2009). Functional analyses of *ANG* variants of ALS (Kieran et al., 2008) and PD (Steidinger et al., 2011) further revealed that WT *ANG* protects neurons from stress-induced cell death whereas the neuroprotective activity is largely lacking in ALS *ANG* variants (Subramanian et al., 2008). Mechanistically, it has been found that 5'-tiRNA^{Ala} conferred motor neurons cytoprotective and pro-survival functions against stress (Ivanov

et al., 2014). Functional studies of ALS variants further revealed that WT ANG, but not the inactive ANG variants, is capable of inducing tiRNAs that strongly inhibit global protein synthesis (Yamasaki et al., 2009). In addition to activation of the AKT and ERK survival signaling pathways (Kieran et al., 2008), these findings strongly suggest that ANG-mediated neuroprotection also comes from tiRNA-mediated translational repression, and that tiRNA production deficiency of ANG variants is an underlying pathogenesis of neurodegenerative diseases.

tiRNA production has also been described in stemness maintenance of primitive hematopoietic stem and progenitor cells (HSPCs). Goncalves et al. reported that tiRNAs are abundantly expressed in HSPCs and that their production is regulated by ANG (Goncalves et al., 2016; Silberstein et al., 2016). ANG is the only RNase demonstrated to mediate tiRNA production (Yamasaki et al., 2009) and was found to co-localize with SGs in HSPCs. Transfection of bioactive 5' tiRNAs resulted in reduced global protein synthesis, increased expression of pro-survival and pro-self-renewal genes, decreased expression of pro-apoptotic genes, and enhanced regenerative capabilities of the long-term hematopoietic stem cells (LT-HSCs) (Goncalves et al., 2016).

Collectively, ANG-mediated tiRNA biogenesis has been shown to have various biological functions, including translational repression, inhibition of apoptosis, and stemness preservation. In response to environmental stress and self-renewing demands, ANG promotes tiRNA biogenesis and thereby endows cytoprotective functions.

1.3.3. ANG regulates the metabolism of other RNAs

ANG is also involved in biogenesis and metabolism of other RNAs. Li et al. reported that ANG promotes prostate CSC stemness through endonucleolytic processing of 5S rRNAs. ANG mediates the production of bioactive 3'-end 5S rRNA

fragments that function to restrict protein translation and preserve prostate CSC stemness (Li et al., 2020). ANG may also cleave microRNAs. In ANG-treated HeLa cells, microRNAs were found to be differentially regulated (Thomas et al., 2018). ANG-mediated microRNA degradation has also been implicated (Sheng and Xu, 2016). Moreover, Skorupa et al. reported a novel paracrine ANG signaling pathway that motor neuron secreted ANG regulates normal astroglia and astrocytoma cells by targeting an unknown group of RNAs (Skorupa et al., 2012). Further research is needed to identify novel ANG substrates, to determine underlying regulatory mechanisms for the targeting specificity, and to elucidate the functionality of the metabolites.

1.3.4. ANG in normal physiology and diseases

ANG, as a secreted RNase, has been detected in nearly all human cell types, tissues, and organs (Weiner et al., 1987). In normal plasma, circulating ANG has a concentration of ~250 mg/L with the liver to be the predominant source (Bläser et al., 1993). ANG is actively taken up by target cells via autocrine and/or paracrine pathways and plays important roles in various physiological and pathological processes (Lyons et al., 2017; Sheng and Xu, 2016).

ANG is highly upregulated in numerous human cancers (Barton et al., 1997; Eberle et al., 2000; Hartmann et al., 1999; Li et al., 1994; Miyake et al., 1999; Montero et al., 1998; Shimoyama et al., 1996; Verstovsek et al., 2001a; Yoshioka et al., 2006) and is involved in virtually all steps of tumorigenesis ranging from cancer cell proliferation and migration, tumor angiogenesis and invasion, cancer stem cell preservation (Li et al., 2020; Yu et al., 2017), as well as the acquisition of resistance to environmental and therapeutic insults (Li and Hu, 2010; Sheng and Xu, 2016).

More specifically, ANG sustains unrestricted proliferation of cancer cells via constantly translocating into nucleus where it stimulates rRNA transcription (Tsuji et al.,

2005). Because ANG controls rRNA biogenesis tempo, it emerges as a permissive factor for angiogenic actions of other angiogenic factors and thus is required for tumor angiogenesis (Kishimoto et al., 2005). In tumor stroma, ANG promotes invasion by various mechanisms (Sheng and Xu, 2016). ANG has been shown to activate protease cascades to promote basement membrane degradation that facilitates migration (Hu et al., 1994). ANG was also found to support GBM cells perivascular invasion by mediating extracellular matrix (ECM) remodeling and vascular association (Yang et al., 2022). Furthermore, hypoxic stress, a hallmark of solid tumors, remarkably elevates ANG expression in tumors (Hartmann et al., 1999; Pilch et al., 2001). ANG-induced tRNA-mediated adaptation/survival mechanism is proposed as a contributor of cancer progression (Li and Hu, 2010). Moreover, ANG was found to regulate the stemness of leukemic and prostate CSCs. ANG internalization blockade significantly decreased leukemic myeloid cell frequency (Yu et al., 2017) and sensitized prostate CSCs to the chemotherapeutic agent, docetaxel (DCX) (Li et al., 2020). The role of ANG in GSCs has not been previously examined and is a topic of this thesis research, which will be described in Chapter 3. ANG influences nearly every facet of tumorigenesis, yet the high level of ANG in the plasma and tumor microenvironment impede direct ANG targeting for effective cancer therapy. Nuclear import blockade with neamine may partially circumvent the caveat (Kishimoto et al., 2005). Targeting ANG cellular uptake by disrupting the binding of ANG to its receptor, PLXNB2 (Yu et al., 2017), offers a feasible avenue for therapeutic purpose, and is a focus of this study.

ANG is downregulated or loss-of-function mutated in neurodegenerative diseases, including ALS (McLaughlin et al., 2010), AD (Kim and Kim, 2012) and PD (Steidinger et al., 2011). These ANG variants have been shown to affect ANG's angiogenic activity, ribonucleolytic capacity, nuclear-cytoplasmic shuttling, or protein stability (Li and Hu, 2010; Prehn and Jirström, 2020; Sheng and Xu, 2016). In the course

of neurite outgrowth and pathfinding, motor neurons rely on ANG-mediated rRNA biogenesis to meet elevated energy demands (Kishikawa et al., 2008). In response to stress, ANG elicits neuroprotective activity towards neural progenitors and postmitotic neurons through a tiRNA-mediated translational repression mechanism (Kieran et al., 2008; Sebastià et al., 2009; Steidinger et al., 2011; Subramanian et al., 2008). Because VEGF plays an essential role in neuroprotection (Storkebaum et al., 2005), ANG abnormalities may also contribute to CNS pathogenesis through an indirect measure, impairing VEGF-stimulated neurovascular perfusion. Lastly, abnormal serum ANG levels have been described in some other pathological conditions, such as inflammatory bowel disease (Koutroubakis et al., 2004), peripheral vascular disease (Burgmann et al., 1996), endometriosis (Suzumori et al., 2004), rheumatoid arthritis (Hosaka et al., 1995), and diabetes (Neubauer-Geryk et al., 2012; Siebert et al., 2010).

1.3.5. Role of ANG in GBM biology

ANG is upregulated in GBM and its expression is inversely correlated to overall survival of GBM patients (Eberle et al., 2000; Hu et al., 2019). A recent gene expression analysis of TCGA data sets further detailed that GBM lesions have a 4-fold higher ANG expression than that of normal brain tissues (**Figure 1.2.A**). Elevated ANG levels significantly worsened the median survival of patients, with 13.1 and 19.1 months for GBMs of high and low ANG expression (**Figure 1.2.B**). In addition, ANG was found to be the most up-regulated angiogenic factor in GBM cells and GSCs upon VEGF blockade (Lucio-Eterovic et al., 2009). ANG has been demonstrated to be the most enriched angiogenic molecule in GBM microvesicles that promotes tumor growth via stimulating GBM cell proliferation and tubule formation by endothelial cells (Skog et al., 2008). Recently, Yang et al. provided the first experimental demonstration that ANG promotes GBM progression by enhancing cell proliferation, tumor angiogenesis, vascular

association, and invasion in both genetic mouse models and athymic mice bearing human GBM xenografts.

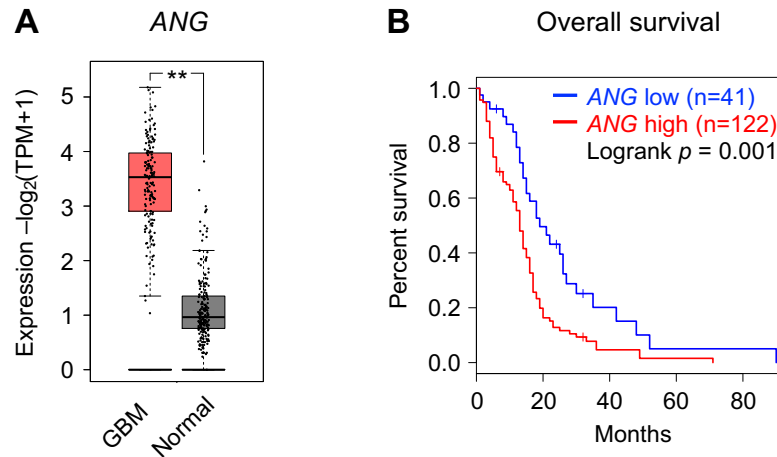


Figure 1.2. ANG is up-regulated in GBM and its levels are inversely correlated to survival of GBM patients.

(A) ANG mRNA in GBM samples and normal brain tissues. Normal (matched TCGA normal brains and GTEx data), n = 207; GBM (TCGA, Cell 2013), n = 163. The Gene Expression Profiling Interactive Analysis (GEPIA2) platform was used for analysis and visualization. Data shown are means $\pm$ SEM. ** $p < 0.01$

(B) The survival plot of GBM patients (TCGA, Cell 2013) with high (red, n = 122) and low (blue, n = 41) expression of ANG. The top 75% was defined as the high, and the bottom 25% was defined as the low in ANG expression among the 163 patients in this cohort. GEPIA2 platform was used for survival analysis and visualization.

Ang1 deficiency significantly prolonged the survival of PDGF-induced pro-neural GBM mice (**Figure 1.3.**). The median survival was 38, 43, and 48 for mice under *Ang1*^{+/+}, *Ang1*^{+/-}, and *Ang1*^{-/-} background, suggesting ANG may influence the survival of these GBM mice in a dose-dependent manner. Mechanistically, ANG was found to mediate ECM remodeling and vascular association of GBM cells by upregulating *MMP9* and *CD24*, respectively (Yang et al., 2022). Both actions are highly linked to perivascular invasion that fuels GBM infiltration (Baker et al., 2014; Cuddapah et al., 2014; Winkler et al., 2009). Moreover, the effect of ANG in GSCs has been described for the first time in this work that ANG promoted GSC tumorsphere formation (Yang et al., 2022).

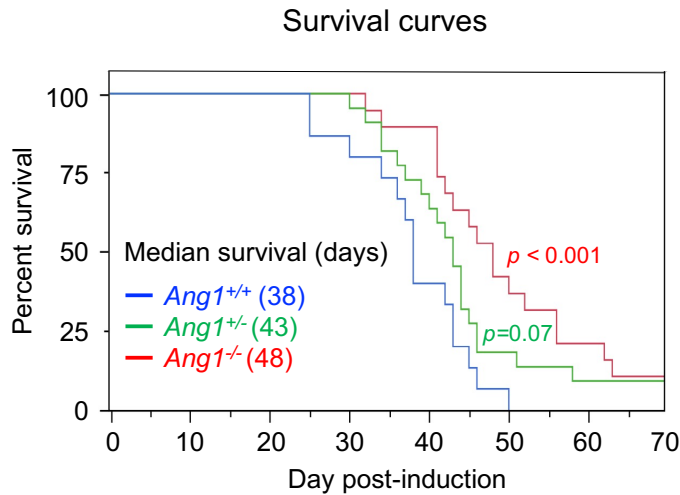


Figure 1.3. *Ang1* deficiency enhances survival of PDGF-induced GBM mice. Survival plots of wild WT (blue, $n = 15$), *Ang* knockout heterozygous (*Ang1*^{+/-}) (green, $n = 22$) and homozygous (*Ang1*^{-/-}) (red, $n = 19$) GBM mice. The numbers shown are median survival. p values are in comparison to the WT group.

To our knowledge, this is the only study that assessed ANG functional contributions in the context of GSCs. Goncalves et al. reported that ANG preserves HSPC primitiveness by mediating tiRNA production (Goncalves et al., 2016). Li et al. revealed that cytoplasmic ANG promotes prostate CSC self-renewal by actively producing bioactive 3'-end fragments of 5S rRNA (Li et al., 2020). Further questions directly stem from these early observations. First, it will be essential to determine ANG subcellular localization in GSCs and the underlying mechanisms by which ANG regulates GSCs. Second, it is of vital importance to assess if targeting ANG receptor can effectively attenuate GSC stemness, given that elevated ANG level in plasma (Bläser et al., 1993) and GBM tumor microenvironment (Eberle et al., 2000; Hu et al., 2019; Skog et al., 2008) poses clinical challenges for ANG neutralization. Third, since GSCs are intrinsically resistant to radio- and chemo- therapeutics (Lathia et al., 2015), it is of great value to examine if ANG uptake blockade can sensitize GSCs to the front-line chemotherapy drug, TMZ.

1.4. PLXNB2 orchestrates diverse biological functions of ANG

PLXNB2, the second member of the B-type Plexin family, is a single-pass transmembrane protein known to mediate neurodevelopment and pattern formation of the vertebrate nervous system (Deng et al., 2007; Friedel et al., 2007). In the developing brain, PLXNB2 expression is widespread, particularly prominent at the ventricular-subventricular zone (V-SVZ) where PLXNB2 regulates the undifferentiated cellular pools of NSCs and neural progenitor cells (NPCs) to avert premature exhaustion (Daviaud et al., 2016; Saha et al., 2012). Beyond the critical developmental window, the expression of PLXNB2 in CNS is restricted to SVZ where NSCs are preserved (Deng et al., 2007; Saha et al., 2012). In CNS malignancies, PLXNB2 is found to be differentially expressed, with an eight-fold greater expression in GBM compared to low-grade astrocytomas (Shinoura et al., 1995). The elevated expression of PLXNB2 has also been described in the primitive and differentiated cell types of gliomas (Huang et al., 2021; Yu et al., 2017).

1.4.1. PLXNB2 ligands, elicited signals, and biological functions

Plxnb2^{-/-} mice display severe developmental abnormalities, including neural tube closure defects, exencephaly, that results in peri-natal lethality in mice of an inbred C57BL/6 background (Deng et al., 2007; Friedel et al., 2007) and a disorganized cerebellar layering and foliation (defective corticogenesis) caused by the retarded migration of cerebellar granule cell precursors (CGPs), a phenotype described in the surviving outbred strains (Deng et al., 2007; Friedel et al., 2007; Hirschberg et al., 2010; Saha et al., 2012). Further, PLXNB2 has been linked to several neurological disorders, such as autism spectrum disorders (Van Battum et al., 2015) and fear-related disorders (Simonetti et al., 2021), in which aberrant semaphorin 4C (SEMA4C) signaling is critically involved. The binding of ligand SEMA4C to PLXNB2 triggers intracellular signaling cascades, mainly via the modulation of small GTPases activity (Deng et al.,

2007; Huang et al., 2021; Worzfeld et al., 2014). Sema4C expression (Maier et al., 2011) as well as an intact GAP domain of PLXNB2 (Worzfeld et al., 2014) have been demonstrated indispensable for cerebellar development. Sema4C-PLXNB2 activated RhoA signaling has been shown to enhance dendritic ramifications and synaptic connectivity in the adult hippocampus, a crucial mechanism for fear memory recall (Simonetti et al., 2021). In CNS malignancy, SEMA4C-PLXNB2 has been demonstrated to regulate biomechanical properties of GSCs to facilitate diffuse infiltration, a process mediated by intracellular Ras-GAP domain (Huang et al., 2021).

In addition to SEMA4C, ANG is another high-affinity ligand for PLXNB2 ($K_d = 0.74$ nM). PLXNB2 serves as a functional receptor to mediate physiological, pathological, and therapeutic functions of ANG in diverse cell types and circumstances. ANG binds to PLXNB2 at the SEMA4C non-overlapping region and elicits distinct mechanistic events. Unlike SEMA4C, the binding of ANG to PLXNB2 does not activate RhoA signaling. Instead, ANG is internalized via PLXNB2-mediated endocytosis and undergoes context-specific subcellular localization to exert transcriptional regulatory or ribonucleolytic functions (Yu et al., 2017). To date, the biological activities of the ANG-PLXNB2 axis has been described in nearly all facets of a cell: proliferation, survival, regeneration, and migration (Goncalves et al., 2016; Li et al., 2020; Yang et al., 2022; Yu et al., 2017). Whether ANG-PLXNB2 regulates RAC and CDC42 activation needs to be fully determined. Additionally, an intact intracellular GAP domain of PLXNB2 is required for SEMA4C to enable developmental (Worzfeld et al., 2014) and cell biomechanical (Junqueira Alves et al., 2021) regulations, and these functions were found independent of RAS inactivation. Future work that elucidates whether ANG also can signal through PLXNB2-GAP for functions will be of great mechanistic significance to understand PLXNB2-mediated signals.

1.4.2. PLXNB2 in GBM biology

PLXNB2 is upregulated in GBM (Le et al., 2015; Shinoura et al., 1995) (**Figure 1.4.A**) and its expression is found to be inversely correlated with patients' survival (**Figure 1.4.B**).

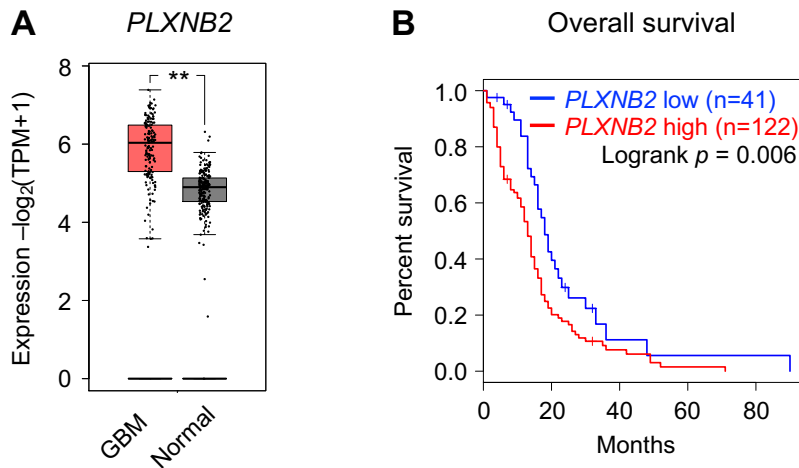


Figure 1.4. *PLXNB2* is up-regulated in GBM and its levels are inversely correlated to survival of GBM patients.

(A) *PLXNB2* mRNA in GBM samples and normal brain tissues. Normal (matched TCGA normal brains and GTEx data), $n = 207$; GBM (TCGA, Cell 2013), $n = 163$. The Gene Expression Profiling Interactive Analysis (GEPIA2) platform was used for analysis and visualization. Data shown are means $\pm$ SEM. $**p < 0.01$

(B) The survival plot of GBM patients (TCGA, Cell 2013) with high (red, $n = 122$) and low (blue, $n = 41$) expression of *PLXNB2*. The top 75% was defined as the high, and the bottom 25% was defined as the low in *PLXNB2* expression among the 163 patients in this cohort. GEPIA2 platform was used for survival analysis and visualization.

PLXNB2 has been demonstrated as a strong promoter of GBM invasion, a process can be triggered by both SEMA4C (Huang et al., 2021; Le et al., 2015) and ANG (Yang et al., 2022) via distinct mechanisms. Through *PLXNB2*-mediated RhoA and Rac1 signaling, SEMA4C regulates cytoskeletal dynamics of GBM cells and thereby promotes invasive growth (Le et al., 2015). *PLXNB2* was also found to enhance the diffusive infiltration of GSCs through a functional engagement of intracellular GAP domain, and this process is presumably triggered by SEMA4C (Huang et al., 2021).

However, it is worth noting that, unlike ANG, SEMA4C is not differentially expressed in between GBM lesions (**Figure 1.5.A**) and normal brain tissues, and as expected, SEMA4C levels in GBMs show no correlation with patient survival (**Figure 1.5.B**).

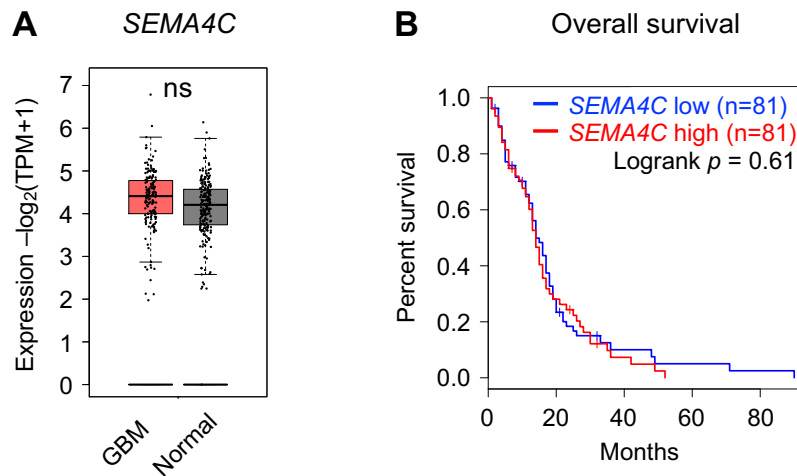


Figure 1.5. *SEMA4C* is un-differentially expressed in between GBM lesions and normal brain tissues and *SEMA4C* levels have no correlation with patient survival. (A) *SEMA4C* mRNA in GBM samples and normal brain tissues. Normal (matched TCGA normal brains and GTEx data), $n = 207$; GBM (TCGA, Cell 2013), $n = 163$. The Gene Expression Profiling Interactive Analysis (GEPIA2) platform was used for analysis and visualization. Data shown are means $\pm$ SEM. ns, not significant. (B) The survival plot of GBM patients (TCGA, Cell 2013) with high (red, $n = 81$) and low (blue, $n = 81$) expression of *SEMA4C*. The top 50% was defined as the high, and the bottom 50% was defined as the low in *SEMA4C* expression among the 163 patients in this cohort. GEPIA2 platform was used for survival analysis and visualization.

ANG is prominently upregulated in GBM and its levels independently predicts unfavorable overall survival of patients. In addition to tumor angiogenesis and ECM remodeling that are applicable for most solid tumor types, PLXNB2 has been shown to mediate ANG uptake in GBM cells that promotes cell proliferation, invasion, and tumorigenic capacity (Yang et al., 2022). GBM is a "stem cell" disease where GSCs promote tumor heterogeneity, adaptability, and ultimate therapy resistance (Prager et al., 2020). In Chapters 3-6, we ask three main questions: [1] does ANG/PLXNB2 regulate

self-renewal properties of GSCs [2] how does ANG/PLXNB2 enable the regulatory functions, and [3] can anti-PLXNB2 therapeutics be used in treating GBM?

1.4.3. GSC lines utilized to study the role of ANG/PLXNB2 in GSCs

To integrate ANG-PLXNB2 axis in a wider molecular network of GSCs, we have obtained three patient-derived GSCs of various molecular subtypes and pathological features from Dr. Hiroaki Wakimoto (MGG8 and MGG18, Massachusetts General Hospital, Boston, MA) and Dr. Brent Cochran (GS6-22, Tufts University, Boston, MA).

More specifically, GS6-22 cells were derived from right temporal primary GBM of an 82-year-old female patient (Sherry et al., 2009). Under an undifferentiated format, GS6-22 cells propagate as tumorspheres (**Figure 1.6.**) and express NSC markers, CD133 and NESTIN. GS6-22 harbors *PTEN* mutation, has an elevated AKT expression (Kulkarni et al., 2018), displays mesenchymal subtype signatures (Behnan et al., 2017; Verhaak et al., 2010) with high expression of genes of the S100A family, necrosis factor super family and NF- κ b pathway (Kulkarni et al., 2018). GS6-22 cells do not express MGMT and are highly sensitive to TMZ with an *in vitro* IC₅₀ of 2.7 μ M (Yuan et al., not published).

MGG8 was isolated from primary GBM tumor that contains primitive neuroectodermal tumor (PNET)-like nodules: dense foci of small round cells with hyperchromatic nuclei and a high nuclear-cytoplasmic ratio (Wakimoto et al., 2012). MGG8 cells propagate as typical sphere structure (**Figure 1.6.**) in an undifferentiated format and express CD133 (Wakimoto et al., 2009). MGG8 shows features of proneural subtype (Behnan et al., 2017; Verhaak et al., 2010): Genetically, MGG8 contains amplifications of *PDGFRA*, *MYCN*, and *MDM2* as well as homozygous deletion of *CDKN2A&B* (Wakimoto et al., 2012). Transcriptionally, MGG8 displays high expression

of oligodendrocytic development genes, such as *PDGFRA*, *NKX2.2*, *OLIG2* and proneural development genes, *SOX2*, *DLL3*, *ASCL1*, *TCF4* (Kulkarni et al., 2018). AKT activation was detected in MGG8 cells (Wakimoto et al., 2012). MGG8 cells are MGMT-negative TMZ-sensitive with an *in vitro* IC₅₀ at 1.69 μ M (Jahan et al., 2017). MGG8 cells were found extremely tumorigenic as 50 cells (cultured under stem cell format) can efficiently repopulate orthotopic xenograft tumors (5/5) in mice (Wakimoto et al., 2009). MGG8 tumors contain undifferentiated cytological features and display a highly invasive phenotype, a butterfly-like diffusive growth pattern (Wakimoto et al., 2009; Wakimoto et al., 2012).

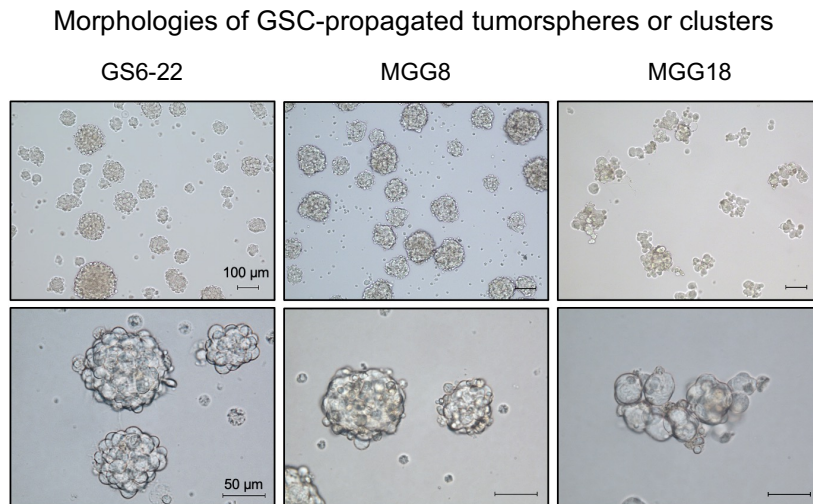


Figure 1.6. Morphologies of GSC-propagated tumorspheres or clusters. Representative tumorspheres or clusters formed by GS6-22, MGG8 and MGG18 GSCs cultured in an undifferentiated format. Scale bar, 100 μ m and 50 μ m.

MGG18 was isolated from primary giant cell GBM and harbors *PTEN* and *CDKN2A* deletion. AKT activation was found in MGG18 cells (Wakimoto et al., 2012). MGG18 is non-MYC cells with elevated homologous recombination activity (Ning et al., 2019). Unlike most GSCs, MGG18 cannot propagate as typical tumorspheres, instead they form loose clusters of cells (**Figure 1.6.**). MGG18 does not express MGMT but

shows prominent resistance to TMZ with *in vitro* IC50 of 445.8 μ M (Jahan et al., 2017). Additionally, MGG18 is insensitive to PARP inhibitors (Ning et al., 2019). MGG18 cells give rise to orthotopic tumors in mice and these xenograft tumors were found to recapitulate phenotypic and genotypic features of the primary giant cell GBM. More specifically, MGG18 tumors display marked cellular heterogeneity and contain giant and bizarre-looking neoplastic cells with multiple hyperchromatic nuclei. MGG18 xenografts contain undifferentiated populations. MGG18 tumors belong to less invasive discrete nodular subtype, displaying as well-circumscribed masses with distinct borders (Wakimoto et al., 2012).

To analyze the role of PLXNB2 in GSC regulation, we generated *PLXNB2* KO clones and pooled populations of all three GSC lines using CRISPR/Cas9 gene editing technology that introduced a frameshift mutation in the *PLXNB2* protein-coding region for gene inactivation. In addition, we generated stable luciferase-expressing GSCs. These cellular models were subsequently employed in *in vitro* and *in vivo* studies described in Chapters 3-6.

Chapter 2: Materials and Methods

2.1. Glioblastoma stem cell culture

GSCs were normally maintained in DMEM/F12 (1:1 mixture, Gibco™) supplemented with B-27 minus vitamin A (1:50, 12-587-010, Gibco™), basic fibroblast growth factor (bFGF, 10 ng/ml, Invitrogen), recombinant human EGF (rhEGF 20 ng/ml, Invitrogen), and 100 units/ml penicillin-streptomycin (15140-122, Thermo Fischer) in low attachment dishes in a humidified atmosphere containing 5% CO₂ at 37°C. When most spheres reached the diameter of 100-150 µm, they were subcultured by dissociating spheres as single cells by Accutase (AT104500, Innovative Cell Tech). Growth factors including bFGF and rhEGF were supplemented into the culture medium twice a week. For GSC adherent culture, glass coverslips were pre-coated with poly-D-lysine & laminin mixture (127-2.5, Sigma), which promotes cell adherence and reduces cellular differentiation.

2.2. Sphere formation, *in vitro* serial passaging, and extreme limiting dilution assay

Sphere formation was mainly performed by plating 1,000 single cells into each well of 96-well plate in 100 µl stem cell culture medium for 7 days. If there was special seeding density, incubation time and use of 24-well plate, we specified the information in result section and figure legends. Cells were treated according to different experimental purposes. On assay termination day, images of per tested wells were captured under 4x objective lens using a Nikon eclipse Ti microscope. The size of spheres was measured with NIS-Elements AR program. Unless it was specified, we considered only spheres with diameter ≥100 µm as the true tumorspheres in this work.

In vitro serial passaging experiment was performed by plating 1,000 fully-dissociated GS6-22 vector and *PLXNB2* knockout cells into each well of 96-well plate in

100 µl stem cell culture medium for 7 days. On day 7, images of per tested wells were acquired, and sphere number and size were thereby analyzed. Total cell number raised from 1,000 cells (per well) was determined using an automated cell counter (Nexcelom). Then, every 1,000 fully-dissociated cells were plated into each well of 96-well plate for the next passaging. For *PLXNB2* knockout cells, serial passaging experiment ended as there was not enough cells to re-initiate a new passage, even for 1 well (less than 1,000 cells). For the vector group, serial passaging experiment was terminated after passage 7.

The workflow of extreme limiting dilution assay (ELDA) was diagramed in Figure 4.7. Briefly, transduction vector and *PLXNB2* knockout cells of GS6-22, MGG8 and MGG18 were seeded at designated number of single cells in sphere media in 96-well plate (8 replicates) for designated number of days. On assay termination day, images of per tested wells were captured and the presence of the true tumorsphere was determined. Seeding density and incubation time for GS6-22: 160, 80, 40, 20, 10, 8, 6, 5, 4, 3, 2, 1 cell per well for 7 days; MGG8: 200, 100, 75, 50, 20, 10, 5, 1 cell per well for 7 days; MGG18: 200, 100, 75, 50, 20, 10, 5, 1 cell per well for 14 days. ELDA data was analyzed and plotted by an online ELDA software <https://bioinf.wehi.edu.au/software/elda/index.html> (Hu and Smyth, 2009).

2.3. Cell number analysis

Cell viability was measured by CellTiter-Glo reagent (G9242, Promega) following manufacturer's instructions. Briefly, experiments were performed with opaque-walled 96-well plates. On the assay termination day, 20 µl CellTiter-Glo reagent was added into the wells to measure the cell viability. Plates were then placed on an orbital shaker and

luminescent signals were stabilized after the 15 min incubation at room temperature.

The luminescence data was acquired with GloMax-Multi Detection System (Promega).

Cell number of vector and *PLXNB2* knockout cells was calculated by fitting each readout on the equation of the cell number standard curve of the respective cell type.

Cell number of some experiments was determined by the automated cell counter (Nexcelom).

2.4. Generation of *PLXNB2* knockout GSCs by CRISPR/Cas9 gene editing technology

We utilized both plasmid- and lentiviral-based delivery systems to establish *PLXNB2* knockout pooled populations and clones for GS6-22 (plasmid), MGG8 (lentiviral) and MGG18 (lentiviral). For plasmid-based gene targeting, we cloned the small guide RNA oligos that target the first coding exon of *PLXNB2* into pSpCas9 backbone vector (62988, PX459, Addgene). Target sequence is TGACGGTCGCCGCCGAGAAC. PX459-sg*PLXNB2* plasmids were transfected into fully dissociated GS6-22 cells using Lipofectamine LTX DNA Transfection Reagent (A12621, Invitrogen) per manufacturer's protocol. The transfection medium was changed after 24 h, and GS6-22 transfectants were selected with 2 µg/ml puromycin (Life Technologies) for 7-10 days. pSpCas9 backbone vector, PX459, was used as the vector control. For lentivirus-based gene targeting, small guide RNA (sg*PLXNB2*) was cloned into LentiCRISPRv2 vector (52961, Addgene). Lentiviral particles were produced by co-transfecting 293T cells with lentivirus plasmid, envelope plasmid pCMV-VSV-G (8454, Addgene), and packaging plasmid psPAX2 (12260, Addgene) using DNA transfection reagent (TransIT-LT1, MIR2304, Mirus). Viral particle-containing cell supernatants were collected twice after transfection (24 and 48 h) and viral particles were concentrated by ultracentrifugation. Fully dissociated MGG8 and MGG18 GSCs were transduced with the

lentivirus and the transduction medium was changed after 48 h. Cells were then subject to puromycin selection at a concentration of 1 µg/ml for 7 days. LentiCRISPRv2 vector was used as the transduction vector control. *PLXNB2* knockout clones were obtained by serial dilution of a puromycin selected pooled population. Successful *PLXNB2* knockout was confirmed by immunoblotting and immunofluorescence staining of protein PLXNB2. Site-specific modification of gene *PLXNB2* was validated by genomic DNA sequencing. Primers used for fragment cloning (PCR product: 1181 bp) and DNA sequencing are 5'CCACCAACACACAGCAGTTC3' (forward) and 5'GCCCAGGTCTCCAGACAGTA3' (reverse). Alignment analysis was performed by SnapGene software.

2.5. GSC adherent growth and *in vitro* cell differentiation assay

In vitro differentiation workflow was diagramed in Figure 4.10.A. To monitor GSC adherent growth, the designated number of cells were cultured adherently on coverslips pre-coated with poly-D-lysine & laminin (127-2.5, Sigma) in stem cell culture medium in the 24-well plate. Differentiation was induced by two approaches: 1) cells (GS6-22: vector and *PLXNB2* knockout cells) were maintained in the plating medium for 7-10 days without growth factors replenishment; 2) cells (MGG18: vector and *PLXNB2* knockout cells) were initially plated with stem cell culture medium. Differentiation was induced by the sequential growth factor depletion (10 days, without growth factors replenishment) and addition of 1% fetal bovine serum for another 15 days. Differentiation induction methods were modified from previously described procedures (Guichet et al., 2013; Park et al., 2017; Pollard et al., 2009).

2.6. RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells or xenograft tumors using TRIzol™ Reagent (ThermoFisher). Tumor tissues were homogenized using a homogenizer for a thorough

lysis. Total RNA (2 µg) was reverse transcribed to cDNA with random or Oligo(dT)₁₈ primers by M-MLV reverse transcriptase (Promega) or SuperScript™ First-Strand Synthesis System (ThermoFisher), per manufacturer's instructions. qRT-PCR analysis was performed on a LightCycler 480 II (Roche) using TB Green Advantage qPCR premix (Takara Bio). Relative expression was determined by the $2^{-\Delta\Delta C_t}$ method, using GAPDH as an internal control. Heat-maps were plotted using log₂ (fold of change) by GraphPad Prism9. The primer set are as follows:

Table 2.1. Human qRT-PCR primer sequence

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
<i>ANG</i>	CTGGGCGTTTTGTTGTTGGTC	GGTTTGGCATCATAGTGCTGG
<i>PLXNB2</i>	TCTGGCAGGGTGCTATATGCT	GAAGGGCTTGTAGAAGATGTCA C
<i>NEUROD1</i>	TCCTTCGATAGCCATTACATC	GCTGCCTTTTGTAAACACGAC
<i>NGN1</i>	CCAGCCACCACTTCAGTGTGAT TT	TATTGTCAGCCGGCTCAAACCG AA
<i>NGN2</i>	TGGGTCTGGTACACGATTGC	GGGTCTCGATCTTGGTGAGC
<i>PAX3</i>	TCCATACGTCCTGGTGCCAT	TTCTCCACGTCAGGCGTTG
<i>PAX6</i>	GGTTGGTATCCGGGGACTTC	TCCGTTGGAAGTATGGAGT
<i>NKX2.2</i>	GGGACGCCGGCAAGA	TAGGTCTGCGCCTTGAGAA
<i>VGLUT1</i>	AGCCGCCGCATCATGT	GCCTCCGCAGTTCATCAACT
<i>ASCL1</i>	CCCAAGCAAGTCAAGCGACA	AAGCCGCTGAAGTTGAGCC
<i>MYT1L</i>	CAATGGAAAGGGATTTTAAGCA	TTTGAGATTATGTACCAACGTTA GATG
<i>CD24</i>	CTCCTACCCACGCAGATTTATT C	AGAGTGAGACCACGAAGAGAC
<i>NEUN</i>	CCAAGCGGCTACACGTCT	GCTCGGTCAGCATCTGAG
<i>OTX2</i>	GCAGAGGTCCTATCCCATGA	CTGGGTGGAAAGAGAAGCTG
<i>MAP2</i>	TAACCAACCACTGCCAGACCT GAA	GCCACATTTGGATGTCACATGG CT
<i>POU3F2</i>	CGGCGGATCAAACCTGGGATTT	TTGCGCTGCGATCTTGTCTAT
<i>DCX</i>	GCCAGGGAGAACAAAGGACTTT	CACCCCACTGCGGATGA
<i>TUBB3</i>	CCTGACAATTTTCATCTTTGGTC AGAGT	GCACCACATCCAGGACCGAAT
<i>OLIG1</i>	CACAGCGGCCCGGAGACTT	CCTGTAGCCCACCAGCTCGTAG A
<i>OLIG2</i>	GCTCCTCAAATCGCATCCA	AAAGGTCATCGGGCTCTG
<i>GFAP</i>	AGAAGCTCCAGGATGAAACC	AGCGACTCAATCTTCCTCTC
<i>PROM1</i>	TGGATGCAGAACTTGACAACGT	ATACCTGCTACGACAGTCGTGG T
<i>CD44</i>	CACGTGGAATACACCTGCAA	GACAAGTTTTGGTGGCACG

<i>NESTIN</i>	CTGGAGCAGGAGAAACAGG	TGGGAGCAAAGATCCAAGAC
<i>CDCP1</i>	CTGAACTGCGGGGTCTCTATC	ATGTTGCTTTCTCGTGGCAGA
<i>L1CAM</i>	TCACGGGCAACAACAGCAACT	CGGCTTCCTGTCAATCATGCT
<i>OCT4</i>	CAGTATCGAGAACCGAGTGAG AG	AAAATCCTCTCGTTGTGCATAGT C
<i>FOXO1</i>	TGGACATGCTCAGCAGACATC	TTGGGTCAGGCGGTTCA
<i>SOX2</i>	GGGAAATGGGAGGGGTGCAAA AGAGG	TTGCGTGAGTGTGGATGGGATT GGTG
<i>FOXO3</i>	TCTACGAGTGGATGGTGC GTT	CGACTATGCAGTGACAGGT
<i>MUSASHI1</i>	GAGACTGACGCGCCCCAGCC	CGCCTGGTCCATGAAAGTGACG
<i>ID2</i>	TTGTCAGCCTGCATCACCAGA G	AGCCACACAGTGCTTTGCTGTC
<i>FOXG1</i>	CCCTGGGACCAGACTGTAAGT GAAC	TGGCGGCTCTTAGAGATAGACT TGTCAG
<i>SOX9</i>	TCTGAACGAGAGCGAGAA	GCGGCTGGTACTTGTAATC
<i>TP53</i>	CAGTCTTGAGCACATGGGAGG	GGCGACTGTCCAGCTTTGT
<i>CDKN1A</i>	GTCAGTGTCTTGTAACCCTTG TG	CGGCGTTTGGAGTGGTAGAAA
<i>CDKN1B</i>	CATTTGGTGGACCCAAAGAC	CTTCTGAGGCCAGGCTTCTT
<i>BAK</i>	GCAGGCTGATCCCGTCC	CTGCGGAAAACCTCCTCTGT
<i>BAX</i>	GCCCTTTTGCTTCAGGGTTT	TGAGACACTCGCTCAGCTTC
<i>BAD</i>	CTTGGGCCCAAGCATGT	TCTGCAGAGCTGGAGTCTTCC
<i>CASP6</i>	ACTGGCTTGTTCAAAGG	CAGCGTGTAACGGAG
<i>CCND1</i>	GCTGCGAAGTGGAACCATC	CCTCCTTCTGCACACATTTGAA
<i>CCNE1</i>	GCCAGCCTTGGGACAATAATG	CTTGCACGTTGAGTTTGGGT
<i>XIAP</i>	CCTGCAGGATTGCCTTCCTAA	TGATGTCTGCAGGTACACAAGT
<i>BCL2</i>	GTGGATGACTGAGTACCTGAA C	GCCAGGAGAAATCAAACAGAGG
<i>GAPDH</i>	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA

2.7. Custom TaqMan™ small RNA assay

5'tiRNA-Gly-GCC RT-qPCR was performed using custom TaqMan™ small RNA assay (assay ID: CTEPTKJ, catalog no. 4398987, ThermoFisher) that specifically detects the target sequence GCAUUGGUGGUUCAGUGGUAGAAUUCUGCCUGC. Total RNA (10 ng for cell samples and 50 ng for tissue samples) was reverse transcribed to cDNA with TaqMan™ MicroRNA Reverse Transcription Kit (catalog no. 4366596, Applied Biosystems) and qRT-PCR was performed using TaqMan™ Universal Master Mix II, no UNG (catalog no. 4440040, Applied Biosystems) on a LightCycler 480 II

(Roche) according to the manufacturer's guidelines. Relative expression was determined by the $2^{-\Delta\Delta C_t}$ method, using GAPDH as an internal control.

2.8. tiRNA transfection

5'-P-tiRNA-Gly-GCC (5'GCAUUGGUGGUUCAGUGGUAGAAUUCUCGCCUGC) was commercially synthesized (IDT). GSCs were transfected with 100 nM of 5'-P-tiRNA-Gly-GCC or control by TransIT[®]-LT1 transfection reagent (MIR 2300, Mirus Bio).

2.9. Paraffin-embedded section preparation and hematoxylin and eosin staining

Brains or tumor lesions were fixed in 10% neutral buffered formalin for at least 48 h at room temperature, prepared as 5- μ m thickness paraffin-embedded sections, and stained with hematoxylin and eosin. Slide images were acquired using a scanner and a Nikon eclipse Ti microscope.

2.10. Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were dewaxed and rehydrated by heating at 60 °C for 60 min followed by incubating in xylene and a graded series of alcohols (100, 95, 70, and 50% ethanol in distilled H₂O). After washing with distilled H₂O, slides were immersed completely in a plastic dipper filled with freshly-made sodium citrate buffer (10 mM sodium citrate, pH 6.0) and boiled for 12 min in a pressure cooker (high pressure) for antigen retrieval. Slides were quickly rinsed with distilled H₂O and endogenous enzymes were blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 10 min at room temperature, followed by washes in distilled H₂O and TBST (0.2% Tween[™] 20). Slides were then blocked in 10% goat normal serum in TBST at room temperature for 1 h and incubated with primary antibodies diluted in 10% goat serum TBST in a humidified chamber at 4 °C in the dark overnight. The primary

antibodies (immunohistochemistry) against human GFAP (mAb25941, R&D) and NeuN (mAb377, Sigma-Aldrich) were used at 5 µg/ml and 1:100. After washing with TBST, sections were incubated with secondary antibodies (VECTASTAIN Elite ABC HRP Kit, PK-6102, Vector Laboratories) for 45 minutes and diaminobenzidine using the DAB Substrate Kit (SK-4100, Vector Laboratories) for 2-5 minutes at room temperature, followed by hematoxylin staining (Modified Mayer's Hematoxylin, ThermoFisher). Slides were mounted with Permount™ medium (Fisher Scientific).

2.11. Immunofluorescence and fluorescence intensity analysis

For paraffin-embedded tissues, sections were dewaxed and rehydrated by heating at 60 °C for 60 min followed by incubating in xylene and a graded series of alcohols (100, 95, 70, and 50% ethanol in distilled H₂O). After washing with distilled H₂O, slides were immersed completely in a plastic dipper filled with freshly-made sodium citrate buffer (10 mM sodium citrate, pH 6.0) and boiled for 12 min in a pressure cooker (high pressure) for antigen retrieval. After washing with distilled H₂O, sections were blocked in 10% goat normal serum in TBST (0.2% Tween™ 20) at room temperature for 1 h and incubated with primary antibodies diluted in 10% goat serum TBST in a humidified chamber at 4 °C in the dark overnight. Primary antibodies (immunofluorescence) are anti-human VWF (1: 100, A0082, Agilent) and Ki67 (1:50, ab9260, Abcam). After washing with TBST, sections were incubated with goat anti-mouse IgG (H+L), Alexa Fluor 488 (A-24920, Invitrogen), goat anti-rabbit IgG (H+L), Alexa Fluor 555 (A-21428, Invitrogen), and goat anti-rabbit IgG (H+L), Alexa Fluor 488 (A-11008, Invitrogen) at 1:500 at room temperature for 1-2 h. The cover slips were mounted on slides with ProLong™ Gold antifade reagent with DAPI (P36931, Invitrogen) and fluorescent images were acquired using a Nikon eclipse Ti fluorescent microscope.

For immunocytochemical analysis, cells were cultured adherently on coverslip coated with poly-D-lysine & laminin (127-2.5, Sigma) and treated according to experimental purposes. Cells were fixed and permeabilized by 1) freshly-made 4% paraformaldehyde solution (15710, Electron Microscopy Science) for 30 mins and 0.3% Triton X-100 in PBS for 7 mins, 2) ice-cold 100% methanol in -20°C for 15 mins. Cells were blocked with 5% normal goat serum or 3% BSA diluted in PBS at room temperature for 1 h and then incubated with primary antibodies in 5% goat serum or 3% BSA PBS overnight at 4°C. Primary antibodies include anti-human ANG (50 µg/ml, mAb 26-2F and 20 µg/ml, polyclonal IgG antibody R113, made-in-house), PLXNB2 (10 µg/ml, mAb100^{high}, made-in-house), fibrillarin (1:1000, ab18380, Abcam), PABP (8 µg/ml, SC18611, Santa Cruz), neurofilament (1:100, 837904, BioLegend), NEUN (1:50, MAB377, EMD Millipore), GFAP (10 µg/ml, MAB25941, R&D Systems), CD44 (1 µg/ml, HPA005785, Sigma), NEUROD1 (1:250, ab205300, Abcam) and NEUROD1 (1:100, ab60704, Abcam). After washing with PBS, samples were incubated with species-appropriate secondary antibodies for 60 minutes at room temperature at a dilution of 1:400. Secondary antibodies include: donkey anti-goat IgG (H+L), Alexa Fluor 555 (A-21432, Invitrogen), rabbit anti-mouse IgG (H+L), Alexa Fluor 488 (A-11059, Invitrogen), goat anti-mouse IgG (H+L), Alexa Fluor 488 (A-24920, Invitrogen), goat anti-rabbit IgG (H+L), Alexa Fluor 555 (A-21428, Invitrogen), and goat anti-rabbit IgG (H+L), Alexa Fluor 488 (A-11008, Invitrogen), goat anti-mouse IgG (H+L), Alexa Fluor 555 (A-28180, Invitrogen). Negative control samples were exposed to species-appropriate IgG (primary antibody) and a corresponding secondary antibody to demonstrate the specific staining. The cover slips were mounted on slides with ProLongTM Gold antifade reagent with DAPI (P36931, Invitrogen) and fluorescent images were acquired using a Nikon eclipse Ti fluorescent microscope.

Fluorescence intensity was quantified with ImageJ software. To calculate the fluorescence intensity. The corrected total cell fluorescence (CTCF) was calculated as $CTCF = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background readings})$. Mean CTCF intensity was calculated by corrected total cell fluorescence (CTCF) divided by respective area. Detailed procedure was described in <https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html>.

2.12. Neurites/neuronal-like processes analysis

Neurites/neuronal-like processes were traced and measured using FIJI (ImageJ) using the simple neurite tracer plugin. The number of terminals was manually counted after tracing. Detailed procedure was described in <http://snyderlab.com/2016/05/25/tracing-neurons-using-fiji-imagej/>.

2.13. TUNEL assay

Apoptosis rate in tumor tissues was determined by *In Situ* Cell Death Detection Kit (11684795910, Roche) per manufacturer's instructions. Paraffin-embedded sections were dewaxed and rehydrated according to previous described procedures. Sections were rinsed in PBS, permeabilized in freshly-prepared permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 8 min, and rinsed again with PBS. Sections were incubated with TUNEL reaction mixture (50 μ l) for 60 min at 37 °C in a humidified chamber in the dark. A negative control was set up simultaneously and processed with the same procedures. The slides were rinsed three times with PBS and mounted with cover glasses using ProLong™ Gold antifade reagent with DAPI (P36931, Invitrogen) and fluorescent images were acquired using a Nikon eclipse Ti fluorescent microscope.

The quantification of each specimen was performed in 40 high-power fields, and cells with green nuclei were identified as positive.

2.14. Protein isolation and immunoblotting

Cells were harvested, washed with PBS, lysed in RIPA lysis and extraction buffer (89900, Thermo Scientific) with 1 μ M DTT and a protease inhibitor cocktail tablet cComplete mini (11836170001, Roche) and centrifuged at 12,000 g for 15 mins at 4°C. The supernatant was used as the cell lysate in immunoblotting blotting. For xenograft tumors, proteins were extracted using TRIzol™ Reagent (ThermoFisher). Tumor tissues were homogenized using a homogenizer for a thorough lysis. Proteins were pelleted from the phenol-ethanol supernatant by isopropanol. Proteins were washed by 0.3 M guanidine hydrochloride in 95% ethanol and solubilized by 1% SDS.

A total of 25-50 μ g protein was separated by SDS-PAGE (7.5% resolving gel for PLXNB2, 10% for mouse IgG) and electro-transferred to a nitrocellulose membrane in Towbin system buffer. The membrane was blocked with 5% fat-free milk in TBST containing 0.1% Tween®20 at room temperature for 1 hr and incubated with the indicated primary antibodies at 4 °C overnight followed by incubation with species-appropriate secondary antibodies (goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP) at room temperature for 30 mins. Bound antibodies were visualized with chemiluminescence SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Images were acquired by image analyzer (ProteinSimple) and analyzed with Image J software. The following primary antibodies were used, anti-human PLXNB2 (5 μ g/ml, mAb17, made-in-house), β -Actin (1:1000, sc-130656, Santa Cruz), and β -Actin (1:1000, sc-47778, Santa Cruz), and anti-mouse IgG(H+L)-HRP (1:5000, 62-6520,

Invitrogen). Secondary antibodies include goat anti-mouse IgG (H+L)-HRP (1:3000, 62-6520, Invitrogen) and goat anti-rabbit IgG (H+L)-HRP (1:3000, 65-6120, Invitrogen).

2.15. Small RNA gel electrophoresis and band intensity quantification

Total RNA was extracted from cells or xenograft tumors using TRIzolTM Reagent and quantified by NanoDrop (Thermo Scientific). Tumor tissues were homogenized using a homogenizer for a thorough lysis. A total of 20-50 µg of RNA was diluted in Novex TBE-Urea sample buffer (LC6876, Invitrogen), heated at 158 F for 5 min, and cooled to room temperature prior to loading. A low molecular weight marker ladder (J76410, Affymetrix) was heated and cooled at the same time. A 15% TBE-Urea gel (EC6885BOX, Invitrogen) was pre-run at 76 V for 60 min and samples were loaded and electrophoresed at 100 V in 1x TBE running buffer for 160 mins using the XCell SureLockTM mini-cell system (Invitrogen). The gel was stained with SYBRTM Gold Nucleic Acid Gel Stain solution (S11494, Invitrogen) for 30 min with agitation and imaged on a blue-light transilluminator. Band intensity was quantified by Image J. Detailed procedure was described in <http://www.navbo.info/DensitometricAnalysys-NIHimage.pdf>

2.16. Small RNA gel extraction and small RNA sequencing

Total RNA was isolated, separated by denaturing urea polyacrylamide gel electrophoresis and stained by SYBRTM Gold solution. Small RNA was extracted from the gel using “Crush and Soak” method. Briefly, small RNA-containing regions were cut out using clean razor blades. Gel slides were then transferred into sterile, round-bottom, nuclease-free microcentrifuge tubes and finely crushed using 25-gauge needles. RNA-containing slides were incubated with ~ 2 volumes of elution solution (500 mM NaOAC, 1 mM EDTA-KOH pH 8.0, 0.1% DEPC) at 4 °C overnight and another 3 hours at room temperature on a nutator. Next, elution supernatants were carefully collected by

centrifugation (5000 g, 2 mins, twice). Gel debris-free supernatants were incubated with 0.1 volume of 3 M NaOAc pH 5.2 and 3 volumes of 100% ethanol at -20 °C for 1 hour to precipitate the RNA. Then, RNA was washed twice with 70% ethanol and collected by centrifugation at 15,000 rpm for 15 mins at 4 °C. Air-dry RNA pellets were resuspended in ultra-pure water and stored in -80 °C.

RNA sequencing and data analysis were performed by Tufts University Core Facility Genomics. Small RNA samples were purified using QIAseq miRNA Library Kit (331502, QIAGEN). The integrity of recovered RNA was assessed with RNA 6000 Pico Kit of the Agilent 2100 Bioanalyzer (5067-1513, Agilent Technologies). RNA quantity was also determined by Agilent 2100 Bioanalyzer. RNA libraries were prepared with QIAseq miRNA Library Kit according to the manufacturer's instructions. RNA samples were sequenced using NextSeq 550 Mid-Output 150 cycles kit (Illumina) and run as a single-read 150 base mode. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads from raw data. Single-end clean reads were aligned to the reference genome, GRCh38 (hg38). The featureCountsOut.hg38 was used to count the reads numbers mapped to each gene of tRNAs, microRNAs, snRNAs, snoRNAs, and rRNAs (Data processed by Dr. Albert Tai). The levels of specific small RNA were calculated based on reads count and presented as RPM (Reads Per Million Reads), a normalized value to the total read of the respective group. Differential expression analysis of two groups was calculated by $\log_2$ $(\text{RPM}_{\text{test}}+1)/(\text{RPM}_{\text{ctrl}}+1)$ and fold change of 2 was set as the threshold for significant differential expression. To get rid of comparison bias introduced by extreme low reads, we focused on small RNA species that possess at least 100 RPM in control treated vector cells. For small RNA sequencing data, all bar graphs and heatmaps were generated by GraphPad Prism9. Fragment alignment graphs were generated by Integrative Genomics Viewer (Broad Institute).

2.17. Generation of luciferase labeled MGG18 vector and *PLXNB2* knockout cells

Lentiviral vector, Pico2-Fluc-mCherry, is a kind gift from Dr. Hiroaki Wakimoto (Massachusetts General Hospital, Boston, MA) (Jahan et al., 2017). Lentiviral packaging was performed by transfection of 293T cells as described (Shah et al., 2008). MGG18 vector and *PLXNB2* knockout cells were transduced with LV-Pico2-Fluc-mCherry. MGG18-Vector-FmC and MGG18-*PB2*KO-FmC were obtained after puromycin selection (1.5 µg/ml), serial cell dilution, and validation of the expression.

2.18. Intracranial GSC implantation and tissue processing

Healthy, 8 weeks old, female athymic nude mice (Nu/J, *Foxn1*^{nu}, The Jackson Laboratory, Bar Harbor, ME, USA) were randomly selected as xenograft recipients (n = 7-8, per group).

To examine the effect of *PLXNB2* knockout on GSC intracranial tumorigenicity, MGG18-Vector-FmC and MGG18-*PB2*KO-FmC GSCs were dissociated into single cells and stereotactically implanted into mouse brains (4 x 10⁵ cells per mouse) at the coordinate: right striatum, 2.2 mm lateral to the midline, 0.5 mm anterior to the bregma, and 2.5-mm deep by a 26s-gauge syringe (80383, Hamilton). The infusion rate (1 µl/minute) was controlled by the NanoJet stereotaxic syringe pump (Chemyx). Body weight of mice were measured 3 times per week, and orthotropic tumor growth was monitored by Fluc bioluminescence (BLI) imaging on indicated days (Figure 6.2.A).

To assess the effect of *PLXNB2* antibody on MGG18 *in vivo* TMZ sensitivity, MGG18-Vector-FmC GSCs (4 x 10⁵ cells per mouse) were stereotactically implanted into the brains of athymic nude mice (n=15) and tumor growth was monitored by Fluc BLI imaging on indicated days (Figure 6.4.). On day 28, most MGG18 tumors were found to reach the appropriate size and blood-brain barrier (BBB) of mouse brains was projected to become compromised for antibody penetration. Based on the size of tumors

(Table 6.1. and Figure 6.5.A), mice were randomized into two treatment groups: 1) TMZ group (25 mg/kg TMZ, n = 7) and 2) TMZ+PLXNB2mAb group (25 mg/kg TMZ + 30 mg/kg PLXNB2 mAb, n = 8). Treatments were given by intraperitoneal (i.p.) injection on day 28 after Fluc BLI imaging. Temozolomide (T2744, TCI) was prepared as 100 mM stock dissolved by dimethyl sulfoxide. PLXNB2 mAb (mAb100^{high}) had a concentration of 4.965 mg/ml buffered with PBS. All mice were monitored daily by health scoring and weighting, and tumor growth was assessed every week by Fluc BLI imaging.

Mice were followed for survival and sacrificed when neurological symptoms became significant or on the experiment termination day. Neurological signs or signs of morbidity include hunched posture, gait changes, lethargy, and significant weight loss. Immediately after animal euthanasia, mouse brains were harvested and rinsed with PBS. Part of each brain was fixed in 10% neutral buffered formalin for histological analysis. Part of each tumor xenograft was snap frozen in liquid nitrogen for molecular analysis. All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Tufts University/Tufts Medical Center. All procedures were performed in accordance with the protocols. Veterinary care was provided on a 24-hour basis by the Tufts Comparative Medicine Services.

2.19. *In vivo* bioluminescence imaging

D-luciferin (LUCK-100, GoldBio) was intraperitoneally given to animals at 125 mg/kg body weight and then mice were anesthetized with isoflurane for imaging analysis. The tumor luciferase images were captured by an IVIS spectrum *in vivo* imaging system (PerkinElmer) at the Tufts Small Animal Imaging Preclinical Testing Facility. Data of *in vivo* bioluminescence imaging was visualized and processed with PerkinElmer *in vivo* imaging software.

2.20. Statistical Analysis

All bar graphs were presented as mean $\pm$ SEM (standard error of mean) and all heatmaps were represented by mean values (GraphPad Prism 9). For comparisons of two experimental groups, unpaired two-tailed Student's *t* tests were employed (Excel). For survival analysis, Kaplan-Meier survival curves were generated by GraphPad Prism 9, and groups were compared using log rank tests. ELDA results were analyzed by the ELDA online software (<http://bioinf. wehi.edu.au/software/elda/>) and Chi-squared test was used to determine the statistical differences. For all analyses, differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns stands for not significant).

Chapter 3: ANG regulates stemness of GSCs

3.1 ANG-regulated stem cell properties of patient-derived GSCs

In order to understand the regulatory function of the ANG-PLXNB2 axis in GSC properties, we first examined the effect of ANG, the ligand, on GSC self-renewal. The sphere-forming capacity of two independent patient-derived GSC lines, GS6-22 (Kulkarni et al., 2018; Sherry et al., 2009) and MGG8 (Wakimoto et al., 2009) were examined in the absence or presence of exogenous ANG. GS6-22 is a mesenchymal subtype GSC carrying *PTEN* mutation with elevated AKT expression, and MGG8 is a pro-neural subtype GSC having *PDGFRA* amplification and *CDKN2A&B* homozygous deletion. They both are able to form tumorspheres. The number of tumor spheres formed from 1,000 GS6-22 and MGG8 was 120 ± 4 and 32 ± 2 , respectively, in the absence of exogenous ANG, which was increased to 163 ± 7 and 46 ± 3 in the presence of 1 $\mu\text{g/ml}$ exogenous ANG, representing 36.2% and 40.1% increase, respectively (**Figure 3.1., A and B**). As the number of spheres reflects the abundance of cells possessing the self-renewal capacity (Lathia et al., 2015), an indication of stemness, these results suggest that ANG was able to enhance the stemness of both GS6-22 and MGG8. Moreover, we found that ANG enhanced tumorsphere formation in a dose-dependent manner (**Figure 3.1.C**), reaching a plateau at 0.3 $\mu\text{g/ml}$, a concentration comparable to the physiological level of ANG in human plasma (Bläser et al., 1993). These results indicate that ANG is able to promote self-renewal potential of GSCs as well as stimulate clonal growth of tumorspheres.

The diameters of the tumorspheres were marginally but statistically significantly enhanced by exogenous ANG, being 132 ± 0.9 and 135 ± 1.3 μm , respectively, for GS6-22 and MGG8 in the presence of 1 $\mu\text{g/ml}$, as compared to 130 ± 0.7 and 130 ± 1.4 μm in the absence of ANG. (**Figure 3.1.D**). The marginally increased sphere size may reflect an enhanced GSC clonal growth. Or tumorsphere cut-off diameter (100 μm) used in this work might mask the difference to some extent.

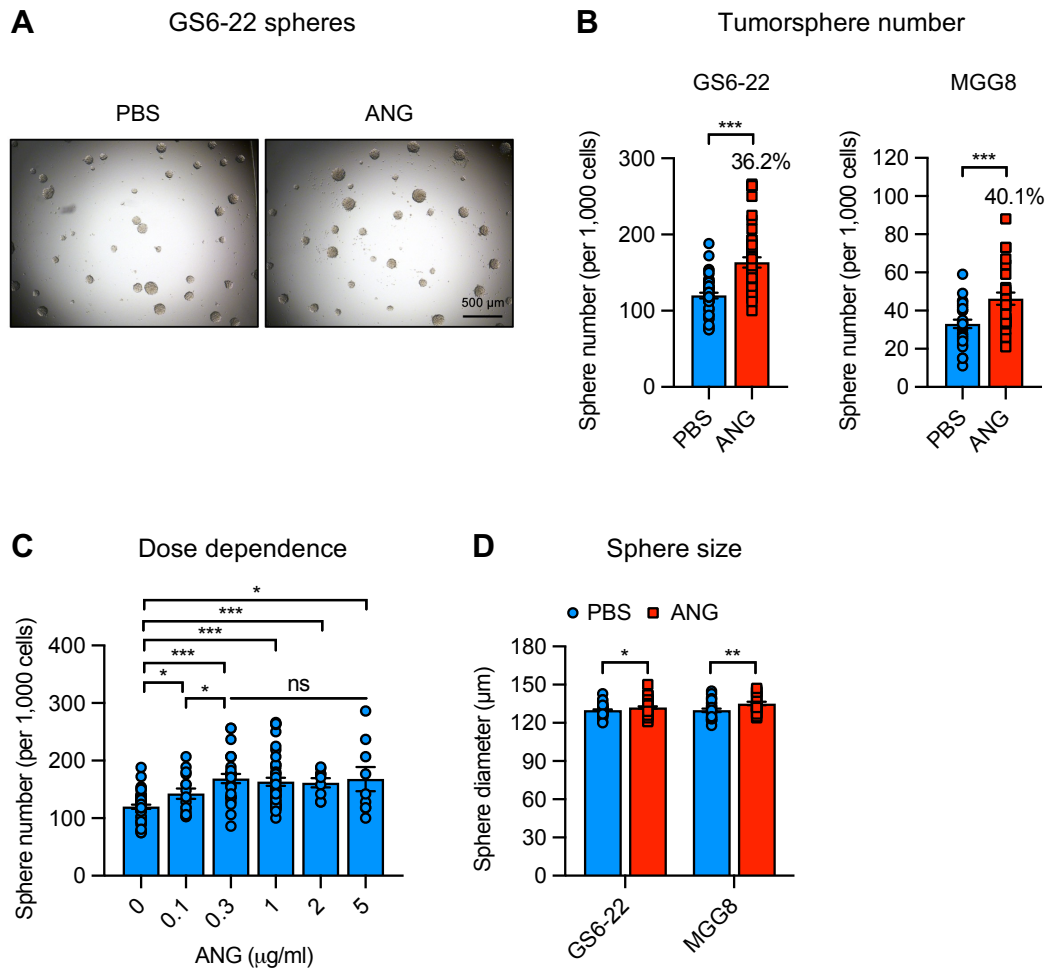


Figure 3.1. ANG promoted tumorspheres of patient-derived GSCs.

(A) Representative images of tumorspheres grown from GS6-22 plated at the density of 1,000 cells per 100 μl culture media per well of 96-well plate in the presence of PBS control or 1 $\mu\text{g/ml}$ ANG for 7 days. Scale bar, 500 μm .

(B) Tumorsphere number of GS6-22 and MGG8 plated at the density of 1,000 cells per well of 96-well plate and cultured in the presence of PBS control or 1 $\mu\text{g/ml}$ ANG for 7 days ($n = 5 - 9$). Note: spheres with diameter $\geq 100 \mu\text{m}$ are considered as tumorspheres.

(C) The number of tumorspheres of GS6-22 plated at the density of 1,000 cells per well of 96-well plate and cultured in the presence of 0, 0.1, 0.3, 1, 2, 5 $\mu\text{g/ml}$ ANG for 7 days ($n = 2 - 8$).

(D) Diameter of tumorspheres derived from GS6-22 and MGG8 plated at 1,000 cells per well of 96-well plate and cultured in the absence or presence of 1 $\mu\text{g/ml}$ ANG for 7 days ($n = 5 - 8$). Each dot represents the mean of diameters of all spheres in a well. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

In addition to promote GSC self-renewal and clonal growth of tumorspheres, ANG was also able to maintain tumorspheres in an undifferentiated state. It is well

known that certain conditions, such as the presence of sufficient stem cell growth factors, including EGF, bFGF, and B27 serum-free supplement, and non-adherent culture dishes, are needed to allow tumorsphere formation and prevent differentiation. In a longer culture period (8-9 days) without growth factor replenishment, GSCs in a tumorsphere underwent morphological changes (**Figure 3.2.A**), reflecting a trend of differentiation into GBM cells, and tended to proliferate in an anchorage-dependent manner. Presence of ANG prevented this morphological change and maintained GSCs in sphere format, indicating its role in perseverance of stemness. However, ANG alone does not allow tumorsphere formation, nor could it substitute EGF or bFGF in tumorsphere culture (**Figure 3.2., B and C**), indicating that the regulatory function of ANG in GSCs requires the participation of stem cell growth factors. In other word, ANG acts as an ancillary factor in regulating GSC properties, allowing a more flexible strategy of targeting in future drug development endeavor.

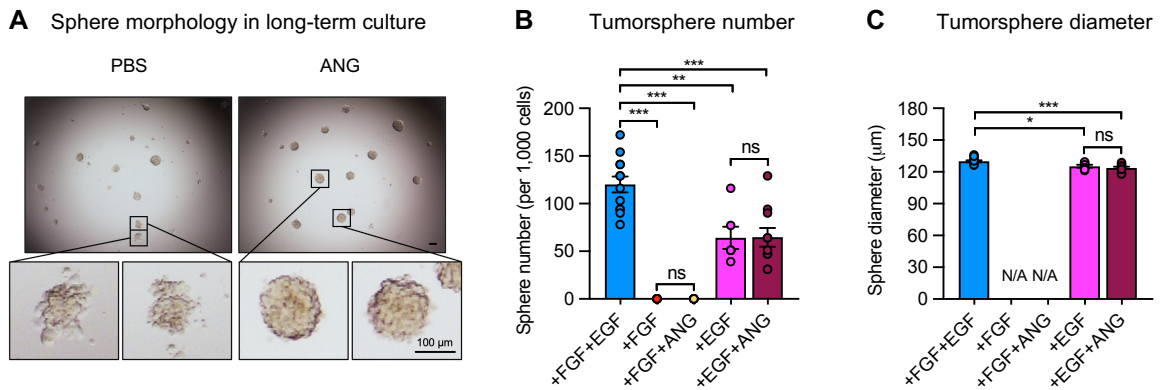


Figure 3.2. ANG sustained GSC stemness.

(A) Representative images of tumorspheres derived from GS6-22 after culture for 8-9 days without medium change or growth factor refreshment in the presence or absence of 2 µg/ml ANG. Scale bar, 500 µm in the main images and 100 µm in the callout boxes. (B-C) Tumorsphere formation of GS6-22 plated at the density of 1,000 cells per well of 96-well plate and treated by 1 µg/ml ANG in the presence or absence of rhEGF or bFGF for 7 days (n = 2). B, tumorsphere number; C, average tumorsphere diameter. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

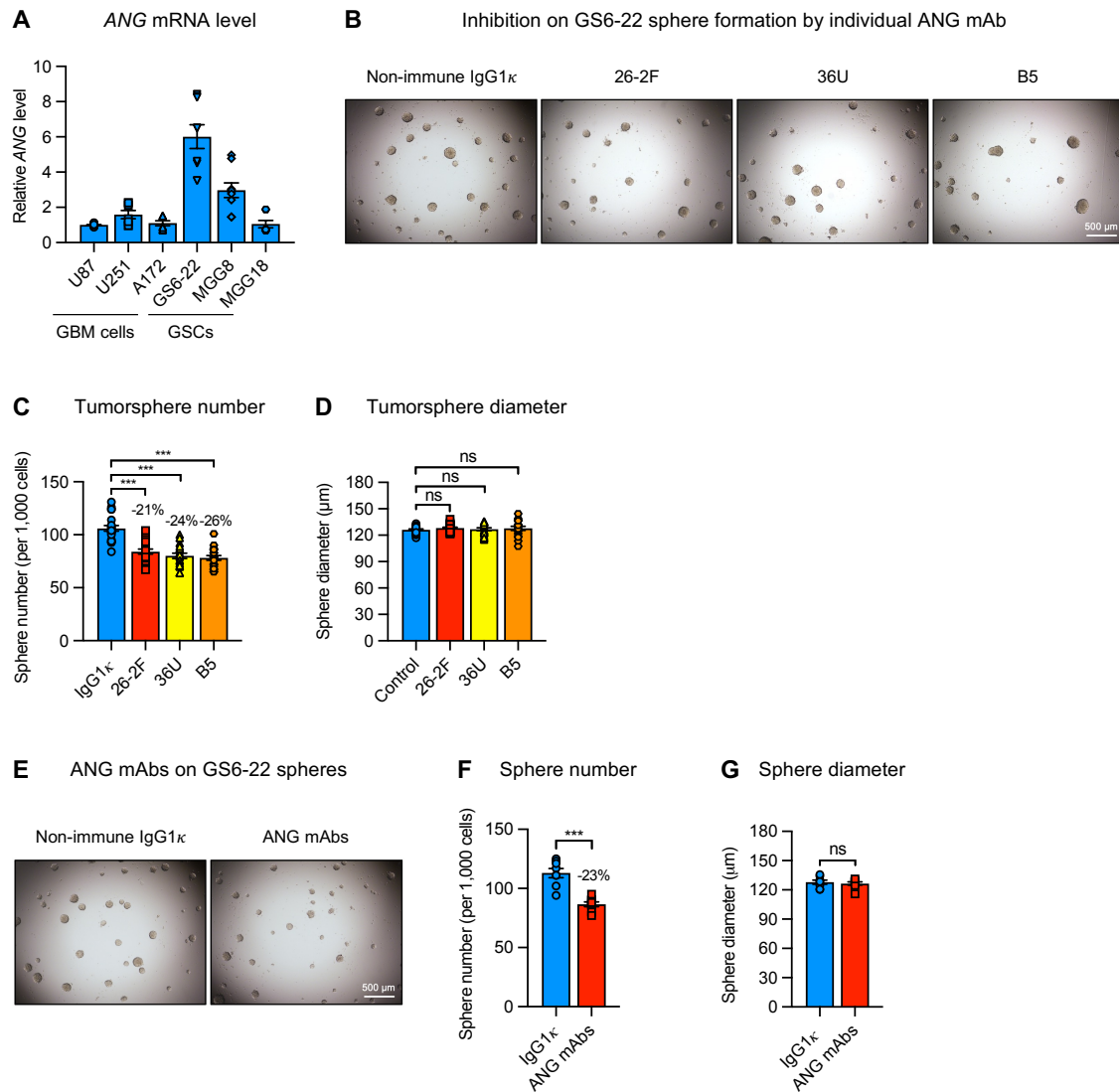


Figure 3.3. ANG mAbs decreased tumorsphere formation of GSCs.

(A) ANG mRNA levels in GBM cells (U87, U251, and A172) and GSCs (GS6-22, MGG8, and MGG18). mRNA levels were determined by qRT-PCR analysis using *GAPDH* as internal control. Bar graphs are relative values to the ANG mRNA level in U87 ($n = 3 - 5$). GBM cells were cultured in DMEM containing 10% FBS. GSCs were cultured in sphere media in a non-adherent format.

(B-D) Tumorsphere formation of GS6-22 at a plating density of 1,000 cells per well of 96-well plate treated by 50 $\mu\text{g/ml}$ ANG mAb (26-2F, 36U, or B5), respectively, or with a non-immune isotype control IgG1- κ for 7 days ($n = 4$). B, representative images; C, tumorsphere number; D, tumorsphere diameter. Scale bar, 500 μm .

(E-G) Tumorsphere formation of GS6-22 at a plating density of 1,000 cells per well of 96-well plate treated by ANG mAb cocktail (100 $\mu\text{g/ml}$ of each, 26-2F, 36U, and B5) or non-immune isotype IgG1- κ for 7 days ($n = 3$). E, representative images; F, tumorsphere number; G, tumorsphere diameter. Scale bar, 500 μm .

*** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

After establishing the role of exogenous ANG in regulating GSC stemness, we next examined the function of endogenous ANG in GSCs. For this purpose, we first examined the expression levels of *ANG* mRNA in three GSC lines and found that they in general express higher levels of *ANG* as compared to the established GBM cell lines (**Figure 3.3.A**). As ANG is a secreted protein and acts in either a paracrine or autocrine fashion, its activity could be neutralized by mAbs that recognize and trap extracellular ANG protein. The effect of three mAbs of human ANG, including clone 26-2F that has been well characterized (Chavali et al., 2003; Fett et al., 1994) and widely used in numerous studies and clones 36U and B5, were examined. As shown in Figure 3.3., the numbers of tumorspheres formed from GS6-22 cultured in the presence of 26-2F, 36U and B5, either alone (50 µg/ml) (**Figure 3.3., B and C**) or in combination (300 µg/ml total concentration) (**Figure 3.3., E and F**) were decreased by 21 - 26% as compared to those of the isotype IgG controls. It is notable that sphere size was not changed upon treatment of ANG mAbs, suggesting that clonal growth of GSCs was not altered under this particular treatment conditions (**Figure 3.3., D and G**).

Lack of effect of ANG mAb treatment on sphere growth could be due to uncomplete neutralization of secreted ANG or could be that the function of endogenous ANG is different from that of exogenous ANG. Taken together, these results demonstrate a regulatory function of ANG in GSC sphere formation.

3.2 ANG regulated GSCs through receptor-mediated events that require an intact ribonucleolytic activity of ANG as well as its nuclear functions

ANG is a small protein of 14 kDa consisting of 123 amino acid residues. Remarkably, three distinct functional sites have been identified, including a receptor binding site at ⁵⁹NKNGNPHREN⁶⁸ (Hallahan et al., 1991; Hu et al., 1991), a ribonuclease triad composed of H13, K40, and H114 (Shapiro et al., 1986), and a nuclear localization

sequence (NLS) of ²⁹IMRRRGL³⁵ (Moroianu and Riordan, 1994). The receptor binding site is required for binding to its cell surface receptor PLXNB2 that mediates ANG endocytosis and activation of signaling pathways, leading to a variety of cellular activities (Yu et al., 2017). The ribonucleolytic site is responsible for RNA processing events to generate small bioactive RNAs, such as tiRNA and the 3'-end of 5S rRNA, that regulate cell survival and stemness (Goncalves et al., 2016; Li et al., 2020). The NLS mediates nuclear import of internalized ANG through the classic nuclear pore route and is essential for ANG nucleolar accumulation where ANG regulates rRNA biogenesis to promote cell growth and proliferation (Tsuji et al., 2005; Xu et al., 2003).

To understand the mechanism of action of ANG in regulating GSC properties, we assessed the effects of ANG variants on tumorsphere formation. Three variants were used for this purpose. The first is R66A in which arginine 66 was replaced by alanine in the receptor binding site so it does not bind to its cell surface receptor PLXNB2 (Yu et al., 2017), the second is K40Q in which the catalytically essential residue lysine 40 was replaced by glutamine so it does not have ribonucleolytic activity (Shapiro et al., 1989), and the third is R33A in which arginine 33 in the NLS was replaced by alanine so it does not undergo nuclear translocation (Moroianu and Riordan, 1994). Figure 3.4 shows that all three types of ANG variants did not promote tumorsphere formation nor did they enhance clonal growth (**Figure 3.4., A and B**), indicating that all three functional sites need to be intact to promote tumorsphere formation.

The indispensable roles of all three domains of ANG are a unique requirement in GSC regulation. It has been reported that nuclear translocation is not required for ANG to enhance the transcript levels of self-renewal genes in CD34+ human cord blood cells (Goncalves et al., 2016). It has also been reported that, upon mutant apolipoprotein A-induced amyloidosis, only the receptor binding capacity is essential for ANG to protect liver hepatocellular carcinoma cells from cell death, and that both nuclear translocation

and ribonucleolytic activity of ANG are dispensable (Del Giudice et al., 2014). GSC is the only cell type so far shown that its regulation requires ANG to have receptor binding ability, nuclear translocation capacity, and ribonucleolytic activity.

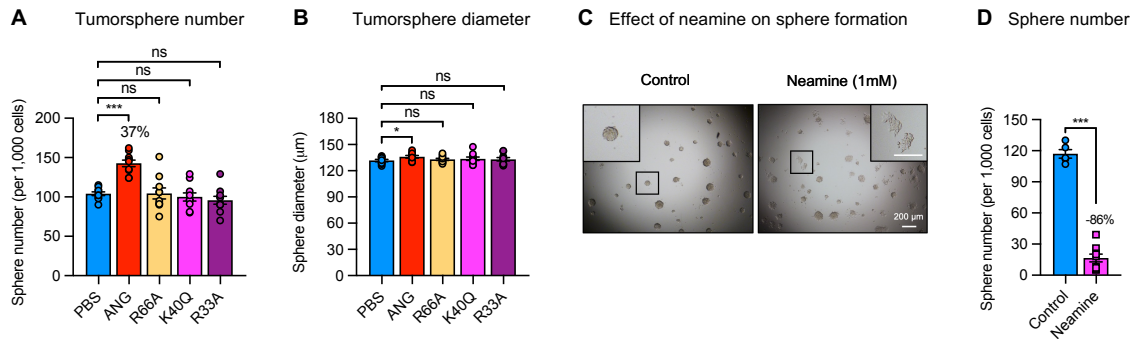


Figure 3.4. Effect of ANG variants and small molecule inhibitors on tumorsphere formation.

(A-B) Tumorsphere number (A) and diameter (B) of GS6-22 plated 1,000 cells per well of 96-well plate and cultured in the presence of PBS control, 1 μg/ml WT, or variant ANG for 7 days (n = 3).

(C-D) Tumorsphere image (C) and number (D) of GS6-22 plated at 1,000 cells per well of 96-well plate and cultured in the presence of PBS control or 1 mM neamine for 7 days (n = 3). Scale bar, 200 μm. Inset, high magnification image of boxed region.

* $p < 0.05$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

To confirm that nuclear translocation of ANG is indeed required, we examined the effect of neamine, a small molecule that blocks ANG nuclear translocation (Kishimoto et al., 2014), on GSC tumorsphere formation and found that sphere formation was decreased by 86% in the presence of 1 mM neamine (**Figure 3.4., C and D**). Taken together, these data demonstrate that ANG regulates GSC tumorsphere formation and clonal growth in a mechanism involving binding to cell surface receptor, nuclear translocation process, and ribonucleolytic events.

3.3 Chapter contributions

Patient-derived GSCs were kindly provided by Dr. Hiroaki Wakimono (Massachusetts General Hospital) and Dr. Brent Cochran (Tufts University).

Experiments in this chapter were performed by Liang Yuan and supervised by Dr. Guo-fu Hu with intellectual assistance from thesis committee members, Dr. Brent Cochran and Dr. Daniel Jay.

Chapter 4: PLXNB2 mediates the functions of ANG in regulating GSCs

4.1 PLXNB2 regulated stem cell properties of patient-derived GSCs

PLXNB2 is the cell surface receptor of ANG (Yu et al., 2017). It is a transmembrane protein originally identified from malignant brain tumors by differential screening (Shinoura et al., 1995) and is known to play a role in neural development (Chen et al., 2012a; Daviaud et al., 2016; Deng et al., 2007; Friedel et al., 2007) and invasive growth of GBM (Le et al., 2015). *PLXNB2* is upregulated in GBM (Le et al., 2015; Shinoura et al., 1995) (**Figure 1.4.A**) and its expression is inversely correlated with patients' survival (**Figure 1.4.B**). *PLXNB2* was recently reported to be a modulator of cell biomechanics. In GBM, *PLXNB2* facilitates diffuse infiltration through influencing cell-cell adhesiveness and durotaxis behaviors of invading cells. This process was found to be triggered upon the binding of SEMA4C that is known to activate the RhoA pathway through the RAS-GAP domain of *PLXNB2* (Huang et al., 2021).

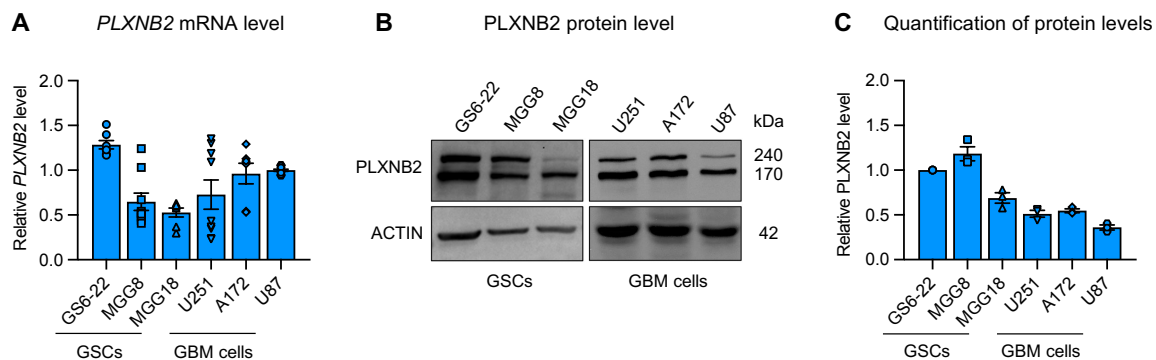


Figure 4.1. *PLXNB2* mRNA and protein levels in GSCs and GBM cells.

(A) *PLXNB2* mRNA levels in GSCs (GS6-22, MGG8, and MGG18) and GBM cells (U251, A172, and U87). mRNA levels were determined by qRT-PCR analysis with *GAPDH* as an internal control (n = 3 - 5). Bar graphs are relative values to the *PLXNB2* mRNA level in GS6-22.

(B-C) *PLXNB2* protein levels in GSCs and GBM cells. B, *PLXNB2* protein levels were determined by immunoblotting, and actin was used as the loading control in the blot; C, relative *PLXNB2* protein levels were determined by ImageJ and normalized to GS6-22.

PLXNB2 was identified as the ANG receptor that is both necessary and sufficient to mediate physiological and pathological functions of ANG (Yu et al., 2017). We have

recently reported that the ANG-PLXNB2 axis promotes GBM progression by enhancing invasion, vascular association, proliferation, and survival of GBM cells (Yang et al., 2022). However, it is unknown if PLXNB2 mediates the function of ANG in regulating GSC properties. To understand the contribution of PLXNB2 in GSC regulation, we set out to examine the consequence of inhibiting PLXNB2 or manipulating its expression in GSCs. First, we examined PLXNB2 expression and found that both PLXNB2 mRNA (**Figure 4.1.A**) and protein (**Figure 4.1., B and C**) were abundantly expressed in GSCs and GBM cell lines. Next, we examined the effect of PLXNB2 mAbs on GSC tumorspheres and found that treatment of GSCs with a pool of three different PLXNB2 mAbs (60 µg/ml) decreased the number of tumorspheres by 25.1% (from 120 ± 6 to 90 ± 5 per 1000 cells) in GS6-22 and by 23.0% (from 33 ± 2 to 25 ± 2 per 1000 cells) in MGG8 (**Figure 4.2., A and B**) without significant effect of sphere diameters (**Figure 4.2.C**), resembling the effect of treatment with ANG mAbs (**Figure 3.3.**). The three mAbs used in this study including mAb17, mAb45, and mAb100, were generated through immunization with a synthetic peptide of the ANG-binding site of PLXNB2 (Yu et al., 2017), HEK293 cells expressing the full-length of human *PLXNB2*, and the extracellular domain of human PLXNB2 (unpublished), respectively. Except mAb17, the epitopes of mAb45 and mAb100 were unknown presently. However, all three are known to bind to PLXNB2 both in immunoblotting and in flow cytometry analysis (unpublished), and each of them individually was able to inhibit tumorsphere formation of GS6-22 cells with comparable activities between each other (**Figure 4.2.D**). Again, no difference on sphere diameters was detected, indicating a lack of effect on clonal growth of GSCs (**Figure 4.2.E**). In addition to the mesenchymal subtype GS6-22 and pro-neural subtype MGG8, we also examined the effect of PLXNB2 mAbs on MGG18, also a mesenchymal subtype of GSC that harbors *PTEN* and *CDKN2A* deletion. MGG18 is highly resistant to TMZ even though it does not express MGMT. It can give rise to orthotopic tumors in mice and

these xenograft tumors recapitulate the phenotypic and genotypic features of the original tumor, a giant cell GBM (Wakimoto et al., 2012). MGG18 cell do not form typical tumorspheres (**Figure 1.6.**) and grow as loosely associated clusters under sphere culture conditions (bFGF + EGF + B27 in non-adherent dishes).

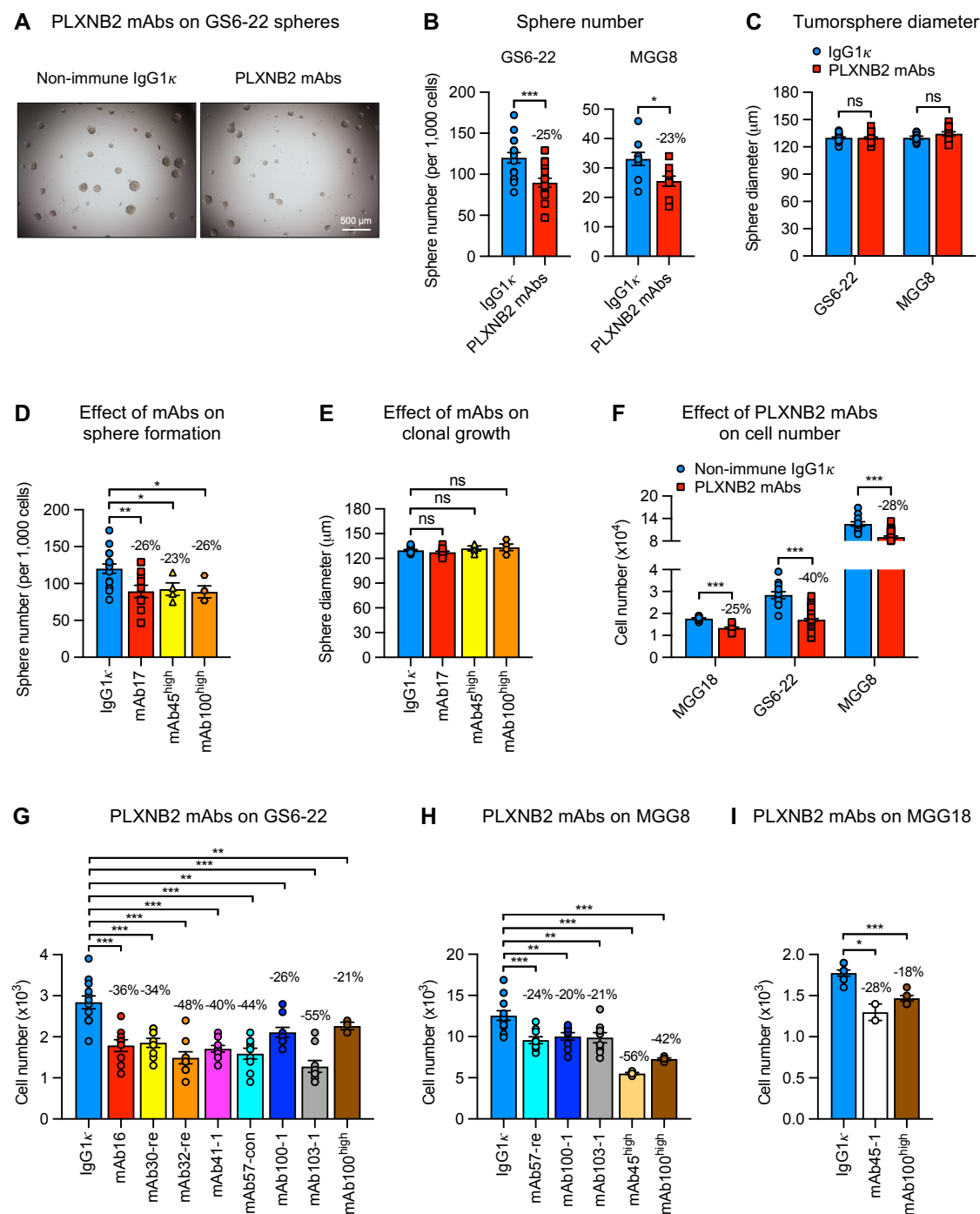


Figure 4.2. PLXNB2 mAbs inhibited GSC tumorsphere formation and decreased proliferation of self-renewable cells.

(A-C) Tumorsphere formation of GS6-22 and MGG8 plated at 1,000 cells per well of 96-well plate treated with 60 µg/ml PLXNB2 mAb cocktail or a non-immune isotype IgG1-κ for 7 days (n = 3 - 4). A, representative images; B, tumorsphere number; C, tumorsphere diameter.

(D-E) Tumorsphere number (D) and diameter (E) of GS6-22 treated with different PLXNB2 mAbs (mAb17, mAb45, or mAb100).

(F) Total cell numbers of GS6-22 (n = 8), MGG8 (n = 5), and MGG18 (n = 8) seeded at 5,000 per well of 96-well plate, treated by 60 µg/ml PLXNB2 mAbs or non-immune isotype IgG1-κ for 7 (GS6-22 and MGG8) or 10 (MGG18) days under undifferentiated culture conditions.

(G-I) Effects of different PLXNB2 mAbs on the proliferation of undifferentiated GS6-22 (G), MGG8 (H), and MGG18 (I). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

Since sphere counting and measurement are technically infeasible, we adapted the total numbers of undifferentiated GSCs as a readout and examined the effect of PLXNB2 mAbs on the proliferation of MGG18 as well as GS6-22 and MGG8, all cultured under undifferentiated conditions. Figure 4.2.F shows that treatment with PLXNB2 mAbs (60 µg/ml) led to a reduction in the total number of MGG18, GS6-22, and MGG8 by 25%, 40%, and 28%, respectively, as compared to treatment with the same concentration of an isotype IgG control. We have also examined the activity of individual PLXNB2 mAbs on proliferation of these three GSC lines cultured under undifferentiated conditions. A total of 8 different mAb clones were tested with GS6-22 (**Figure 4.2.G**), 5 were tested with MGG8 (**Figure 4.2.H**), and 2 were tested in MGG18 (**Figure 4.2.I**). Treatment with these different clones of PLXNB2 mAbs, resulted in a decrease of total cell numbers by 21 - 55% in GS6-22 (**Figure 4.2.G**), 21 - 56% in MGG8 (**Figure 4.2.H**), and 18 - 28% in MGG18 (**Figure 4.2.I**).

Taken together, these results demonstrate that treatment with PLXNB2 mAbs decreased sphere-forming capacity of GSCs resulting in a reduction of self-renewable cell numbers, implicating a role of PLXNB2 in GSC stemness.

4.2 Establishment of *PLXNB2* knockout GSCs by CRISPR/Cas9 gene editing

To unequivocally determine the role of PLXNB2 in GSC regulation, we set out to generate *PLXNB2* knockout (KO) GSCs using CRISPR/Cas9 gene editing technology.

The Cas9 targeting region and the surrounding sequence of *PLXNB2* is shown in Figure

4.3. Successful editing at this site, indicated in black (**Figure 4.3.**), will introduce a frameshift in the coding region and resulted in gene inactivation.

Part of *PLXNB2* coding region PAM TARGET SEQUENCE CLEAVAGE SITE

ATGTCCTGTCTCTGATGAGTTGGTGGTTCTGCTGTAGCCATCGGCCTCGTGTGCCCGAGCGCTGGCGGTGCCACCCCTCCCACAGGAGGTCC
TCAGGAAGCACGGAGACATCGAGAATCTCGATGCTGGCCTGGAGCAGAAACTGAGCTGGACCACTGGAGCCTCGGTGAGGGGGTGCGGGGGGCA
ATGGCACTGACGCTCTGGCCGCTGACCTCTGCTGGGCTGCTGGGCGCAGGTGCCAGCTCGAGGCCCGCAGGCTGAACCTCTTCGCGACGAGGA
AAGAGCTGAACCACTGGCTGTGGATGAGGCCTCAGGCGTGTGTACTCTGGGCGGGTGAATGCCCTTACCAGCTGGATGCGAAGCTGCAGCT
GGAGCAGCAGGTGGCCACGGCCCGCCCTGGACAACAAGAAAGTGCACGCCGCCATCGAGGCCAGCCAGTGCCTAGAGGCTGAGATGACGTGA
CAATGTCAACCACTGCTGCTGCTCGACCTCCCAGGAAGCGCTGTGGAGTCGCGCAGCTCTTCAAGGGCATCTGCGCTCTGCGGCCCTGA
GCAACATCTCCCTCGCGCTGTTTACGAGGACGGCAGCGGGGAGAAGTCTTCTGTCGCCAGCAATGATAGGGCGTGGCCACAGTGGGGCTGCT
GAGCTCCACGGTCTGGTGGTGACCGCTGCTGTTTGGGGCAAGGCAATGGGCCACAGCACAAGCCGATCATCTGTGAGCATCTGGCTGTGGT
ACCGCACTGACAGCAGGAGGCTTTGAAGCCTACAGCGGACCGCCACTCAAGCCGCGGTACTCTGCCAACACACAGCAAGATCTGTGGCG
GCCCTCGAGGACGCGCCCTACGCTCTTCTTTGCTTCAACAGCAGGACAAGCACCAGCCGCGGGAACCCGACAGCTGTGCTGGCAGCATGTGCAGAGA
AGACCCCACTACTACTCTCTGAGATGGACCTGCAGTCCGGGACCCCGACTCACCGCGCTGCCTTTGGCACTGCTGCGCCCTCCG
TGGCTGCGCTGGCTCTGGCAGGGTGCTATATGCTGTCTTCAGACAGACAGCCGAGCAGTGGGGGGCCGGTGGCGGCCTGTGCCTGTTCCG
GCTGACAAGCTGACCGCAAGTAGGAGCGCAACCGCAACCGCTGTACACAGCACCGGAGCCCGGTGACATCTTCTACAAGCCCTTCCACG
GCGATATCAAGTGGCGCGGCCACGCGCGGGCTCAGCAAGAGCTTCCATGTGGCTCGAGACCTGCCCTACCGCTGGGACGCGCGCAG
GGCTCAGAGGCACAGCCGTGCTGCAGCGTGGAGGCCCTGAACCTACCGG**CCGTGACGTCGCCCGCCGAGAAC**CAACCCACAGCTGTGCTTTTCTGGG
CACCTCTGATGGCCGGATCTCAAGGTGTACCTCACCCAGATGGCACTCTCAGATACGCTCTATGCTTGTGGAGATAAACAGAGACTCA
GCGCGACCTGGTACTGTCTGAGAGACTGGGACGCTGTACGCCATACCCAGGACAAGTGTTCCGGCTGCGGTGTCAGGAGTGCTGAGTCA
CCGACGTGACCCAGTCCGCGCACTCCGAGGACCCCTACCTCGGCTGGTGGCTGCTGTCGAGGAGCAGTACGCCGGAGGCCGATGTCGCGG
GCCGAGGAGCCAGCACTGCTGTGTGAGGCGAAGCAAGTCTGCTGGTGGCTCACCACGCGCCACGCCAAGAACATGAGCGCGCGGCCAG
GGGGAGGTGCAGCTGACCGTCAAGCCCTCCTCGCTGCCCTGAGCAGGAGGAGGACAGTTGCTGTGCCTTTTGGGGAGTCGCGGCCACACCCGCC
CGCTGGAGGGCGAGGCCGTACTGCAACTCCCCAAGCACTCCCGCTACACCGCCAGGCCAGTGGCGGTGACCATCCAGCTCT
CCTTAGCAGGAGCAACATCTTCTCAGTCTACAGACTACCCCTCTACAGACTGCGCCAGGCCATGAGCTGAGGAGAACCTGGCTGCACTCT
CTCGGTGAGCAACCGCTGGAACCTGCAGTGGGACCTGCCTACCAAGAGTGGCGGAGGCTCGCCCAACCTGAGGACGGCATGCTCGCTGCC
CATACTGGAGGACAGCTGTGCCAGTTCTCTGGGACCGACGCCCTGGTGAATCCCATGAACCAAGACAGATGTGAACCTCCAGGGCAAG

Figure 4.3. Schematic representation of *PLXNB2* gene targeting by CRISPR/Cas9 gene editing strategy.

Yellow highlights for *PLXNB2* coding region (part); blue highlights for protospacer adjacent motif (PAM); target sequence (on the first coding exon of *PLXNB2*) is shown in bold red font; black highlights for Cas9 cleavage site.

Plasmid-based transfection was used in GS6-22 and lentiviral-based apparatus was used in MGG8 and MGG18 to deliver single guide RNA (sgRNA) and Cas9 into the cells. Multiple *PLXNB2* KO clones were established for GS6-22, MGG8, and MGG18 cells. These single clones, as well as pooled populations, were used in this study to avoid clonal peculiarity. Genomic DNA sequencing analysis confirmed the site-specific modification of *PLXNB2* gene (**Figure 4.4.A**). A complete loss of PLXNB2 protein expression was validated by immunoblotting and immunofluorescence analysis (**Figure 4.4., B, C, and D**).

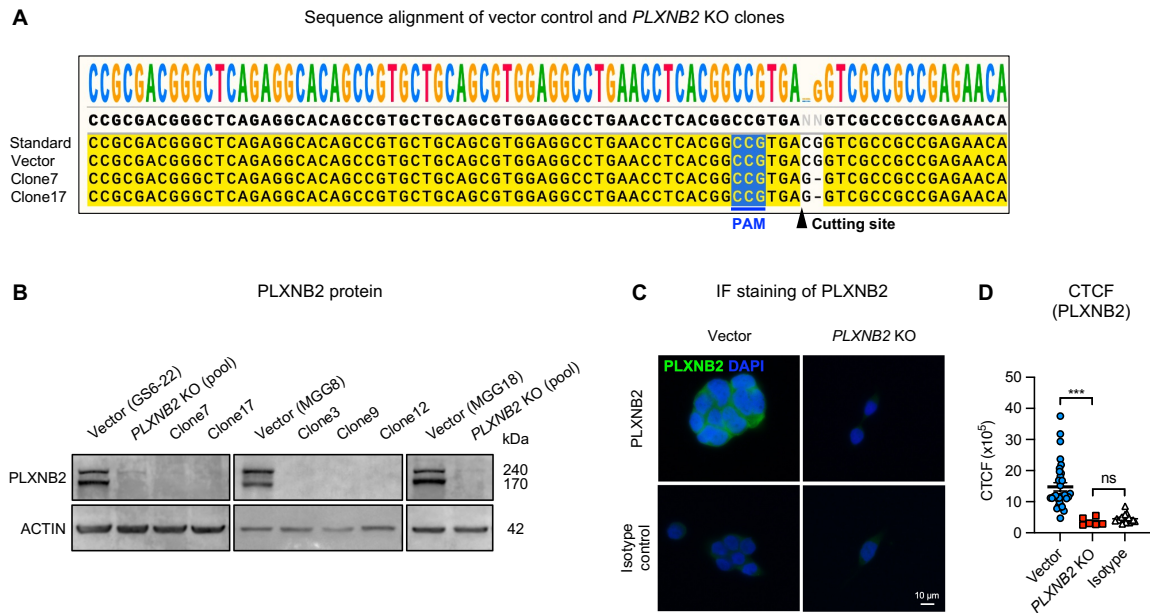


Figure 4.4. Validation of *PLXNB2* knockout in GSCs at genomic and protein levels.

(A) Cas9-editing region sequence of *PLXNB2* from vector control, *PLXNB2* knockout clones 7 and 17 aligned to the genome sequence of *PLXNB2* at this region. PAM is highlighted in blue color and Cas9 cutting site marked by black arrow. Sequence alignment was performed by SnapGene viewer software.

(B) Immunoblotting of PLXNB2 protein in transfection vector GSCs, *PLXNB2* knockout pooled populations, and *PLXNB2* knockout clones of GS6-22, MGG8 and MGG18. Actin was used as the loading control.

(C) Immunofluorescence staining of PLXNB2 protein in vector control and *PLXNB2* knockout MGG18 cells. PLXNB2 and nuclei were stained by mAb100 and DAPI. Scale bar, 10 μ m.

(D) The corrected total cell fluorescence (CTCF) was calculated using ImageJ software. The number of cells analyzed is 32 (vector control), 6 (*PLXNB2* knockout) and 18 (isotype control), respectively.

*** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

4.3 *PLXNB2* ablation abolished ANG cellular uptake, decreased GSC self-renewal, and accelerated stem cell exhaustion

PLXNB2 KO markedly impaired tumorsphere formation in all three GSC lines (Figure 4.5. A, E, I). In GS6-22 cells, two individual KO clones and a pooled population were tested. The number of tumorspheres formed from 1,000 cells of clone 7, clone 17, and the pooled population were 36 ± 4 , 31 ± 3 , and 31 ± 5 , respectively, representing

73.6%, 77.4%, and 77.1% decrease as compared to that formed from the same number of vector control (136 ± 5) (**Figure 4.5.B**). The size of the tumorspheres was also significantly decreased upon *PLXNB2* KO as indicated by diameters of the spheres, which was $139 \pm 1 \mu\text{m}$ in the vector control and was $123 \pm 3 \mu\text{m}$ (-12%) in clone 7, $128 \pm 1 \mu\text{m}$ (-8%) in clone 17, and $122 \pm 2 \mu\text{m}$ (-12%) in the pooled population (**Figure 4.5.C**).

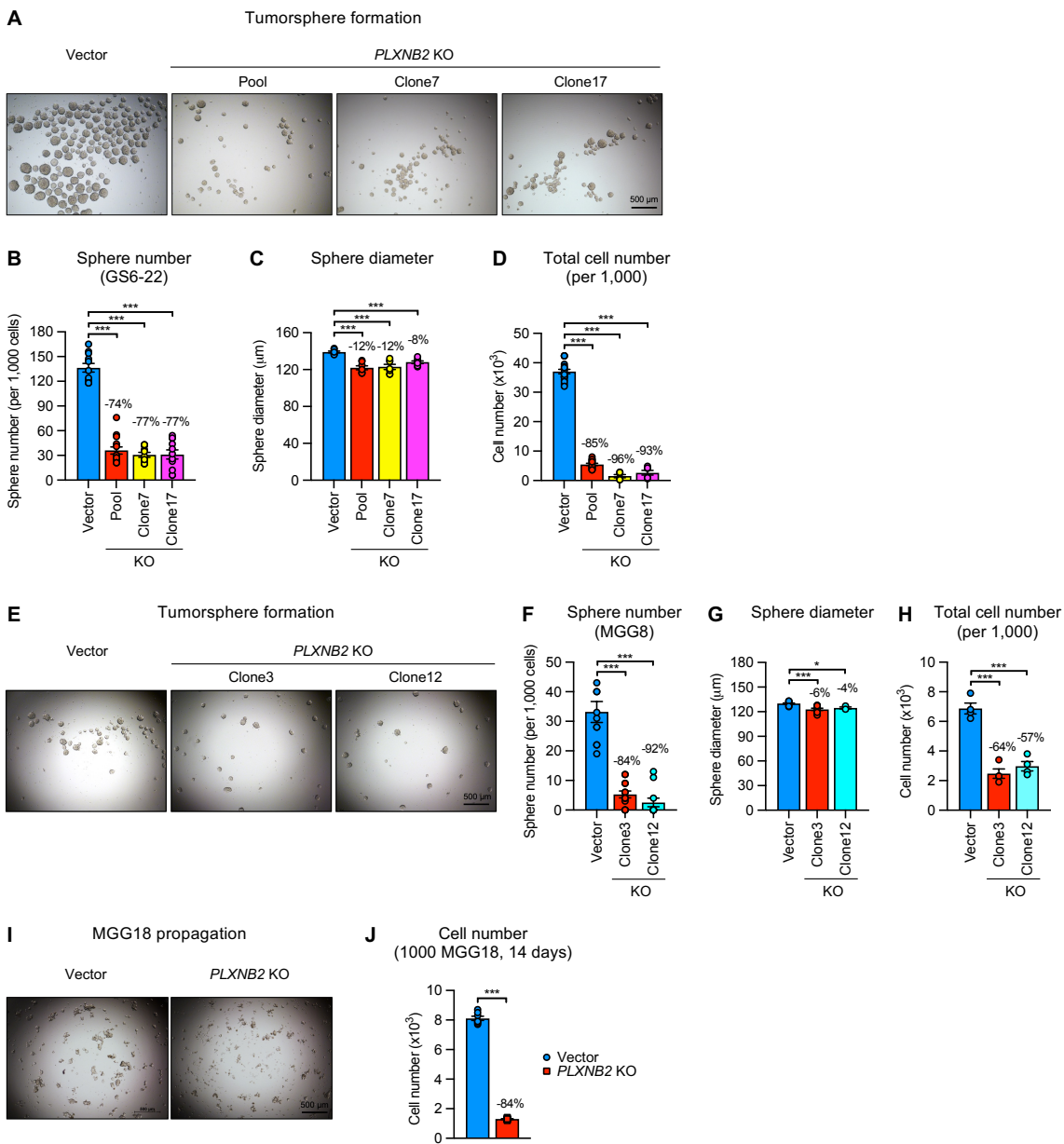


Figure 4.5. *PLXNB2* knockout drastically attenuated GSC tumorsphere formation and cell proliferation.

(A) Representative tumorsphere images of GS6-22 transduction vector control GSCs, *PLXNB2* knockout pooled population, clone 7, and clone 17. Fully-dissociated cells were plated at 1,000 cells per well of 24-well plate for 7 days. Scale bar, 500 μ m.

(B-C) Analysis of tumorsphere number (B) and tumorsphere diameter (C) (n = 3).

(D) Cell number analysis (n = 3). The number of cells was manually counted after tumorsphere dissociation.

(E-G) Tumorsphere formation of MGG8 transfection vector control GSCs and *PLXNB2* KO clone 3 and 12 at 1,000 cells per well of 96-well plate for 7 days (n = 3). E, representative images; F, tumorsphere number; G, tumorsphere diameter. Scale bar, 500 μ m.

(H) Cell number analysis (n = 3 - 11). The number of cells was manually counted after tumorsphere dissociation.

(I-J) Effects of *PLXNB2* knockout on MGG18 propagation. The total cell number derived from 1,000 MGG18 was analyzed by CellTiter-Glo[®] luminescent cell viability assay on day 14 (n = 7). I, representative images; J, cell number. Scale bar, 500 μ m.

p* < 0.05, **p* < 0.001, ns = not significant by unpaired two-tailed Student's *t* test.

A decrease in both number and size of the tumorspheres resulted in a drastic decrease in cell numbers of *PLXNB2* KO cells cultured under sphere-forming conditions. On day 7, tumorspheres were dissociated and total cell numbers were counted. Figure 4.5.D shows that 1,000 vector control GS6-22 gave rise to $37,011 \pm 754$ cells within 7 days, however, 1,000 pooled, clone 7, and clone 17 of *PLXNB2* KO cells gave rise to $5,492 \pm 1,435$, $1,508 \pm 523$, and $2,663 \pm 794$, respectively, in the same time period, representing an 85, 96, and 93% decrease in progenies (**Figure 4.5.D**). In other word, loss of *PLXNB2* expression decreased the self-renewal activities of GS6-22 by 7- to 25-fold. Similar results were obtained with MGG8 and MGG18 cells. Two individual clones were selected and analyzed in MGG8. As shown in Figure 4.5.E-H, *PLXNB2* KO in MGG8 decreased tumorsphere numbers by 84 - 92% (**Figure 4.5.F**), sphere diameters by 4 - 6% (**Figure 4.5.G**), which resulted in an overall reduction of cell numbers by 57 - 64% (**Figure 4.5.H**). With MGG18, only pooled population of *PLXNB2* KO cells were analyzed as these GSCs do not form typical tumorspheres (**Figure 4.5.I**). Figure 4.5.J shows that loss of *PLXNB2* expression decreased proliferation of undifferentiated MGG18 cells by 6-fold.

PLXNB2 is the cell surface receptor of ANG that mediates internalization of ANG, an essential step for ANG to elicit its biological functions in a variety of cell types (Yu et al., 2017). We thus examined the effect of *PLXNB2* KO on cellular uptake of ANG as well as the activity of ANG in promoting tumorsphere formation. Immunofluorescence staining with the human ANG-specific polyclonal antibody R113 revealed that addition of exogenous ANG significantly increased cellular ANG in vector controls of both GS6-22 (**Figure 4.6.A**) and MGG18 (**Figure 4.6.C**) cells (top 2nd panels) but not in *PLXNB2* KO cells (**Figure 4.6., A and C**, top 4th panels), suggesting that PLXNB2 mediates ANG uptake in GSCs. In the vector control cells, exogenous ANG was found to be co-localized with Fibrillarin that was stained to locate nucleolus (**Figure 4.6., A and C**, bottom 2nd panels), indicating that exogenously added ANG underwent nuclear translocation and accumulated in the nucleolus. The finding that exogenous ANG was accumulated in the nucleolus is unique to GSCs as previous reports have shown that ANG was translocated to stress granules rather than to the nucleus in primitive HSPCs (Goncalves et al., 2016) and in prostate CSCs (Li et al., 2020). The different subcellular localization of ANG in GSCs as compared to HSPCs and prostate CSCs could reflect intrinsic unique properties of GSCs. One plausible explanation could be that the GSCs used in this study were not in their most primitive state (quiescent CSCs) and were CSCs of the proliferating pool, a status resembling progenitor cells. Indeed, ANG was reported to undergo nuclear translocation in myeloid progenitor cells in the hematopoietic lineage (Goncalves et al., 2016). No matter what the actual cause for nuclear translocation of ANG in GSCs, these results are consistent with the finding that ANG R33A variant, which is defective in nuclear translocation, failed to promote tumorsphere formation (**Figure 3.4.A**). It is also notable that endogenous ANG was detectable, mainly in the cytoplasm with minor nuclear staining, of both vector control (**Figure 4.6., A and C**, top 1st panel) and *PLXNB2* KO cells (**Figure 4.6. A and C**, top 3rd

panel). These fluorescence signals could result from the staining of newly synthesized ANG that is one the way of being secreted. It could also be that cells could internalize minor amount of ANG in a *PLXNB2*-independent manner.

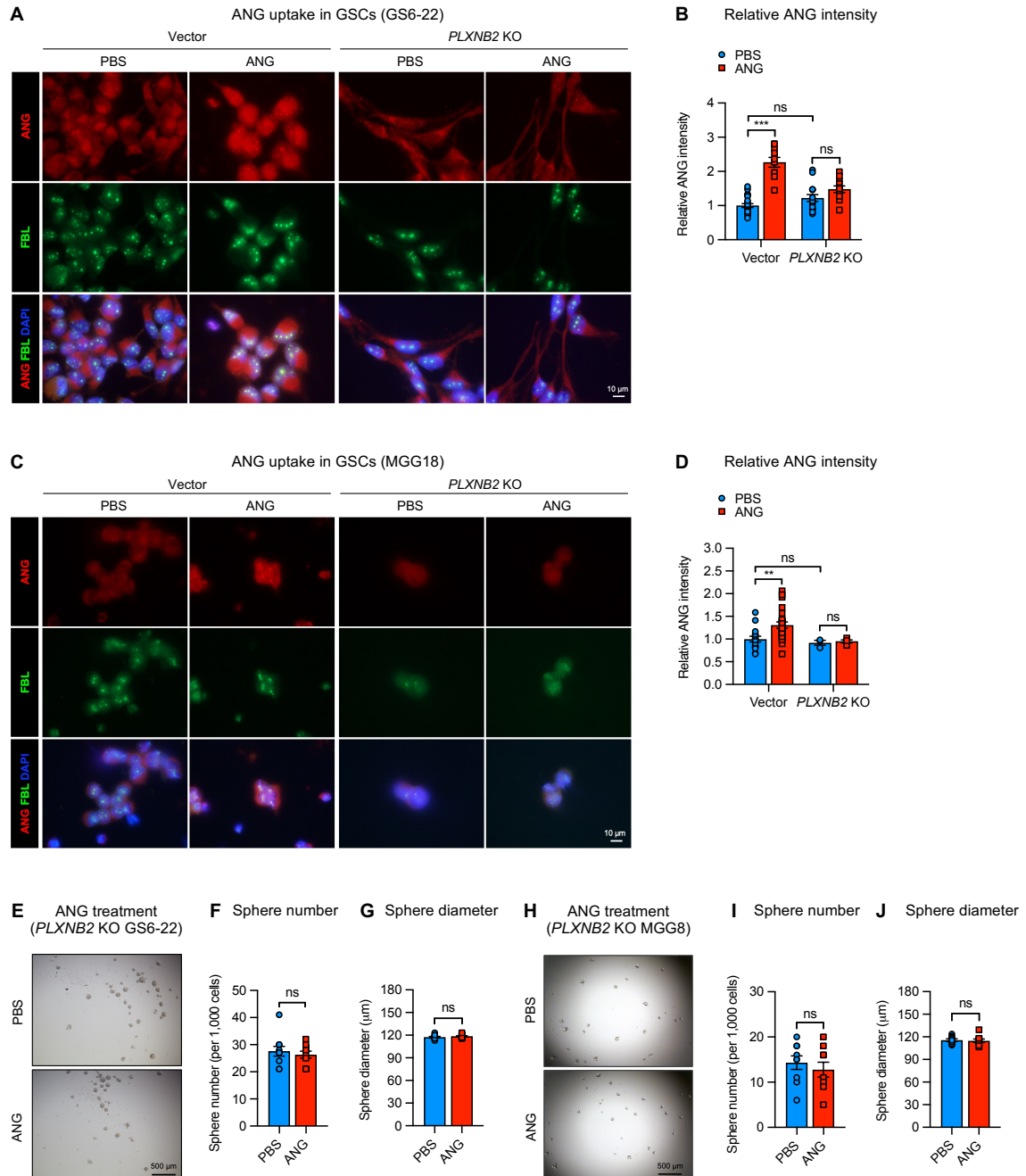


Figure 4.6. *PLXNB2* ablation abolished ANG cellular uptake as well as ANG-stimulated tumorsphere formation.

(A) Immunofluorescence staining of ANG in transduction vector control and *PLXNB2* knockout GS6-22 cells. Cells were grown on poly-D-lysine coated coverslip with stem

cell media for 5 days. For exogenous ANG treatment group, cells were incubated with 1 $\mu\text{g/ml}$ ANG for 30 mins and then fixed with ice cold methanol, blocked, and stained with anti-human ANG antibody (R113) and anti-human Fibrillarin antibody. Nuclei were stained by DAPI. Scale bar, 10 μm .

(B) The CTCF for ANG was calculated using ImageJ and presented as the relative value normalized to PBS-treated vector control cells. The number of cells analyzed was 21 (vector + PBS), 16 (*PB2KO* + PBS), 10 (vector + ANG), and 11 (*PB2KO*+ANG).

(C) Immunofluorescence staining of ANG in MGG18 vector control and *PLXNB2* knockout cells. Scale bar, 10 μm .

(D) The CTCF for ANG was presented as the relative value normalized to PBS-treated vector cells. The number of cells analyzed was 16 (vector + PBS), 3 (*PB2 KO* + PBS), 24 (vector + ANG), and 4 (*PB2 KO* + ANG).

(E-G) Tumorsphere formation of 1,000 *PLXNB2* knockout GS6-22 cells treated with 1 $\mu\text{g/ml}$ ANG or PBS for day 7 ($n = 3$). E, representative images; F, tumorsphere number; G, tumorsphere diameter. Scale bar, 500 μm .

(H-J) Tumorsphere formation of 1,000 *PLXNB2* knockout MGG8 cells treated by 1 $\mu\text{g/ml}$ ANG or PBS for day 7 ($n = 3$). E, representative images; F, tumorsphere number; G, tumorsphere diameter. Scale bar, 500 μm .

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

Consistent with the finding that *PLXNB2* KO impaired cellular uptake of ANG in GSCs, ANG no longer promoted tumorsphere formation of *PLXNB2* KO cells. The number and the size of tumorspheres derived from *PLXNB2* KO GS6-22 (**Figure 4.6., E-G**) and MGG18 (**Figure 4.6., H-J**), in the presence and absence of ANG were essentially the same, confirming the role of *PLXNB2* in mediating the function of ANG in regulating GSC properties.

After establishing the role of *PLXNB2* on GSC regulation, we next assessed the effect of *PLXNB2* KO on stem cell frequency. Extreme limiting dilution analysis (ELDA) was used for this purpose (**Figure 4.7.A**), which has been widely used and shown to be a reliable method to determine how much real stem cells exist in a given population (Hu and Smyth, 2009). As shown in Figure 4.7.B, the seeding density required for forming true tumorspheres was significantly higher for *PLXNB2* KO cells as compared to those of the corresponding controls in all three GSC lines. The stem cell frequency was calculated according to an online ELDA software at

<https://bioinf.wehi.edu.au/software/elda/index.html> and shown to be 22.5 ± 2.6 , 5.8 ± 0.2 ,

and 2.1 ± 0.3 in the vector control cells of GS6-22, MGG8, and MGG18, respectively, which were reduced to 1.7 ± 0.5 , 0.9 ± 0.2 , and 0.7 ± 0.2 , respectively, in the corresponding *PLXNB2* KO cells, representing a decrease by 92.4, 83.1, and 66.7% upon *PLXNB2* ablation in the three GSC lines, respectively (**Figure 4.7.C**).

Determination of a true stem cell frequency in MGG18 cells was not realistic as they do not form typical spheres as mentioned above. The numbers shown in Figure 4.7.B reflect the best estimation of appearance of a sphere-like structure in the well after 14 days of culture.

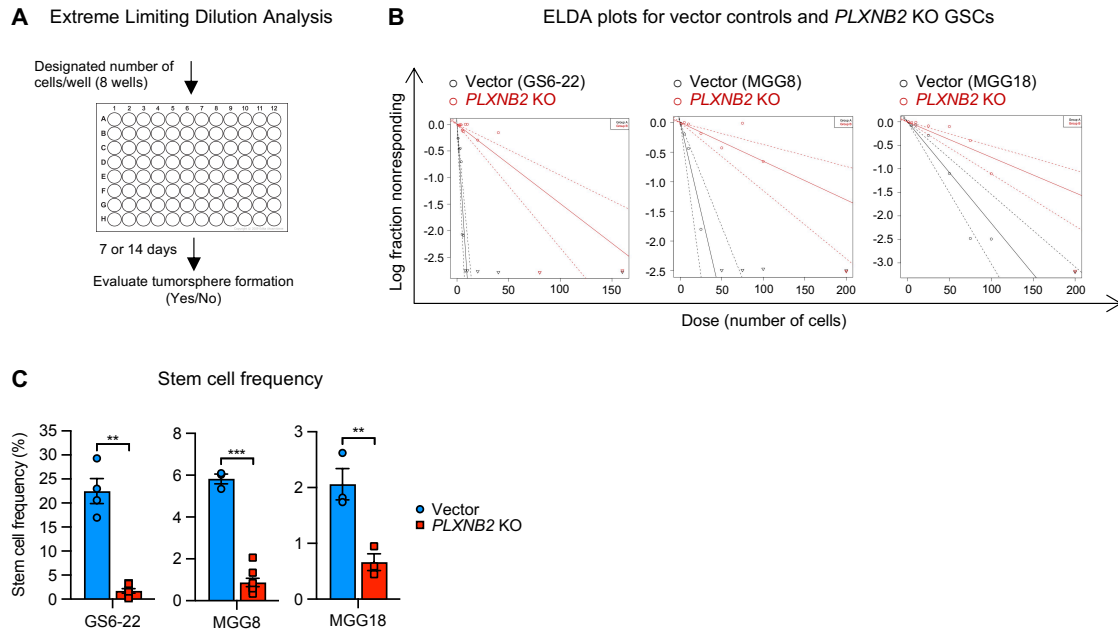


Figure 4.7. *PLXNB2* ablation decreased stem cell frequency.

(A) A schematic diagram for ELDA. Transduction vector control and *PLXNB2* KO cells of GS6-22, MGG8 and MGG18 were seeded at designated number of single cell suspension in sphere media in wells of 96-well plate (8 replicates) for designated number of days ($n = 3 - 4$). On the termination day, only spheres with diameter $\geq 100 \mu\text{m}$ are considered as tumorspheres. Seeding density and incubation time for GS6-22: 160, 80, 40, 20, 10, 8, 6, 5, 4, 3, 2, 1 cell per well for 7 days; MGG8: 200, 100, 75, 50, 20, 10, 5, 1 cell per well for 7 days; MGG18: 200, 100, 75, 50, 20, 10, 5, 1 cell per well for 14 days. Given MGG18 cannot propagate as organized tumorspheres, the diameter of MGG18 cluster was used for analysis.

(B) Representative ELDA plots for transduction vector control and *PLXNB2* KO cells of GS6-22, MGG8 and MGG18. Dotted lines represent the 95% confidence intervals. Plots were generated by <http://bioinf.wehi.edu.au/software/elda/>.

(C) Quantification of stem cell frequency for transduction vector control and *PLXNB2* KO cells of GS6-22, MGG8 and MGG18 ($n = 3 - 4$).

A decrease in stem cell frequency upon *PLXNB2* KO was accompanied by a faster stem cell exhaustion as determined by *in vitro* serial passaging experiments. As shown in Figure 4.8.A, 1,000 vector control GS6-22 were able to give rise to a decent number of tumorspheres up to seven passages (142 ± 6 , 152 ± 4 , 66 ± 5 , 67 ± 4 , 52 ± 3 , 55 ± 3 , 50 ± 3 for passage 1 to 7, respectively). A marked reduction in sphere number was found between passages 2 and 3 for unknown reason, but the sphere-forming ability was not significantly changed between passages 3 and 7. At passage 7, there were still 50 spheres formed from 1,000 cells, indicating 35.2% perseverance of stem cell number as compared to the 142 spheres formed from the same number of seeding cells at passage 1. In contrast, *PLXNB2* KO clones displayed rapid and progressive decrease of sphere-forming abilities during serial passaging, and a complete loss after passage 3 for clone 7 and after passage 4 for clone 17 (**Figure 4.8.A**). It is also notable that the size of tumorspheres formed from vector control GSCs remained unchanged along serial passaging whereas those formed from *PLXNB2* KO clones progressively decreased along passaging (**Figure 4.8.B**). It has been reported that only self-renewing cells can maintain sphere-forming potentials in multiple generation (Qin et al., 2012). We thus calculated the theoretical number of total self-renewing GSCs derived from 1,000 vector control, *PLXNB2* KO clone 7 and clone 17 of GS6-22 (**Figure 4.8.C**). A total of 2.35×10^{10} self-renewing GSCs could have been propagated from 1,000 vector control GS6-22 in 49 days after 7 serial passages, however, all stem cells in clone 7 and clone 17 would have exhausted after passage 3 and 4. The peak numbers of self-renewing GSCs reached on day 7 (passage 1) in clone 7 were 1,508, and on day 14 (passage 2) in clone 17 were 9,588, respectively, which was 25- and 157-fold lower as compared to 37,300 (passage 1) and 1,509,407 (passage 2) in the vector control within the same time period at the corresponding passage number. These results demonstrate that *PLXNB2* ablation not only decreases stem cell frequency, but also accelerates GSC exhaustion.

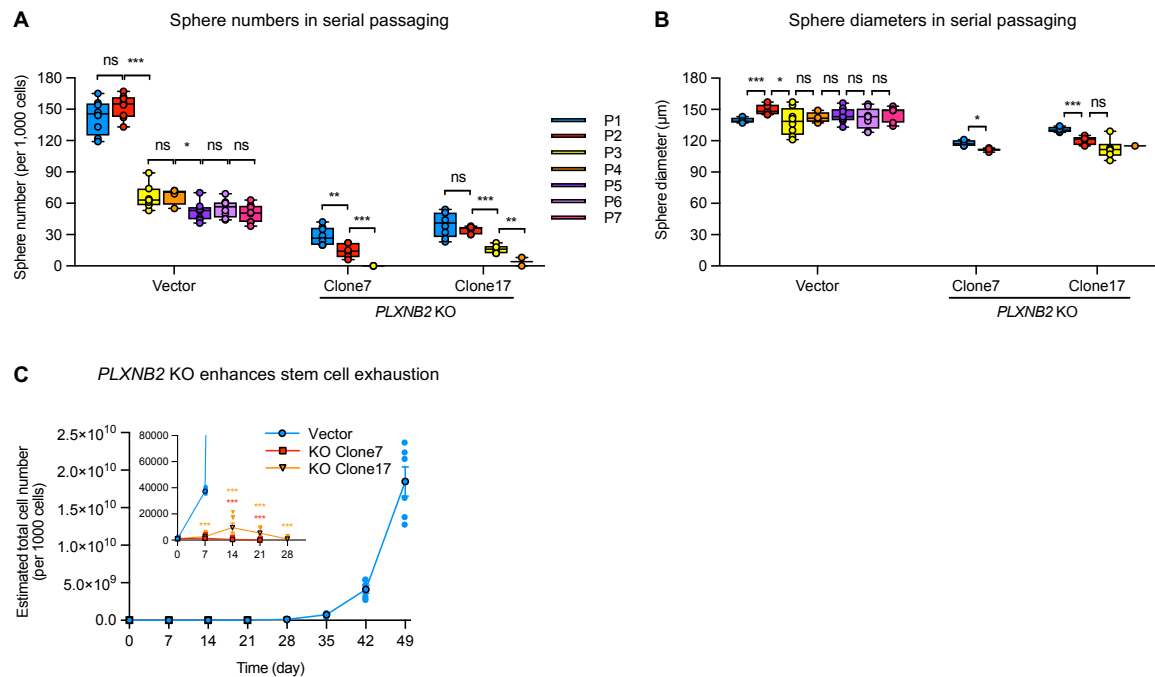


Figure 4.8. *PLXNB2* ablation accelerated GSC exhaustion.

(A-B) Effect of *PLXNB2* knockout on GSC tumorsphere formation along serial passinging (7 passages total). GS6-22 transduction vector control and *PLXNB2* KO clones (7 and 17) were plated at density of 1,000 per well in sphere media for 7 days/per passage (n = 3). A, tumorsphere number; B, tumorsphere diameter.

(C) The estimated numbers of cells risen from 1,000 vector control, *PLXNB2* KO clone 7, and clone 17 along serial passinging. The inset shows the estimated cell numbers from day 0 to 28. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

Together, these data provide a feasibility to target ANG/*PLXNB2* axis to abolish ANG cellular uptake, suppress GSC self-renewal, and accelerate stem cell exhaustion.

4.4 *PLXNB2* inhibition attenuated GSC adherent growth of GSCs and promoted a neuronal differentiation-associated transcriptional profile

In order to understand how *PLXNB2* ablation leads to decrease of stem cell frequency and acceleration of stem cell exhaustion, we performed a qRT-PCR array analysis of 40 genes that are commonly used to assess multipotent neural stem cell maintenance, proliferation, and differentiation. As shown in Table 4.1, the most

significantly differential expression between vector control and pooled population of *PLXNB2* KO GS6-22 cells were found in the class of stemness regulator and marker, such as *CDCP1*, *SOX2*, *NESTIN*, and *PROM1*, and in the class of differentiation genes, such as *NEUROD1*, *PAX6*, *FGFR4*, and *ASCL1*.

Table 4.1. qRT-PCR array analysis of human neural stem cell markers

Genes	Vector control	Pooled <i>PB2</i> KO	Fold Changes
	Cycle No.	Cycle No.	
<i>ABCG2</i>	30.77	25.61	33.97
<i>PROM1</i>	34.35	34.8	0.70
<i>GFAP</i>	26.46	26.93	0.69
<i>NUMB</i>	22.62	22.08	1.38
<i>RXRA</i>	22.55	22.22	1.19
<i>ASCL1</i>	30.14	29.35	1.64
<i>CDCP1</i>	29.62	31.48	0.26
<i>SLC2A1</i>	23.46	23.48	0.94
<i>OTX2</i>	31.15	32.82	0.30
<i>SOX1</i>	22.44	21.85	1.43
<i>CTNNB1</i>	20.43	20.29	1.05
<i>CTCR4</i>	30.56	30.84	0.78
<i>ID2</i>	21.7	21.17	1.37
<i>PAX3</i>	28.11	29.65	0.33
<i>SOX2</i>	21.33	22.71	0.37
<i>BMI1</i>	22.34	21.78	1.40
<i>FABP7</i>	17.68	17.66	0.96
<i>MSI1</i>	25.59	25.09	1.34
<i>PAX6</i>	29.9	25.85	15.74
<i>SOX9</i>	19.43	18.85	1.42
<i>SMARCA4</i>	20.5	20.22	1.15
<i>FGFR4</i>	27.01	25.11	3.55
<i>NES</i>	29.71	30.8	0.45
<i>PDGFRA</i>	30.81	30.15	1.50
<i>MSX1</i>	21.85	22.51	0.60
<i>CDH2</i>	19.87	19.43	1.29
<i>FOXD3</i>	30.67	31.03	0.74
<i>NEUROD1</i>	29.68	24.35	38.21
<i>PRKCZ</i>	31.35	31.03	1.19
<i>TRAF4</i>	21.09	20.87	1.11
<i>CALCR</i>	35.89	34.1	3.29
<i>FZD9</i>	29.1	28.31	1.64
<i>NOG</i>	32.24	32.84	0.63
<i>ROR2</i>	35.52	36.41	0.51
<i>VIM</i>	13.14	13.84	0.58
<i>FUT4</i>	26.52	26.46	0.99
<i>GATA2</i>	28.51	27.64	1.74
<i>NOTCH1</i>	28.11	28.16	0.92
<i>RUNX1</i>	25.25	26.44	0.42
<i>ZIC1</i>	24.07	22.77	2.34
ScienCell Research Laboratories, GK113			

We therefore examined the effect of *PLXNB2* ablation on differentiation- or stemness-related genes in all three GSC lines in a greater detail, including 19 differentiation transcription factors and markers (**Figure 4.9.A**), 13 stemness transcription factors and markers (**Figure 4.9.B**), and 11 cell death and cell cycle-related genes (**Figure 4.9.C**).

In the mesenchymal GS6-22 and MGG18 cells, *PLXNB2* ablation increased the expression of neuronal differentiation transcriptional factors and markers, including *NEUROD1*, *NGN1*, *NGN2*, *PAX3*, *PAX6*, *NKX2.2*, *VGLUT1*, *MYT1L*, *ASCL1*, and *MAP2* (**Figure 4.9.A**). In the proneural subtype of GSC MGG8, proneural development genes, such as *SOX2*, *DLL3*, *ASCL1*, *TCF4*, and oligodendrocytic development genes, such as *PDGFRA*, *NKX2.2*, *OLIG2* are already high (Kulkarni et al., 2018). *PLXNB2* ablation further elevated the transcription of *NGN2*, *NKX2.2*, and *DCX* (**Figure 4.9.A**). Analyses of self-renewal genes revealed a universal decrease of the transcript levels of this class of genes in all three GSC lines upon *PLXNB2* KO with the most significant changes observed in *CD44*, *NESTIN*, *CDCP1*, and *L1CAM* (**Figure 4.9.B**). In case of cell death and cell cycle regulators, *PLXNB2* KO significantly elevated expression of cell cycle inhibitors, such as *TP53* and *CDKN1A*, decrease of cell cycle promoters and pro-survival genes, such as *CCNE* and *XIAP* (**Figure 4.9.C**). We also performed cell cycle analyses to examine the cell cycle distribution of GS6-22 upon *PLXNB2* ablation. We found that *PLXNB2* loss did not affect the proportions of cells at G0/G1 and S phases (**Figure 4.9., D and E**). Instead, it remarkably decreased the proportion of cells at G2/M phase from 12% in *PLXNB2*-expressing cells to 5% in *PLXNB2* KO cells. At the same time, it largely increased the apoptotic cell proportion from 6% in *PLXNB2*-expressing cells to 12% in *PLXNB2* KO cells. This observation suggests that, upon *PLXNB2* ablation, most GS6-22 cells are unable to engage in proper cell division, instead, they undergo apoptosis. In sum, *PLXNB2* knockout promoted GSCs a pro-neuronal differentiation transcriptional

profile while suppressing the expression of stemness- and survival- associated regulators. *PLXNB2* loss promotes cell apoptosis, probably resulting from an impaired cell division following G2/M phase.

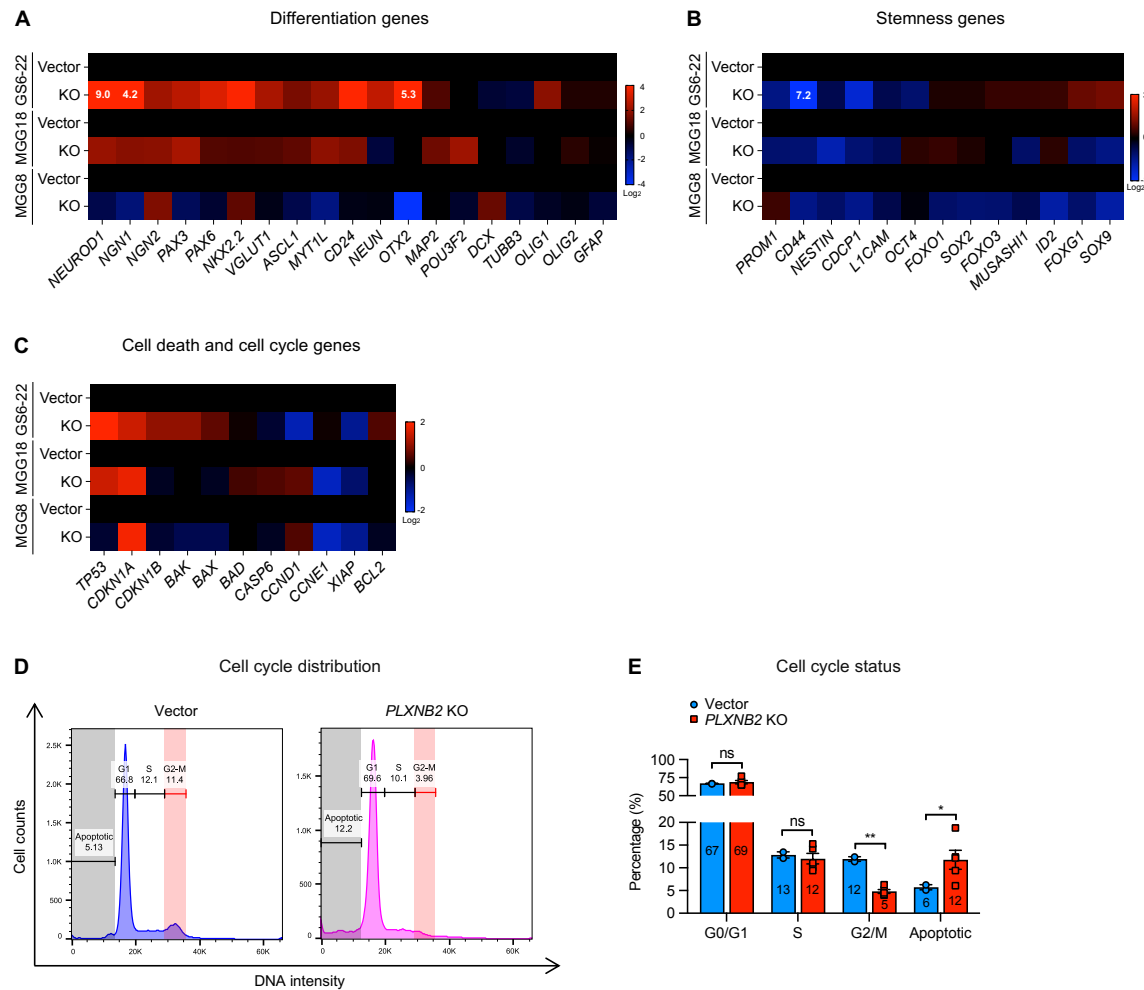


Figure 4.9. *PLXNB2* knockout promoted a neuronal differentiation-associated gene expression profile, attenuated stemness-associated gene expression, and altered the expression of cell cycle and cell death regulators.

(A) Heatmap showing the expression of neuronal (*NEUROD1*, *NGN1*, *NGN2*, *PAX3*, *PAX6*, *NKX2.2*, *VGLUT1*, *ASCL1*, *MYT1L*, *CD24*, *NEUN*, *OTX2*, *MAP2*, *POU3F2*, *DCX* and *TUBB3*), oligodendrocyte (*OLIG1*, *OLIG2*) and glial (*GFAP*) genes in vector control or *PLXNB2* knockout cells of GS6-22, MGG18 and MGG8 under sphere culture conditions. Expression levels were determined by qRT-PCR (n = 3). Heatmaps represent the relative mRNA level of each gene normalized to the value of the respective vector control cells.

(B) Heatmap showing the expression of stemness-associated genes (*PROM1*, *CD44*, *NESTIN*, *CDCP1*, *L1CAM*, *OCT4*, *FOXO1*, *SOX2*, *FOXO3*, *MUSASHI1*, *ID2*, *FOXG1*

and SOX9) in vector control or *PLXNB2* knockout cells of GS6-22, MGG18 and MGG8 under sphere culture conditions. Expression levels were determined by qRT-PCR (n = 3). Heatmaps represent the relative mRNA level of each gene normalized to the value of the respective vector control cells.

(C) Heatmap showing the expression of cell death/survival and cell cycle-associated genes (*TP53*, *CDKN1A*, *CDKN1B*, *BAK*, *BAX*, *BAD*, *CASP6*, *CCND1*, *CCNE1*, *XIAP*, *BCL2*) in vector control or *PLXNB2* knockout cells of GS6-22, MGG18 and MGG8 under sphere culture conditions. Expression levels were determined by qRT-PCR (n = 3). Heatmaps represent the relative mRNA level of each gene normalized to the value of the respective vector control cells.

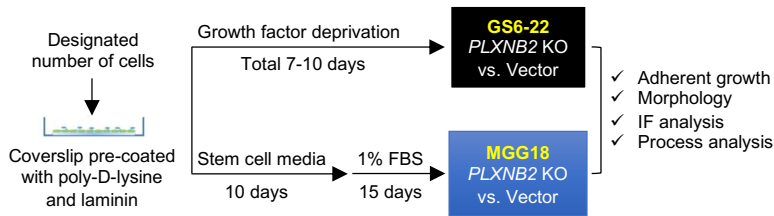
(D) Representative histograms of vector control and *PLXNB2* KO GS6-22 cells in the G0/G1, S, G2/M, and apoptotic phases (by percentage).

(E) Quantitative analysis of distribution of the cells in each phase (n = 2 - 5). Each bar represents mean $\pm$ SEM.

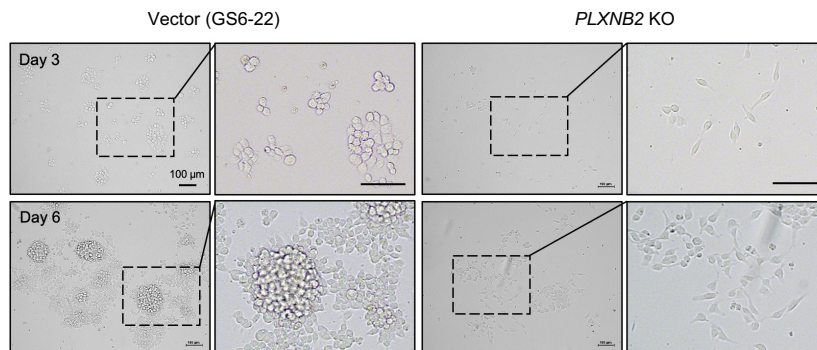
* $p < 0.05$, ** $p < 0.01$, ns = not significant by unpaired two-tailed Student's t test.

The findings that *PLXNB2* KO in GSCs resulted in up-regulation of pro-neuronal transcriptional factors prompted us to study the effect of *PLXNB2* ablation on GSC differentiation. Deprivation of stem cell growth factors (bFGF and EGF) from or addition of 1% fetal bovine serum (FBS) to GSCs cultured adherently on poly-D-lysine & laminin-coated surface were two approaches commonly used to induce stem cell differentiation (Guichet et al., 2013; Park et al., 2017; Pollard et al., 2009), and were applied in our study (**Figure 4.10.A**). In a 6-day adherent culture in the absence of stem cell growth factor replenishment, vector control GS6-22 cells remained circular/rounded morphology and formed spherical colonies as cells populated adherently (**Figure 4.10.B**, left panels), whereas *PLXNB2* KO GS6-22 cells adapted into bipolar or multipolar cells containing thin and elongated neurite-like structures, indications of a neuronal differentiation process (**Figure 4.10.B**, right panels). Moreover, there was an 87% reduction in cell numbers on the 3rd day of culture in *PLXNB2* KO as compared to that of the vector control (**Figure 4.10.C**), indicating that *PLXNB2* ablation dramatically decreased the growth and survival of GSCs in adherent culture. The inhibitory effect could not be accurately assessed afterwards since vector control cells quickly populated and formed three-dimensional colonies.

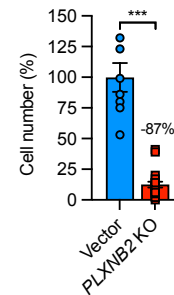
A Schematic diagram of *in vitro* cell differentiation assay



B GS6-22 morphology in adherent culture



C GS6-22 cell number in adherent culture



D MGG18 morphology in adherent culture

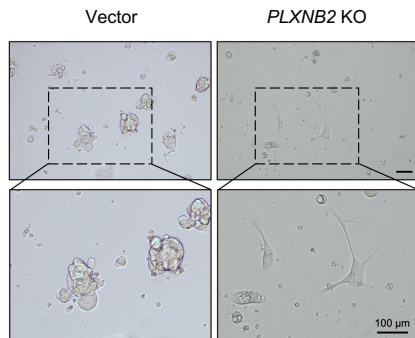


Figure 4.10. *PLXNB2* knockout attenuated GSC adherent growth and resulted in drastic morphological changes associated with differentiation.

(A) Schematic representation of *in vitro* cell differentiation assays.

(B) Representative images of vector control and *PLXNB2* knockout GS6-22 cells growing adherently in stem cell culture media on day 3 and 6 (without growth factor replenishment) ($n = 4$). Highlighted regions by dashed line are magnified on the right. *PLXNB2* knockout massively attenuated adherent growth and resulted in drastic morphological changes of affiliated cells associated with differentiation. Scale bar, 100 μm .

(C) Effects of *PLXNB2* knockout on adherent proliferation on day 3. The number of cells were manually counted by analyzing high magnification images ($n = 8 - 20$). Data shown were derived from four independent experiments. Cell number was shown as percentage (%) normalized to vector controls.

(D) Representative images of vector control and *PLXNB2* knockout MGG18 cells growing adherently under differentiation-inducing conditions (sequential growth factor depletion and addition of 1% FBS) (n = 3). Highlighted regions by dashed line were magnified at the bottom. *PLXNB2* knockout led to a massive reduction in cell number and acquisition of neuronal-like cell morphology. Scale bar, 100 μ m.

*** $p < 0.001$ by unpaired two-tailed Student's t test.

MGG18 cells did not survive in growth factor-deprived adherent culture, so they were cultured on poly-D-lysine and laminin-coated surface in full sphere-forming medium for 10 days followed by another 15-day culture in the presence of 1% FBS. Figure 4.10.D shows that the vector control MGG18 cells were able to maintain a sphere-like morphology under this condition, whereas *PLXNB2* KO MGG18 underwent drastic morphological change as indicated by appearance of multipolar cells with long neurite processes.

These results suggest that *PLXNB2* KO may promote neuronal differentiation while simultaneously diminishing stem cell frequency and accelerating stem cell exhaustion, indicating a robust effect on the stemness of GSCs.

To confirm that *PLXNB2* KO promoted neuronal lineage differentiation of GSCs, we performed immunofluorescence analyses of neurofilament, a pan-neuronal/axonal marker, NEUN, a neuronal maturation marker, and glial fibrillary acidic protein (GFAP), an astroglial lineage protein, in vector control and *PLXNB2* KO GS6-22 cells. As expected, we observed much stronger expression of neurofilament and NEUN in *PLXNB2* KO cells as compared to the vector cells (**Figure 4.11.A**, 1st and 2nd panels). The expression of GFAP was unchanged between vector control and *PLXNB2* KO cells (**Figure 4.11.A**, 4th panel). In contrast, the expression of CD44, a CSC marker also known to promote CSC phenotype and radio-resistance (Anido et al., 2010; Bao et al., 2008; Bhat et al., 2013; Brown et al., 2017), was significantly reduced upon *PLXNB2* ablation (**Figure 4.11.A**, 3rd panel). Figure 4.11.B is the quantification of fluorescence signals by CTCF analysis from multiple images (n = 27 - 124) showing that *PLXNB2*

ablation resulted in 91- and 42- percent increase in neurofilament and NEUN, a 67 percent decrease in CD44, and no changes in GFAP.

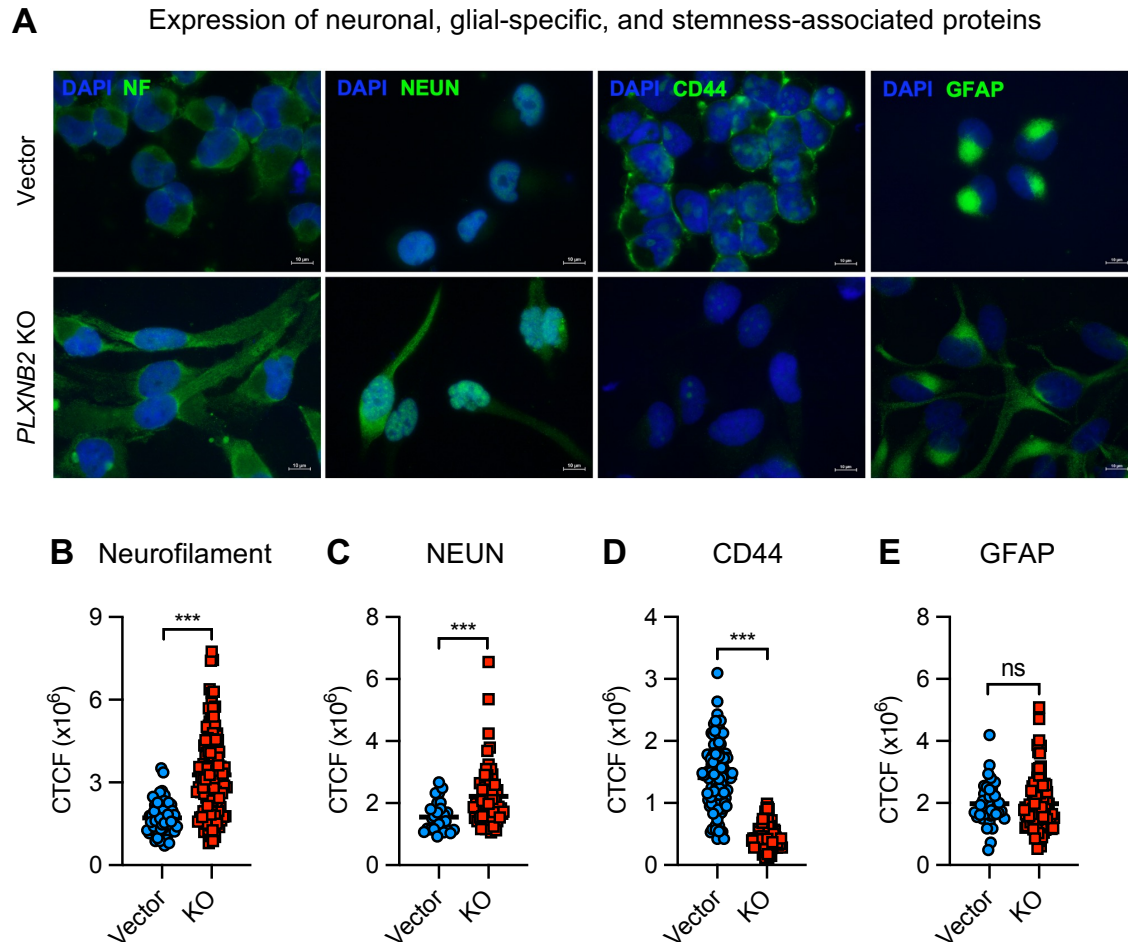


Figure 4.11. *PLXNB2* knockout elevated the expression of neuronal differentiation regulators.

(A) Immunofluorescence staining of neurofilament (pan axonal, cocktail), NEUN, CD44, and GFAP in vector control and *PLXNB2* KO GS6-22 cells that were adherently cultured in stem cell medium for 7 days without growth factor replenishment (a pro-differentiation condition). Nuclei were stained by DAPI. Scale bar, 10 μ m.

(B-E) The CTCF was calculated using ImageJ. B, neurofilament (vector, n = 58; *PB2* KO, n = 124). C, NEUN (vector, n = 27; *PB2* KO, n = 58). D, CD44 (vector, n = 112; *PB2* KO, n = 104). E, GFAP (vector, n = 39; *PB2* KO, n = 90).

***p < 0.001, ns = not significant by unpaired two-tailed Student's t test.

Next, we examined the frequency of vector control and *PLXNB2* KO GS6-22 cells that have neuronal-like process by immunofluorescence staining of neurofilament

(Figure 4.12.A). The percentage of cells containing neurofilament-positive processes was 18% and 97% in vector control and *PLXNB2* KO cells, respectively, indicating a 5.4-fold increase in neuronal processing formation upon *PLXNB2* ablation **(Figure 4.12.B)**. Further, there was a progressive increase in the percentage of cells with longer neuronal-like processes in *PLXNB2* KO cells. In a given total population of 102 cells, the number of cells with the process length < 30, 30 - 100, and >100 μm was 20, 1, and 0 for vector control cells, and was 8, 88, and 5 for *PLXNB2* KO cells. Figure 4.12.C shows the process length distribution in these cells.

We have also examined the expression of NEUROD1, a pivotal neurogenic transcription factor known to promote differentiating neural precursors into neurons (Bertrand et al., 2002) and neuronal differentiation of GSCs (Guichet et al., 2013), and found that its expression in *PLXNB2* KO cells was elevated by 2.5-fold as compared to vector control **(Figure 4.12., D and E)**.

We have shown above in Figure 4.2 that treatment with *PLXNB2* mAbs resulted in a decrease in self-renewing activities of GSCs. In order to know if *PLXNB2* mAbs also induce GSC differentiation, we examined the effect of *PLXNB2* mAbs on NEUROD1 expression in MGG18 cells, and found that NEUROD1 expression in MGG18 cells, cultured on poly-D-lysine and laminin-coated coverslips, was elevated upon treatment with *PLXNB2* mAb (60 $\mu\text{g}/\text{ml}$) **(Figure 4.12.F)** by 3.4-fold **(Figure 4.12.G)**. Moreover, treatment with *PLXNB2* mAb induced a morphological change with enhanced neuronal-like processing formation **(Figure 4.12.F)**. The percentage of the cells with neuronal processes was increased from $8 \pm 3\%$ in the absence of *PLXNB2* mAb to $50 \pm 5\%$ in its presence **(Figure 4.12.H)**. The length of the neuronal-like processes was also increased from 38 ± 3 to 52 ± 0.4 μm upon treatment of *PLXNB2* mAb **(Figure 4.12.I)**. Together, these data demonstrate that *PLXNB2* inhibition, either by gene ablation or by mAb treatment impaired adherent growth of GSCs and induced neuronal-like differentiation.

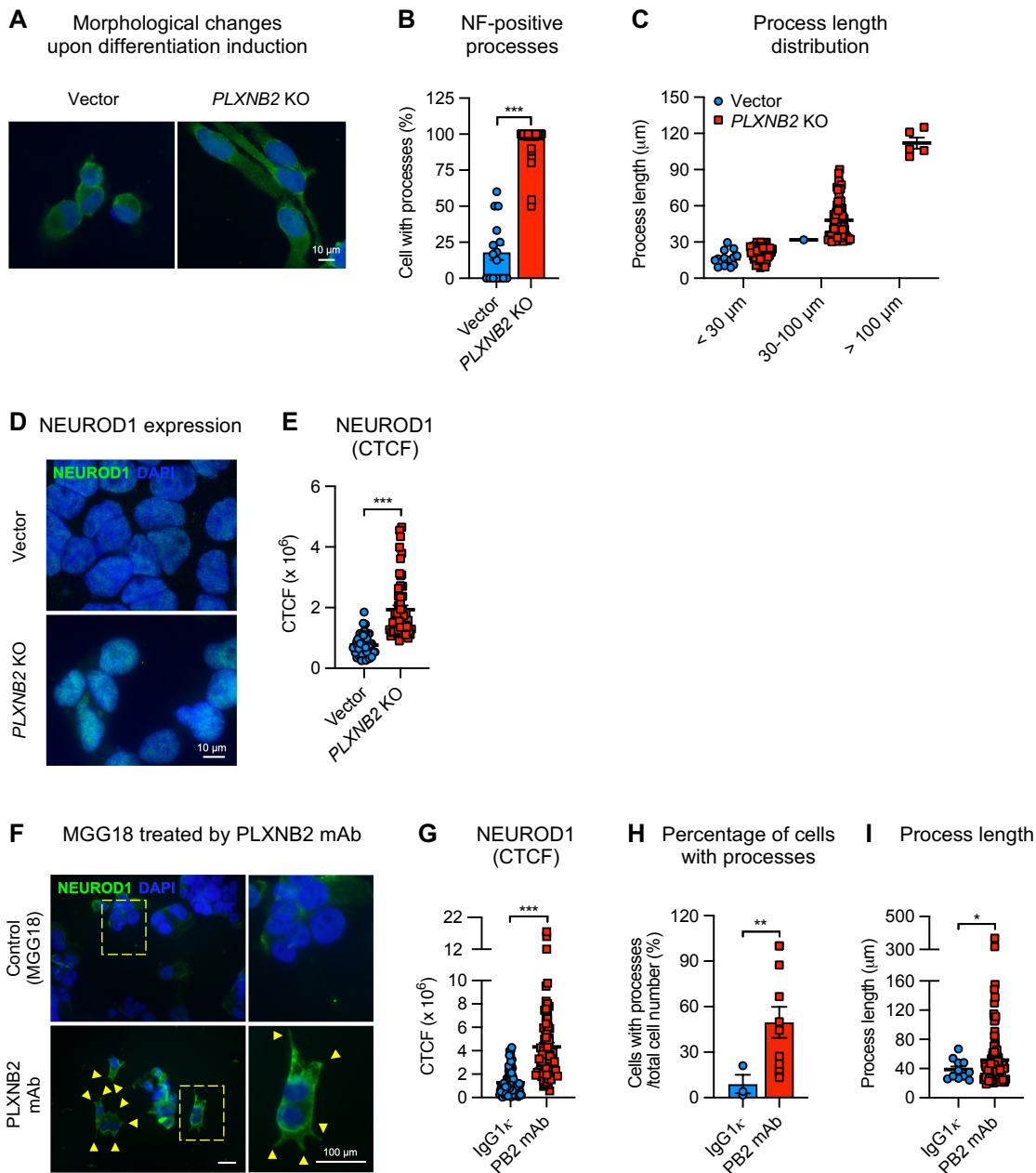


Figure 4.12. *PLXNB2* knockout promoted GSCs a neuronal-like morphology and elevated the expression of NEUROD1, a pivot neurogenic transcription factor.

(A) Representative morphologies of vector control and *PLXNB2* knockout GS6-22 cells that were adherently cultured in stem cell medium for 7 days without growth factor replenishment (a pro-differentiation condition). Neurofilament (green) and nuclei (blue) were stained. Scale bar, 10 μ m.

(B) Quantification of process-containing cells. The number of images analyzed is 16 (vector) and 53 (*PB2* KO). Data shown were mean $\pm$ SD of three independent experiments.

(C) Process length analysis. The numbers of cells analyzed are 102 (vector) and 102 (*PB2*KO). Process length was analyzed using FIJI with Simple Neurite Tracer plugin.

(D) Immunofluorescence staining of NEUROD1 in vector control and *PLXNB2* knockout GS6-22 cells that were adherently cultured in stem cell medium for 7 days without growth factor replenishment. Nuclei were stained by DAPI. Scale bar, 10 μ m.

(E) The CTCF was calculated using ImageJ. The number of cells analyzed is 63 (vector) and 56 (*PB2KO*).

(F) Immunofluorescence staining of NEUROD1 in MGG18. MGG18 GSCs were adherently cultured in stem cell medium and treated with PLXNB2 antibodies (mAb30 or mAb16, 60 μ g/ml) or control (non-immune isotype IgG1- κ) for 7 days. Highlighted regions by dashed line were magnified on the right. PLXNB2 inhibition resulted in drastic morphological changes and induction of protrusion/processes (arrowhead). Nuclei were stained by DAPI. Scale bar, 100 μ m. Images shown are representative of two independent experiments.

(G) The CTCF was calculated using ImageJ software. The number of cells analyzed is 102 (control) and 80 (*PB2* mAbs).

(H) Quantification of process-containing cells. The number of images analyzed is 3 (control) and 11 (*PB2* mAbs).

(I) Quantification of process length. The number of processes analyzed is 11 (control) and 127 (*PB2* mAbs). Process length was analyzed using FIJI with Simple Neurite Tracer plugin.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired two-tailed Student's t test.

4.5 Chapter contributions

All experiments were done by Liang Yuan and supervised by Dr. Guo-fu Hu.

Chapter 5: ANG/PLXNB2 axis regulates GSCs through RNA metabolism

5.1 Subcellular localization of ANG in GSCs

Double immunofluorescence staining using ANG-specific polyclonal antibody (R113) and a mAb against fibrillarin, a nucleolar marker, was performed to examine subcellular distribution of ANG in GSCs. Endogenous ANG was present both in the cytoplasm and the nucleus with cytoplasm having higher amount of ANG than the nucleus (**Figure 5.1.A**). Quantitative analyses show that $58 \pm 3\%$ of cell-associated ANG was localized in the cytoplasm and $42 \pm 2\%$ was in the nucleus (**Figure 5.1.B**). Addition of exogenous ANG increased the level of both cytoplasmic and nuclear ANG (**Figure 5.1.C**) without change in the ratio between the two (**Figure 5.1.B**). Nuclear ANG, both of the endogenous and exogenous origin, was found to be co-localized with FBL (**Figure 5.1.A**), a component of nucleolar small nuclear ribonucleoproteins (snRNPs) and small nucleolar ribonucleoproteins (snoRNPs), involving in ribosomal RNA processing and RNA splicing.

Double immunofluorescence staining of ANG and poly(A)-binding protein (PABP), a SG marker, was also performed to determine if cytoplasmic ANG is localized in SG, a phenomenon that has been linked to cell survival (Emara et al., 2010; Ivanov et al., 2011; Yamasaki et al., 2009) and stemness (Goncalves et al., 2016; Li et al., 2020). Surprisingly, cytoplasmic ANG was found not to be co-localized with PABP, instead, there was co-localization of ANG with PABP in the nucleus, in particular in the presence of exogenous ANG (**Figure 5.1.D**). In GSCs, PABP tends to accumulate in the nucleus, a phenomenon still lacking sufficient explanation (Burgess and Gray, 2012; Gray et al., 2015). The function of nuclear stress granules have been reported (Sandqvist and Sistonen, 2004) but the function remains unknown. The finding that ANG is co-localized with PABP in the nuclear region may be a novel regulatory mechanism of ANG in regulating GSC properties.

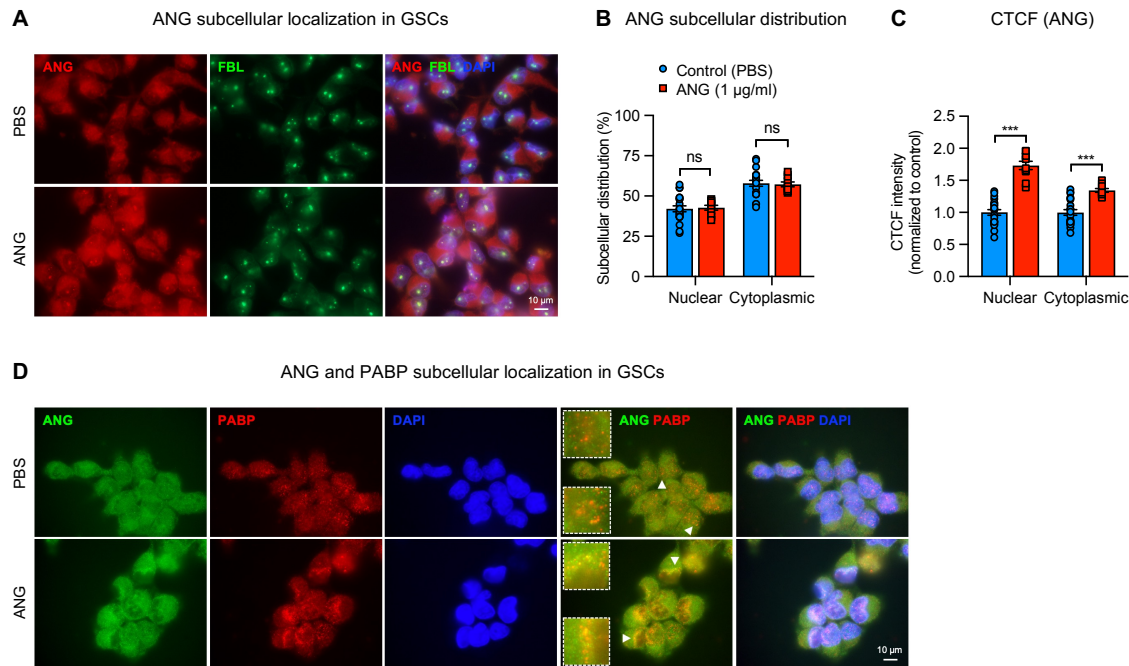


Figure 5.1. ANG subcellular localization in GSCs.

(A) Immunofluorescence staining of ANG and fibrillarlin (FBL) in GS6-22. Cells were grown on poly-D-lysine-coated coverslip in stem cell medium for 5 days. For exogenous ANG treatment, cells were incubated with 1 µg/ml ANG for 30 min and then fixed with ice cold methanol, blocked, and stained with anti-human ANG polyclonal IgG (R113) and monoclonal anti-human FBL IgG. Nuclei were stained by DAPI. Scale bar, 10 µm. Images shown are representatives of three independent experiments.

(B) The ratios of nuclear and cytoplasmic ANG in the presence or absence of ANG (1 µg/ml) treatment. The number of cells analyzed is 21 (PBS) and 10 (ANG).

(C) The CTCF for ANG was calculated using ImageJ and presented as the relative value normalized to respective PBS-treated cells.

(D) Immunofluorescence staining of ANG and PABP in GS6-22 treated with ANG (1 µg/ml) or control (PBS). Cells were stained with monoclonal anti-human ANG IgG (26-2F) and polyclonal anti-human poly(A)-binding protein IgG. Insets (dotted line box) represent high magnification images of the regions indicated by arrowheads. Nuclei were stained by DAPI. Scale bar, 10 µm. Images shown were representatives of two independent experiments.

*** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

5.2 ANG and PLXNB2 regulated small RNA biogenesis in GSCs

The ANG-PLXNB2 axis has been shown to regulate the stemness of primitive cell types through distinct RNA biogenesis. In HSPCs, ANG was found to be accumulated in cytoplasmic SGs and mediate tiRNA biogenesis (Goncalves et al., 2016). These tiRNAs restrict protein synthesis and thereby preserve the regenerative

potentials of HSPCs. In prostate CSCs, ANG was shown to actively produce another class of small RNA, the 3'-end fragments of 5S rRNA, that enhance stemness-related gene transcription and promote tumorsphere formation (Li et al., 2020). To know if the ANG-PLXNB2 axis regulates small RNA biogenesis in GSCs, we first assessed the bulk small RNA production in transduction vector control and in *PLXNB2* KO GSCs in the presence or absence of exogenous ANG. We observed that GS6-22 and MGG18 vector control cells produced a considerable amount of small RNAs between 20-60 nt as shown by small RNA gel electrophoresis and SYBR Gold staining (**Figure 5.2.A**, 1st and 5th lanes). The amounts of small RNA in *PLXNB2* KO cells were substantially lower than the corresponding vector controls (**Figure 5.2.A**, 2nd and 6th lanes). Addition of exogenous ANG resulted in a strong band between 35-40 nt in the vector control GSCs (**Figure 5.2.A**, 3rd and 7th lanes). Quantitative analyses indicate that the amount of small RNAs between 20-60 nt was reduced by 3.3- and 7.2- fold in GS6-22 and MGG18 cells upon *PLXNB2* KO (**Figure 5.2.B**), and that between 35-40 nt was reduced by 2.0- and 5.4-fold (**Figure 5.2.C**), respectively. Addition of exogenous ANG elevated the abundance of small RNA between 35-40 nt by 38.6- and 4.7-folds (**Figure 5.2.D**) and that between 20-60 nt by 9.8- and 1.9-fold (**Figure 5.2.E**), respectively, in vector control GS6-22 and MGG18. In contrast, addition of exogenous ANG had no effect on the abundance of small RNA between 35-40 nt (**Figure 5.2., F and G**) but slightly enhance the production of small RNA between 20-60 nt by 28 and 73%, respectively (**Figure 5.2.H**), in *PLXNB2* KO GS6-22 and MGG18 cells. These results demonstrate a role of the ANG-PLXNB2 axis in the production of small RNA in GSCs, in particular the RNA species between 35-40 nt. The reasons for ANG to slightly enhance the production of small RNA in the ranges of 20-35 plus 40-60 nt in *PLXNB2* KO cells (**Figure 5.2., F and G**) was not clear. One possibility would be that ANG could still be up-taken in *PLXNB2* deficient GSCs by fluid-mediated endocytosis or pinocytosis, and mediate RNA metabolism. This

mechanism of ANG internalization has been reported in other cells including astroglia (Skorupa et al., 2012) and neuroblastoma cells (Ferguson and Subramanian, 2018).

Collectively, these observations suggest that ANG regulates RNA metabolism in GSCs by actively producing a population of 35-40 nt small RNA species and that PLXNB2 is indispensable for mediating the functions of ANG in this process.

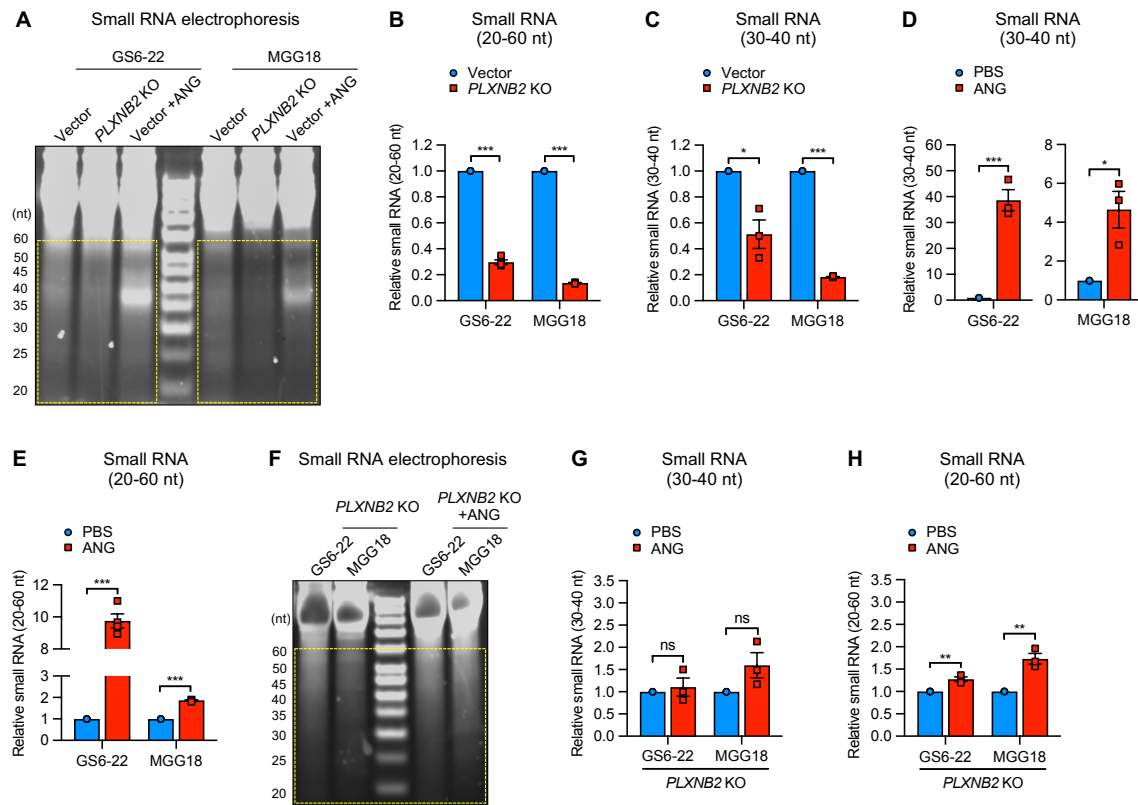


Figure 5.2. ANG/PLXNB2 differentially regulated small RNA production in patient-derived GSCs.

(A-E) Small RNA levels in GS6-22 and MGG18 upon ANG stimulation (1 μ g/ml ANG, 1 day) or *PLXNB2* knockout. A total of 20 μ g RNA of each sample was analyzed by 15% denature polyacrylamide gel electrophoresis and SYBR Gold staining (n = 3). Band intensity was determined by ImageJ and presented as relative values normalized to respective vector control cells. A, representative gel image; B and E, intensity at the region of 20-60 nt; C and D, intensity at the region of 35-40 nt.

(F-H) Effect of ANG on small RNA production in *PLXNB2* knockout GS6-22 and MGG18 (n = 3). Band intensity was determined by ImageJ and presented as relative values normalized to PBS-treated cells. F, representative gel image; G, intensity at the region of 35-40 nt; H, intensity at the region of 20-60 nt. * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant by unpaired two-tailed Student's t test.

5.3 The ANG-PLXNB2 axis regulated tiRNA production in GSCs

To determine the identity of the small RNAs regulated by the ANG-PLXNB2 axis in GSCs, we performed small RNA sequencing analysis of the RNA species smaller than 60 nt extracted from the RNA gel. A total of eight samples, including both vector controls and *PLXNB2* KO of GS6-22 and MGG18 with and without treatment of exogenous ANG. Around 10 million sequences (2.8 - 13.5 millions, see details in Table 5.1.) were read in each sample. Among these reads, tRNA fragments (tRF) accounted for 5.4 and 1.5% of all small RNAs in the vector controls of GS6-22 and MGG18, and 2.6 and 2.0% in the *PLXNB2* KO cells (**Figure 5.3. and Table 5.1.**). Addition of exogenous ANG increased the percentage of tRF in small RNAs to 18.4 and 14.3% in the vector controls of GS6-22 and MGG18, respectively, indicating a 242 and 836% increase (**Figure 5.3. and Table 5.1.**).

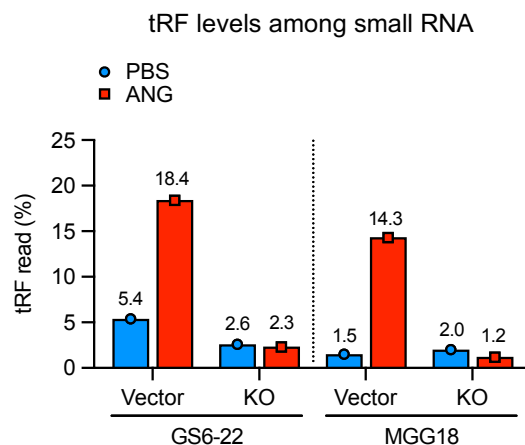


Figure 5.3. tRNA fragment reads percentage.

The percentage of tRNA fragment reads (alignment rate with tRNA) was calculated by the sum of tRNA reads divided by total reads. Small RNAs (≤ 60 nt) were extracted from a 15% denature polyacrylamide gel by “Crush and Soak” method. RNA libraries were prepared with QIAseq miRNA Library Kit. RNA samples were sequenced using NextSeq 550 Mid-Output 150 cycles kit (Illumina) and ran as a single-read 150 base mode. Paired-end clean reads were aligned to the reference genome, GRCh38 (hg38).

However, exogenous ANG failed to elevate tRF abundance in *PLXNB2*-ablated cells (**Figure 5.3. and Table 5.1.**). Actually, exogenous ANG decreased the percentage

of tRF in *PLXNB2* KO cells from 2.6 to 2.3% in GS6-22 cells and from 2.0 to 1.2% in MGG18 cells, respectively (**Figure 5.3. and Table 5.1.**). This slight decrease may be attributed to the increase of other small RNA species as shown in Figure 5.2.H.

Together, these results demonstrate that tRFs were abundantly produced in GSCs and that ANG enhanced tRF expression in a *PLXNB2*-dependent manner.

Table 5.1. Single-end clean reads by small RNA sequencing

	GS6-22				MGG18			
	Vector control		<i>PLXNB2</i> knockout		Vector control		<i>PLXNB2</i> knockout	
	PBS	ANG	PBS	ANG	PBS	ANG	PBS	ANG
Total read count	12163118	10313411	9566229	13561375	12820575	12334771	2844999	11620525
Total tRNA fragment reads	655864	1901473	245267	310127	195713	1763366	56232	134489
tRNA reads percentage (%)	5.4	18.4	2.6	2.3	1.5	14.3	2.0	1.2
Percentage of change (%)		242%		-11%		836%		-41%
tRNA reads per million reads	53922	184369	25639	22868	15266	142959	19765	11573
ANG treatment: 1 µg/ml for 1 day, PBS as control								

To identify the tRFs that are regulated by ANG/*PLXNB2*, we performed fragment alignment analysis using the UCSC tRNA database (<http://gtrnadb.ucsc.edu>). We focused on tRNA species that have ≥ 100 reads per million in PBS-treated vector control cells with the threshold of 2 Log₂ scale in each direction ($\log_2 [(RPM_{\text{test}}+1)/(RPM_{\text{ctrl}}+1)] \geq 2$ or ≤ -2) to identify changes upon *PLXNB2* KO or addition of exogenous ANG. We found that ANG treatment increased the production of 11 and 15 tRF species by over 2 Log₂ scales in the vector controls of GS6-22 and MGG18, respectively, but not in the *PLXNB2* KO cells (**Figure 5.4., A and B**) (**Table 5.2. and Table 5.3.**). Among those ANG-regulated tRFs, 5 species, including those derived from tRNA-Gly-GCC-2,3,5, tRNA-Val-CAC-2, tRNA-Lys-CTT-2, were found in both GSC lines, suggesting a significant overlap of ANG-regulated tRFs in different GSC lines. It is also interesting to note that the tRF species that were upregulated by ANG in vector controls were mostly downregulated in *PLXNB2* KO cells, indicating a common function of endogenous and exogenous ANG, both were mediated by *PLXNB2*.

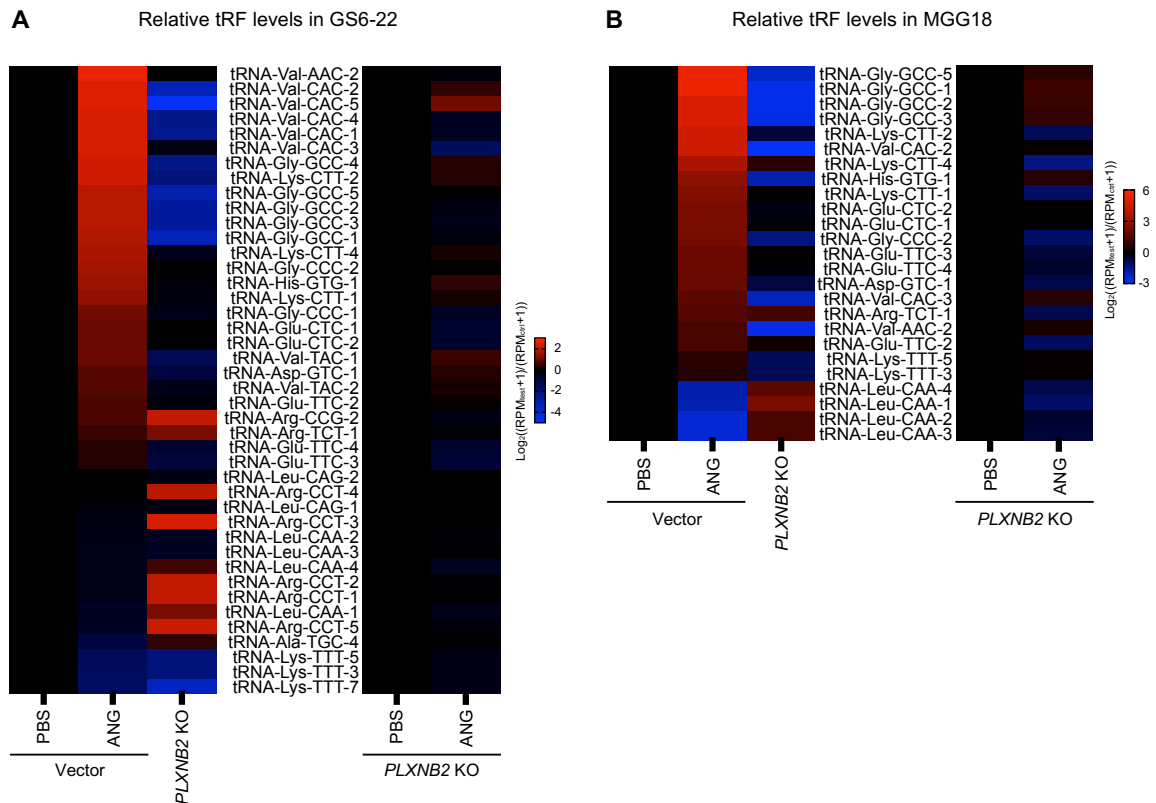


Figure 5.4. The effect of ANG stimulation or *PLXNB2* knockout on tRF levels.

(A) Heatmaps showing the effect of ANG stimulation or *PLXNB2* knockout on tRF levels in GS6-22 cells. tRNA species with less than 100 RPM in PBS-treated vector control cells were not included. Each value presents $\text{Log}_2((\text{RPM}_{\text{test}}+1)/(\text{RPM}_{\text{ctrl}}+1))$ for the indicated conditions.

(B) Heatmaps showing the effect of ANG stimulation or *PLXNB2* knockout on tRF levels in MGG18 cells. tRNA species that having less than 100 RPM in PBS-treated vector control cells were not included. Each value presents $\text{Log}_2((\text{RPM}_{\text{test}}+1)/(\text{RPM}_{\text{ctrl}}+1))$ for the indicated conditions.

Table 5.4 and Table 5.5 list the most abundant 12 tRF species identified in the two GSC lines, among them 10 were shared in both GSC lines. These top 12 tRFs account for 93.7 and 88.9% of the total tRF reads in vector controls of GS6-22 and MGG18, respectively. Exogenous ANG increased the abundance of these top 12 tRFs to 97.9% and 96.5% of the total tRF reads (**Table 5.4 and Table 5.5**). In the *PLXNB2* KO cells, the percentage of these top 12 tRFs were reduced to 71.2% in GS6-22 and to 50.1% in MGG18.

Table 5.2. tRNA fragment levels in GS6-22

GS6-22				
		Vector		PLXNB2 KO
		Log2 (ANG+1/PBS+1)	Log2 (KO+1/VC+1)	Log2 (ANG+1/PBS+1)
1	tRNA-Val-AAC-2	2.7	-0.1	-0.2
2	tRNA-Val-CAC-2	2.5	-3.3	0.4
3	tRNA-Val-CAC-5	2.5	-4.9	1.1
4	tRNA-Val-CAC-4	2.4	-2.3	-0.5
5	tRNA-Val-CAC-1	2.4	-2.4	-0.5
6	tRNA-Val-CAC-3	2.4	-0.3	-1.5
7	tRNA-Gly-GCC-4	2.3	-2.3	0.3
8	tRNA-Lys-CTT-2	2.3	-2.0	0.3
9	tRNA-Gly-GCC-5	2.0	-3.1	-0.1
10	tRNA-Gly-GCC-2	2.0	-2.7	-0.3
11	tRNA-Gly-GCC-3	2.0	-2.7	-0.4
12	tRNA-Gly-GCC-1	1.9	-3.3	-0.2
13	tRNA-Lys-CTT-4	1.8	-0.4	0.2
14	tRNA-Gly-CCC-2	1.7	0.0	0.0
15	tRNA-His-GTG-1	1.6	-0.2	0.4
16	tRNA-Lys-CTT-1	1.5	-0.2	0.2
17	tRNA-Gly-CCC-1	1.1	-0.4	-0.5
18	tRNA-Glu-CTC-1	1.0	0.0	-0.7
19	tRNA-Glu-CTC-2	1.0	0.0	-0.7
20	tRNA-Val-TAC-1	1.0	-1.3	0.5
21	tRNA-Asp-GTC-1	0.8	-1.0	0.3
22	tRNA-Val-TAC-2	0.8	-0.4	0.2
23	tRNA-Glu-TTC-2	0.7	-0.2	0.1
24	tRNA-Arg-CCG-2	0.7	2.1	-0.3
25	tRNA-Arg-TCT-1	0.5	1.2	-0.1
26	tRNA-Glu-TTC-4	0.3	-0.7	-0.7
27	tRNA-Glu-TTC-3	0.3	-0.9	-0.8
28	tRNA-Leu-CAG-2	0.0	-0.3	-0.1
29	tRNA-Arg-CCT-4	-0.1	2.0	0.0
30	tRNA-Leu-CAG-1	-0.2	-0.3	-0.1
31	tRNA-Arg-CCT-3	-0.3	2.4	0.0
32	tRNA-Leu-CAA-2	-0.3	-0.5	-0.2
33	tRNA-Leu-CAA-3	-0.4	-0.5	-0.1
34	tRNA-Leu-CAA-4	-0.4	0.6	-0.5
35	tRNA-Arg-CCT-2	-0.4	2.1	-0.1
36	tRNA-Arg-CCT-1	-0.4	2.1	-0.1
37	tRNA-Leu-CAA-1	-0.5	1.2	-0.4
38	tRNA-Arg-CCT-5	-0.5	2.2	-0.2
39	tRNA-Ala-TGC-4	-1.0	0.4	-0.1
40	tRNA-Lys-TTT-5	-1.4	-2.0	-0.3
41	tRNA-Lys-TTT-3	-1.5	-2.0	-0.3
42	tRNA-Lys-TTT-7	-1.5	-3.5	-0.3
SUM	Log2 (Test+1/Ctrl+1) >=2	11	6	0
SUM	Log2 (Test+1/Ctrl+1) <=-2	0	13	0

Table 5.3. tRNA fragment levels in MGG18

MGG18				
		Vector		PLXNB2 KO
		Log2 (ANG+1/PBS+1)	Log2 (KO+1/VC+1)	Log2 (ANG+1/PBS+1)
1	tRNA-Gly-GCC-5	5.4	-2.2	0.7
2	tRNA-Gly-GCC-1	5.4	-2.7	1.1
3	tRNA-Gly-GCC-2	4.9	-2.7	1.0
4	tRNA-Gly-GCC-3	4.9	-2.6	0.9
5	tRNA-Lys-CTT-2	4.6	-0.5	-0.8
6	tRNA-Val-CAC-2	4.6	-2.9	0.2
7	tRNA-Lys-CTT-4	3.6	0.7	-1.3
8	tRNA-His-GTG-1	2.9	-1.9	0.6
9	tRNA-Lys-CTT-1	2.6	0.0	-1.0
10	tRNA-Glu-CTC-2	2.4	-0.2	0.1
11	tRNA-Glu-CTC-1	2.4	-0.1	0.0
12	tRNA-Gly-CCC-2	2.3	-1.3	-1.0
13	tRNA-Glu-TTC-3	2.1	0.1	-0.5
14	tRNA-Glu-TTC-4	2.1	-0.1	-0.4
15	tRNA-Asp-GTC-1	2.0	-0.6	-0.7
16	tRNA-Val-CAC-3	1.7	-2.1	0.6
17	tRNA-Arg-TCT-1	1.6	1.2	-0.7
18	tRNA-Val-AAC-2	1.3	-2.6	0.4
19	tRNA-Glu-TTC-2	1.3	0.3	-0.9
20	tRNA-Lys-TTT-5	0.7	-0.8	0.2
21	tRNA-Lys-TTT-3	0.6	-0.8	0.2
22	tRNA-Leu-CAA-4	-1.8	1.7	-0.7
23	tRNA-Leu-CAA-1	-1.9	2.4	-1.0
24	tRNA-Leu-CAA-2	-2.4	1.3	-0.4
25	tRNA-Leu-CAA-3	-2.4	1.3	-0.5
SUM	Log2 (Test+1/Ctrl+1) >=2	15	1	0
SUM	Log2 (Test+1/Ctrl+1) <=-2	2	7	0

Table 5.4. Top tRF-producing tRNAs in GS6-22 upon ANG stimulation or *PLXNB2* ablation

GS6-22				
Percentage (%)				
tRF	Vector+ PBS	Vector+ ANG	PB2 KO +PBS	PB2 KO +ANG
tRNA-Gly-GCC	56.3	66.1	16.7	15.9
tRNA-Val-CAC	10.0	16.0	5.2	3.9
tRNA-Lys-TTT	6.5	0.7	3.1	3.1
tRNA-Lys-CTT	5.1	5.4	7.4	9.6
tRNA-Glu-CTC	4.9	2.9	10.5	7.6
tRNA-Gly-CCC	3.1	2.1	5.2	4.6
tRNA-Leu-CAA	1.6	0.4	4.6	4.0
tRNA-Asp-GTC	1.5	0.8	1.6	2.3
tRNA-Glu-TTC	1.5	0.7	2.4	2.8
tRNA-Arg-CCT	1.2	0.3	11.2	12.4
tRNA-Val-AAC	1.2	2.1	2.2	2.2
tRNA-Val-TAC	0.9	0.6	1.1	1.6
Other	6.3	2.1	28.8	30.2
Top12	93.7	97.9	71.2	69.8
Total	100	100	100	100

Table 5.5. Top tRF-producing tRNAs in MGG18 upon ANG stimulation or *PLXNB2* ablation

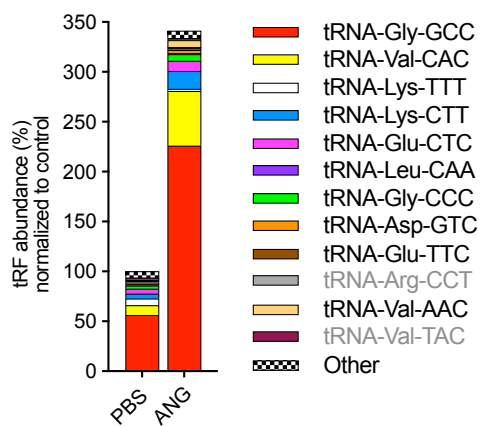
MGG18				
Percentage (%)				
tRF	Vector+ PBS	Vector+ ANG	PB2 KO +PBS	PB2 KO +ANG
tRNA-Gly-GCC	11.0	41.0	1.5	4.6
tRNA-Val-CAC	15.9	24.1	2.3	5.7
tRNA-Lys-TTT	3.3	0.6	1.5	2.6
tRNA-Lys-CTT	9.8	10.0	9.5	7.3
tRNA-Glu-CTC	16.6	9.5	11.8	20.4
tRNA-Gly-CCC	5.3	3.1	1.8	2.2
tRNA-Leu-CAA	3.4	0.1	9.1	9.3
tRNA-Asp-GTC	4.4	1.9	2.3	2.4
tRNA-Glu-TTC	8.4	2.6	7.7	7.3
tRNA-Gly-TCC	1.5	0.5	1.3	0.9
tRNA-Val-AAC	7.8	2.1	1.0	2.1
tRNA-His-GTG	1.5	1.2	0.3	0.8
Other	11.1	3.5	49.9	34.6
Top12	88.9	96.5	50.1	65.4
Total	100	100	100	100

Among the top 12 tRF species that were upregulated by ANG and downregulated upon *PLXNB2* KO, tRNA-Gly-GCC fragments were found to be the most abundant and most upregulated small RNA by ANG (**Figure 5.5, A and B**). Treatment with exogenous ANG (1 µg/ml) increased tRNA-Gly-GCC fragment reads by 302% and 3,376% in the vector control GS6-22 and MGG18, respectively, as compared with those of PBS-treated counterparts (**Figure 5.5, A and B**). Consistently, *PLXNB2* KO decreased the reads of tRNA-Gly-GCC by 86% in GS6-22 and 83% in MGG18 as compared to the vector control cells (**Figure 5.5, C and D**). Notably, ANG treatment did not stimulate the production of tRNA-Gly-GCC in *PLXNB2* KO GS6-22 cells (**Figure 5.5.E**). ANG treatment weakly increased tRNA-Gly-GCC levels in *PLXNB2* KO MGG18 cells (**Figure 5.5.F**). Taken together, these results demonstrate that the ANG-*PLXNB2* axis selectively enriched a subset of tRFs in GSCs and that tRNA-Gly-GCC was the most significantly regulated species.

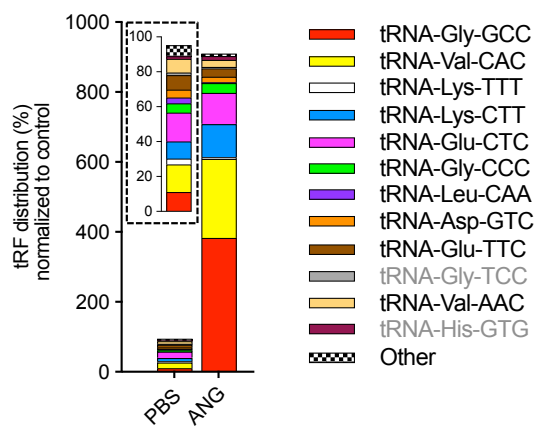
Fragment endpoint analysis showed that 85 and 51% of the tRNA-Gly-GCC fragments are 34 nt in length in GS6-22 and MGG18, respectively (**Figure 5.6. and Table 5.6.**), and are from the 5'-end of tRNA, corresponding to the 5'-tiRNA.

Among the various tRFs, tiRNA is the species that has been shown to be derived by cleavage at the anti-codon loop. ANG is the only endoribonuclease that has been identified to produce tiRNA (Fu et al., 2009). Consistently, we found that the percentage of 5'tiRNA-Gly-GCC (34 nt) was increased to 90 and 91%, respectively, in GS6-22 and MGG18 cells upon treatment of ANG (**Figure 5.6., A and B, and Table 5.6.**). Besides the 34 nt 5'tiRNA-Gly-GCC, 2 fragments of 28 nt and 52 nt from the 5'-end of tRNA-Gly-GCC were also identified, both of them mainly appeared in the *PLXNB2* KO cells. The significance of these two tRFs is currently unknown.

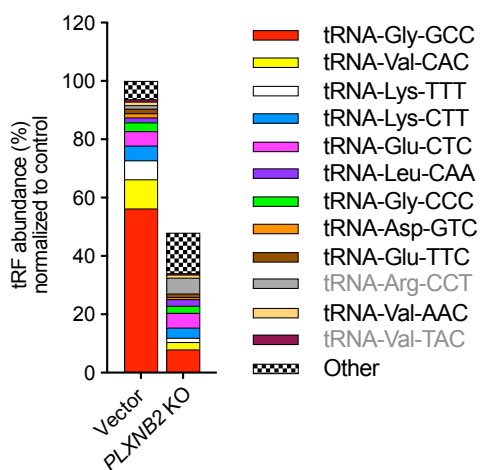
A tRF abundance
(GS6-22)



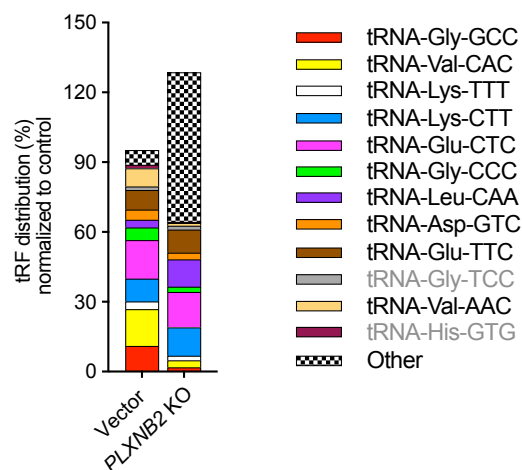
B tRF abundance
(MGG18)



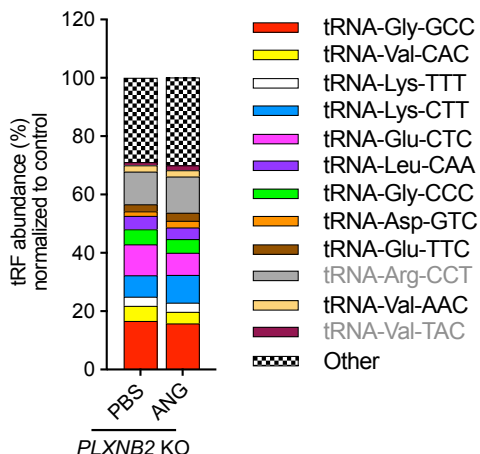
C tRF abundance
(GS6-22)



D tRF abundance
(MGG18)



E tRF abundance
(GS6-22)



F tRF abundance
(MGG18)

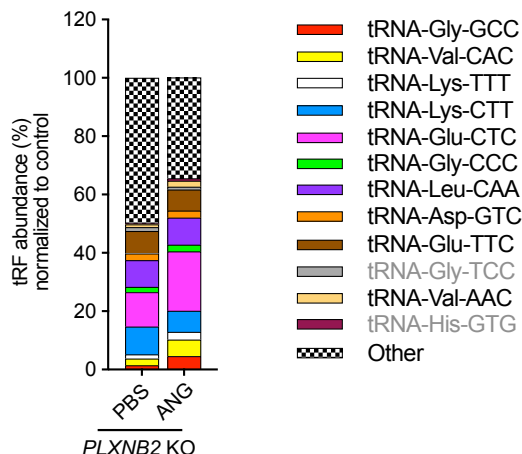


Figure 5.5. The effect of ANG stimulation or *PLXNB2* knockout on tRF abundance. (A-F) Bar graph showing the relative abundance of tRFs under the indicated conditions. Data were presented as the relative values normalized to the respective controls. The 12 most abundant tRFs (in control and treated groups) were labeled in different colors, among them the tRF species that occurred in GS6-22 but not in MGG18, or vice versa, were indicated by gray color. For panel B, the bar for control group was magnified in the inset.

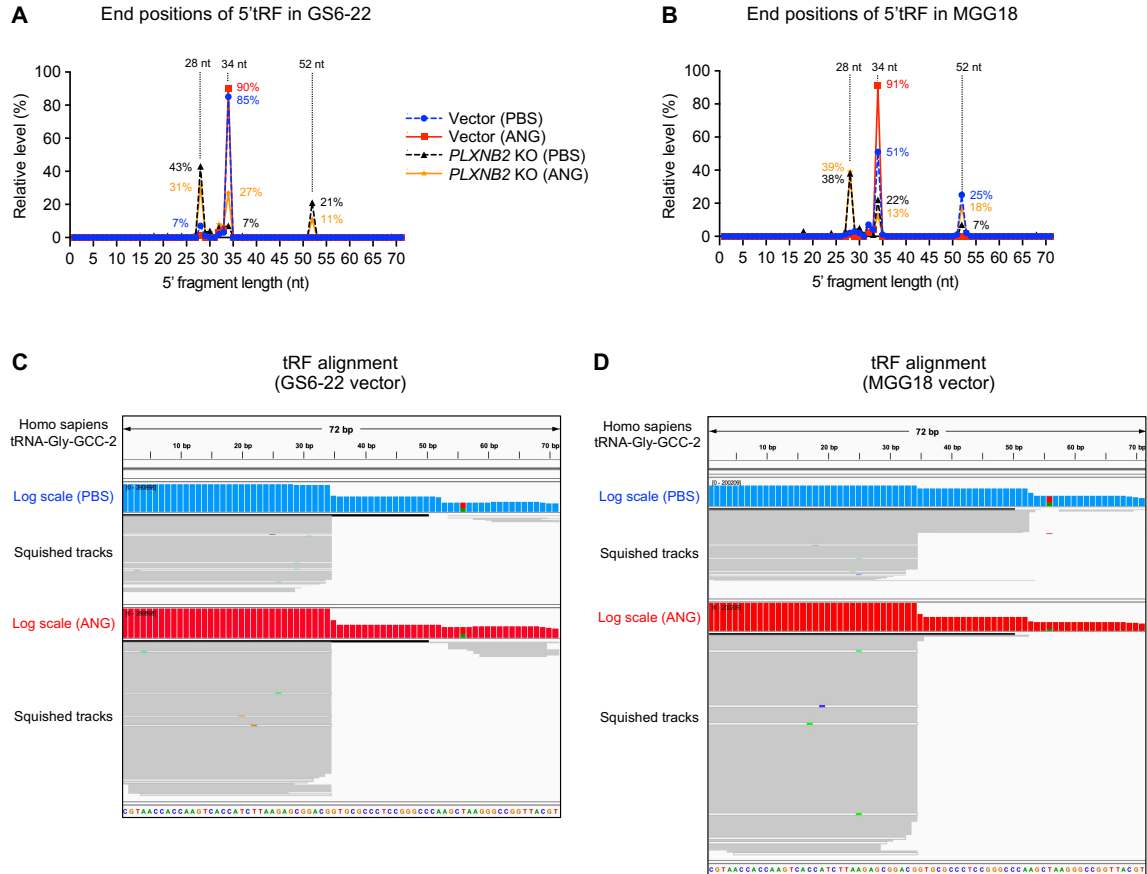


Figure 5.6. ANG/*PLXNB2* regulated the production of 5' tiRNA in GSCs. (A-B) Distribution of 5' fragment end position of tRNA-Gly-GCC-2 in vector control or *PLXNB2* knockout cells in the presence or absence of ANG. A, GS6-22; B, MGG18. (C-D) An overview of all tRFs of the indicated group aligning to tRNA-Gly-GCC-2. Fragment alignment graphs were generated by Integrative Genomics Viewer (Broad Institute). The height of each small bar represents the reads (log scale) of the respective nucleotide. Squished tracks illustrate tRF species and their relative abundance. C, GS6-22; D, MGG18.

Table 5.6. 5' tRNA fragment end site distribution (by percentage)

tRNA-Gly-GCC-2 (GS6-22)		SUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
	VC+PBS	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	1	0	0	2	3	85	0	0
	VC+ANG	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3	4	90	0	0
	KO+PBS	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	2	43	3	4	0	6	6	7	1	0
	KO+ANG	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	2	31	3	3	0	8	7	27	1
tRNA-Gly-GCC-2 (MGG18)		SUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
	VC+PBS	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	3	2	0	7	4	51	1	0
	VC+ANG	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	4	91	0	0	
	KO+PBS	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	2	0	0	3	38	3	5	1	7	1	22	0	0
	KO+ANG	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	39	4	4	1	7	3	13	0	0
tRNA-Gly-GCC-2 (GS6-22)			37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	
	VC+PBS		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	VC+ANG		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	KO+PBS		1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	KO+ANG		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
tRNA-Gly-GCC-2 (MGG18)			37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	
	VC+PBS		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	VC+ANG		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	KO+PBS		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	KO+ANG		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

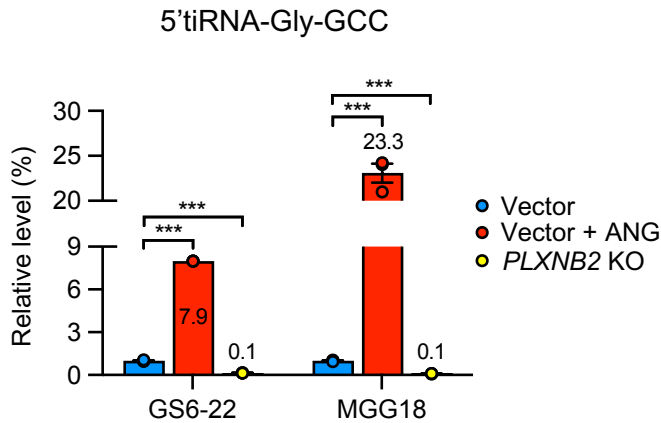


Figure 5.7. ANG/PLXNB2 regulated the production of 5'tiRNA-Gly-GCC in GSCs.

Relative 5'tiRNA-Gly-GCC levels in GS6-22 or MGG18 vector control cells upon ANG stimulation or *PLXNB2* knockout (n = 3). 5'tiRNA-Gly-GCC levels were determined by qRT-PCR using TaqMan™ small RNA assay that specifically detects the target sequence GCAUUGGUGGUUCAGUGGUAGAAUUCUCGCCUGC.

****p* < 0.001 by unpaired two-tailed Student's *t* test.

To validate small RNA sequencing results, we performed TaqMan™ small RNA assay that employs a target-specific looped-primer to synthesize customized cDNAs and thereby enables the detection of interested small RNA by real-time polymerase chain reaction (PCR). The target sequence of "5'-GCAUUGGUGGUUCAGUGGUAGAAUUC UCGCCUGC-3'" (34 nt) was used to specifically quantify 5'tiRNA-Gly-GCC levels. We

found that ANG treatment drastically elevated the levels of 5'tiRNA-Gly-GCC in the vector controls of GS6-22 and MGG18 by 7.9- and 23.3-fold, respectively, whereas *PLXNB2* ablation resulted in marked reduction of 5'tiRNA-Gly-GCC levels by 10-fold in both GSCs (**Figure 5.7**).

5.4 5'tiRNA-Gly-GCC mediated ANG-induced self-renewing functions in GSCs

To determine if 5'tiRNA-Gly-GCC is, at least partially, responsible for ANG/*PLXNB2*-mediated GSC self-renewal, we transfected 5'-phosphorylated tiRNA-Gly-GCC, the active form of tiRNA in promoting cell survival and stemness, in *PLXNB2* KO GSCs to see if it could restore the activity that was lost due to *PLXNB2* gene ablation. Transfection of active tiRNA-Gly-GCC (100 nM) significantly enhanced tumorsphere formation as indicated by 248% increase in tumorsphere number and 13% increase in sphere diameter (**Figure 5.8., A, B, and C**).

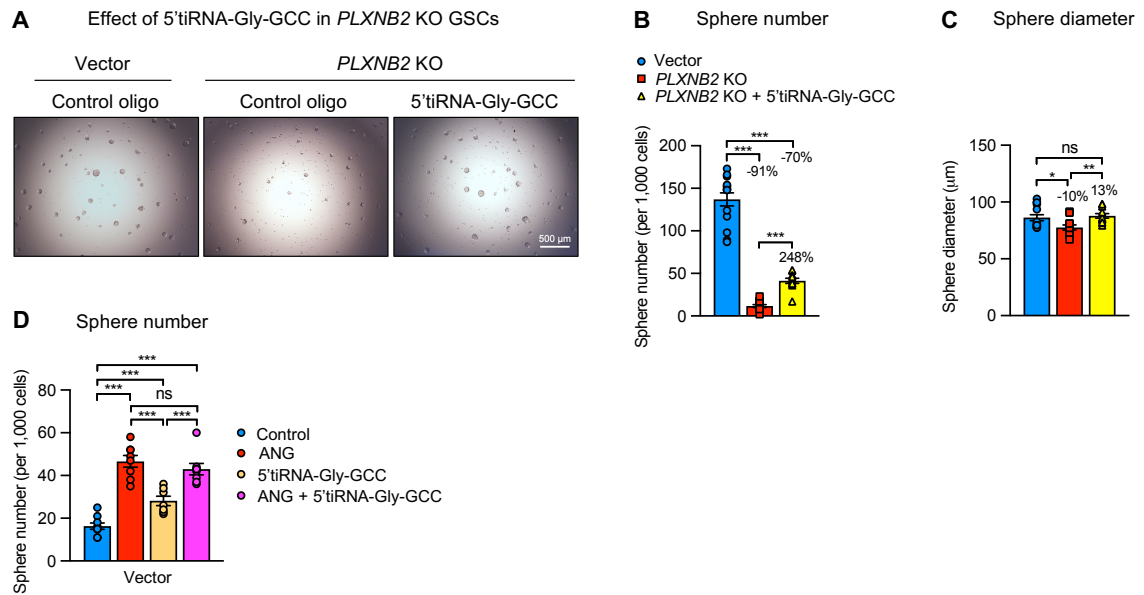


Figure 5.8. 5'tiRNA-Gly-GCC mediated ANG-induced self-renewing functions in GSCs.

(A-C) Sphere formation of 1,000 *PB2*KO GS6-22 cells transfected with 5'-P-tiRNA-Gly-GCC (100 nM) or control by TransIT-LT1 transfection reagent. Vector control cells were also transfected with control oligo by transfection reagent. Cells were maintained in stem

cell culture medium for 7 days. Given the evident toxic effect of the transfection agent on *PB2* KO cells, we set the cut-off sphere diameter at 50 μm . A, representative images; B, tumorsphere number; C, tumorsphere diameter. Scale bar, 500 μm . (D) Sphere formation of 1,000 GS6-22 vector control cells under indicated conditions for 7 days. ANG treatment (1 $\mu\text{g}/\text{ml}$), transfection with 5'-P-tiRNA-Gly-GCC (100 nM), or combination ($n = 3$). The cut-off tumorsphere diameter was set at 100 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant by unpaired two-tailed Student's t test.

Because of the severe toxicity of the transfection reagent in *PLXNB2* KO cells that prevents sphere growth to sizes with diameters over 100 μm , we counted all spheres with a diameter ≥ 50 μm in these experiments. It is notable that tiRNA-Gly-GCC transfection was unable to completely restore the sphere-forming capacity of *PLXNB2* KO cells to the level of vector control GSCs as shown in Figure 5.8.A and Figure 5.8.B that the number of tumorsphere formed was still only 30% of the vector control even though the diameter of spheres was comparable to that of vector control (**Figure 5.8.C**). This is not surprising because tiRNA-Gly-GCC is only one of the species generated by ANG-*PLXNB2*. It is likely that the other tRFs and/or other small RNAs also played a role in mediating the regulatory function of ANG-*PLXNB2* in GSCs. It is also possible that *PLXNB2* has ANG-independent regulatory functions in GSC stemness. These results demonstrate that tiRNA-Gly-GCC was an effector of ANG-*PLXNB2* axis and a mediator of tumorsphere formation of GSCs.

Next, we examined the effect of tiRNA-Gly-GCC on vector control of GS6-22 and compared its activity with that of ANG in promoting tumorsphere formation. Figure 5.8.D shows that transfection of 5'-P-tiRNA-Gly-GCC (100 nM) was able to promote tumorsphere formation to a degree that is 60% of that induced by 1 $\mu\text{g}/\text{ml}$ of exogenous ANG. As described above, tiRNA-Gly-GCC did not 100% recapitulate the effect of ANG, probably due to the function of other small RNAs that are also mediated by ANG or due to tiRNA-independent activity of ANG, such as the nuclear functions of ANG. Significantly, tiRNA-Gly-GCC did not further promote tumorsphere formation on top of

ANG treatment (**Figure 5.8.D**). The lack of additive or synergistic effect between ANG and tiRNA-Gly-GCC indicates that they operated in the same pathway in promoting tumorsphere formation of GSCs. Taken together, these data suggest that ANG-PLXNB2 regulates GSC properties, at least partially, through tiRNA-Gly-GCC.

We also examined the effect of tiRNA-Gly-GCC on a panel of stemness-related genes that have been shown to be downregulated upon *PLXNB2* KO (**Figure 4.9.B**). First, we examined the dynamic changes of the expression of these genes in response to ANG stimulation and found that a subgroup of these genes were stimulated to express by ANG in early time points between 5 to 30 min. This group of early responders include *SOX2* (Hemmati et al., 2003), *OCT4* (Schoenhals et al., 2009), *PROM1* (Singh et al., 2003), and *CDCP1* (Bühring et al., 2004). Their expression was stimulated by ANG in early time points and then subsided at day 7 (**Figure 5.9.A**).

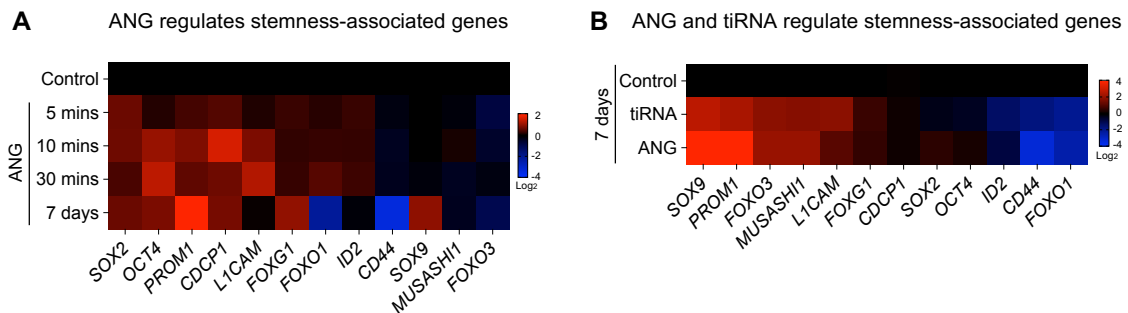


Figure 5.9. ANG and active tiRNA regulate stemness-related genes in GSCs.

(A) Heatmap showing the expression of stemness-related gene in GS6-22 treated by ANG (1 μ g/ml) for 5, 10, and 30 mins. Expression levels were determined by qRT-PCR (n = 3). The heatmap represents the relative mRNA level of each gene normalized to that in PBS treated cells.

(B) Heatmap showing the expression of stemness-related gene in GS6-22 treated by ANG (1 μ g/ml) or transfected with 5'-P-tiRNA-Gly-GCC (100 nM) for 7 days. Expression levels were determined by qRT-PCR (n = 3). The heatmap represents the relative mRNA level of each gene normalized to that in PBS treated transfection control cells.

Another group of stemness-related genes, including *SOX9* and *FOXG1* are later responders that were not stimulated to express by ANG within 30 min but was

expressed on day 7. The significance of these early and later responders and their respective role in ANG-stimulated sphere formation is unclear at present. To compare the effect of ANG and tiRNA-Gly-GCC, we focused on the expression level of these stemness-related genes on day 7 and found that 5'tiRNA-Gly-GCC largely phenocopied the effect of ANG (**Figure 5.9.B**), supporting the notion of the above paragraph that ANG-PLXNB2 regulates GSC properties through tiRNA-Gly-GCC.

Together, these data demonstrate that ANG promoted GSC stemness through an elevated production of bioactive tiRNAs, primary 5'tiRNA-Gly-GCC, and this process was mediated by PLXNB2.

5.5 ANG-PLXNB2 axis regulated the metabolism of microRNAs, snRNAs, snoRNAs, and rRNAs in GSCs

To reveal if ANG/PLXNB2 axis regulates other RNA metabolism, we investigated levels of microRNA as well as fragments derived from snRNAs, snoRNAs, and rRNA in GSCs upon ANG stimulation or *PLXNB2* ablation. MicroRNAs are ~22 nucleotides single-strand non-coding RNAs that have important roles in cell differentiation, survival, and proliferation. MicroRNAs function through targeting the complementary sequence on messenger RNA (mRNA) to induce translational inhibition or mRNA degradation (Rupaimoole and Slack, 2017). ANG has been reported to have ribonucleolytic activities on microRNAs (Sheng and Xu, 2016; Thomas et al., 2018; Weng et al., 2012). We therefore analyzed microRNA abundance from our small RNA sequencing reads and found that ANG treatment led to a marked reduction in microRNA levels by 54% in GS6-22 and 81% in MGG18 cells (**Figure 5.10 and Table 5.7**), suggesting a role of ANG in microRNA degradation.

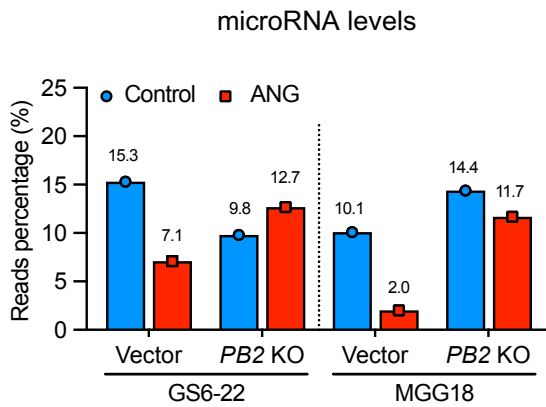


Figure 5.10. The microRNA levels in vector control or *PB2*KO GSCs in the presence or absence of exogenous ANG.

The microRNA level of the indicated group was calculated by total microRNA reads divided by total reads. Paired-end clean reads were aligned to the reference genome, GRCh38 (hg38).

Table 5.7. The microRNA levels in vector control or *PB2*KO GSCs in the presence or absence of exogenous ANG

	GS6-22				MGG18			
	Vector control		<i>PLXNB2</i> knockout		Vector control		<i>PLXNB2</i> knockout	
	PBS	ANG	PBS	ANG	PBS	ANG	PBS	ANG
Total read count	12163118	10313411	9566229	13561375	12820575	12334771	2844999	11620525
Total microRNA reads	1861200	729079	938468	1720527	1299212	242605	409134	1356296
microRNA reads percentage (%)	15.3	7.1	9.8	12.7	10.1	2.0	14.4	11.7
Percentage of change (%)		-54%		29%		-81%		-19%
microRNA reads per million	153020	70692	98102	126870	101338	19668	143808	116716
ANG treatment: 1 µg/ml for 1 day, PBS as control								

To avoid comparison bias introduced by extreme low reads, our analyses focused on microRNA species that possessed at least 100 reads per million total reads occurred in vector control cells. We set the cut-off fold at 2 log₂ scale in each direction as previously described and found that ANG strongly decreased the levels of miRNA25, miRNA29A and miRNA598 in both GSC lines (**Figure 5.11 and Table 5.8**). In MGG18 GSCs, the abundance of additional 43 microRNAs was found to be significantly reduced by ANG (**Figure 5.11 right panel, and Table 5.8**).

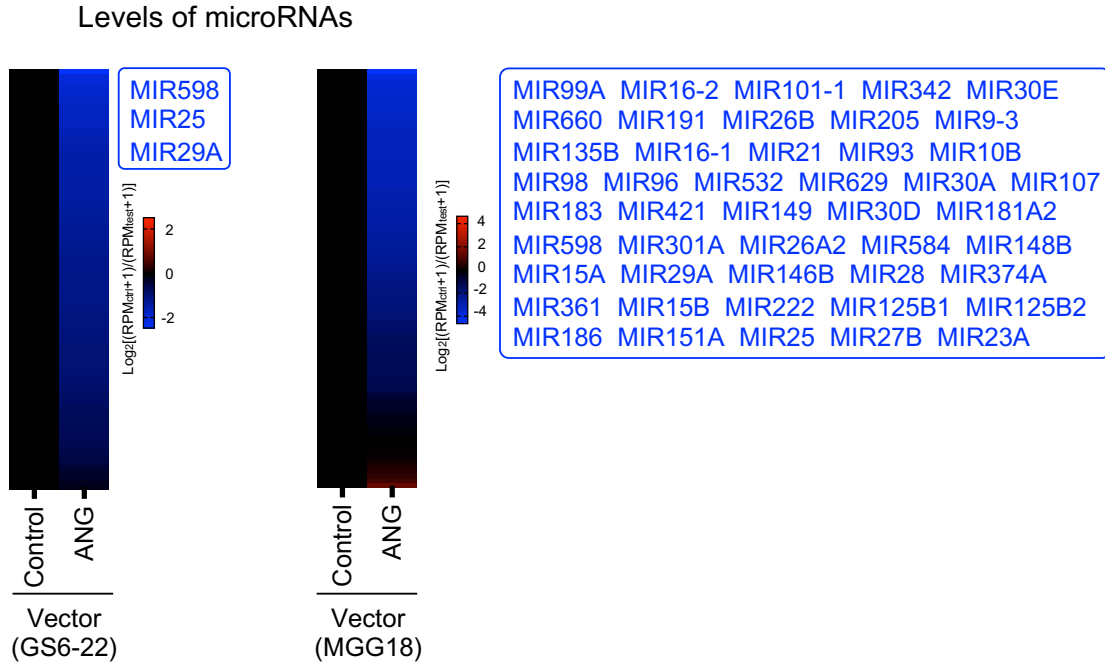


Figure 5.11. The effect of ANG stimulation on microRNAs.

Heatmaps showing the effect of ANG stimulation on microRNA levels in GS6-22 cells (left panel) and MGG18 cells (right panel). Each value presents $\text{Log}_2((\text{RPM}_{\text{test}}+1)/(\text{RPM}_{\text{ctrl}}+1))$ for the indicated conditions. MicroRNAs that have less than 100 RPM in PBS-treated group were not included. Heatmaps were arranged by placing the most downregulated microRNAs on the top according to the values in the ANG-treated group. MicroRNAs that have $\text{Log}_2((\text{RPM}_{\text{test}}+1)/(\text{RPM}_{\text{ctrl}}+1)) \leq -2$ were listed in the text box.

Even though that *PLXNB2* KO did not provide the opposite regulation on the levels of these microRNAs, addition of exogenous ANG did not significantly alter the levels of any microRNAs in *PLXNB2*-null cells (**Table 5.8.**), suggesting that *PLXNB2* mediates the function of exogenous ANG in regulating microRNAs. It is unclear at present for the reason that *PLXNB2* KO did not alter microRNA levels in the absence of exogenous ANG. One of the hypotheses, which needs to be tested experimentally, is that a certain level of ANG concentration is needed to degrade microRNA, which could only be reached by taking up exogenous ANG. The level of ANG required for tiRNA production could be lower, so the amount of internalized endogenous ANG was enough for this purpose. In any events, these observations indicate that ANG takes part in microRNA metabolism in GSCs, and this process is mediated by *PLXNB2*.

Table 5.8. Levels of different microRNAs in vector control or *PB2*KO GSCs in the presence or absence of exogenous ANG

GS6-22					MGG18			
	Vector	<i>PB2</i> KO	GS6-22			Vector	<i>PB2</i> KO	MGG18
	ANG/PBS	ANG/PBS	KO/VC			ANG/PBS	ANG/PBS	KO/VC
MIR598	-2.48	0.26	0.20	1	MIR99A	-4.64	0.87	-0.82
MIR25	-2.04	0.44	-0.87	2	MIR16-2	-3.93	-1.82	2.33
MIR29A	-2.03	-0.04	0.74	3	MIR101-1	-3.65	-0.39	0.21
MIR15B	-1.93	0.54	0.06	4	MIR342	-3.64	0.32	-0.34
MIR532	-1.91	0.63	-0.21	5	MIR30E	-3.56	-0.99	-0.94
MIR339	-1.84	-0.42	0.20	6	MIR660	-3.54	-0.29	-1.85
MIR100	-1.83	-0.25	0.35	7	MIR191	-3.50	0.20	-1.67
MIR500A	-1.76	-0.38	-0.22	8	MIR26B	-3.45	1.84	-0.28
MIR125B1	-1.75	-0.14	1.00	9	MIR205	-3.43	-0.33	1.10
MIR3200	-1.74	0.39	-0.82	10	MIR9-3	-3.41	-0.90	-0.29
MIR99A	-1.74	-0.31	1.04	11	MIR135B	-3.36	-0.22	-2.25
MIR28	-1.73	0.30	0.14	12	MIR16-1	-3.21	-1.67	1.50
MIR26B	-1.62	1.34	-0.22	13	MIR21	-3.21	0.13	0.40
MIR374A	-1.60	1.78	0.40	14	MIR93	-3.18	-0.10	-2.12
MIR10B	-1.55	0.81	-0.09	15	MIR10B	-3.15	1.01	-0.74
MIR501	-1.53	-0.75	-0.18	16	MIR98	-3.15	1.31	0.75
MIR191	-1.52	0.27	-1.39	17	MIR96	-3.13	-0.72	1.89
MIR107	-1.45	0.75	-0.99	18	MIR532	-3.13	-0.19	-0.01
MIR217	-1.45	1.02	2.49	19	MIR629	-3.11	-0.69	1.50
MIR379	-1.44	0.72	-2.17	20	MIR30A	-3.04	-0.64	-0.36
MIR660	-1.44	0.40	-0.39	21	MIR107	-3.02	-0.59	-0.58
MIR342	-1.44	-0.23	-0.16	22	MIR183	-3.00	0.52	0.75
MIR9-3	-1.44	1.13	-0.78	23	MIR421	-2.98	0.78	-0.97
MIR411	-1.44	1.37	-2.47	24	MIR149	-2.98	-0.93	1.68
MIR340	-1.44	0.93	0.35	25	MIR30D	-2.97	-0.09	-1.80
MIR106B	-1.43	-0.04	0.63	26	MIR181A2	-2.87	-0.51	0.86
MIR183	-1.42	-0.19	-0.62	27	MIR598	-2.84	0.02	0.46
MIR125B2	-1.39	0.24	0.16	28	MIR301A	-2.80	-0.45	-2.12
MIR421	-1.36	0.75	-1.26	29	MIR26A2	-2.78	0.36	-1.53
MIR151A	-1.35	0.42	-0.54	30	MIR584	-2.78	-0.81	-0.83
MIR185	-1.33	0.44	-0.69	31	MIR148B	-2.75	-0.77	1.09
MIR324	-1.31	0.01	1.01	32	MIR15A	-2.71	-0.06	-1.80
MIR23A	-1.29	0.98	0.12	33	MIR29A	-2.69	-0.14	1.12
MIR130B	-1.28	0.38	0.93	34	MIR146B	-2.66	0.44	-1.50
MIR1180	-1.27	-0.25	0.01	35	MIR28	-2.56	0.61	1.39
MIR204	-1.27	0.43	-1.09	36	MIR374A	-2.53	0.88	0.46
MIR409	-1.22	0.28	-3.19	37	MIR361	-2.52	0.59	0.27
MIR361	-1.20	0.46	-0.21	38	MIR15B	-2.47	0.69	-0.03
MIR149	-1.18	0.03	-0.79	39	MIR222	-2.46	-0.72	2.48
MIR93	-1.17	0.88	-1.59	40	MIR125B1	-2.38	-0.64	3.10
MIR629	-1.17	0.34	-0.51	41	MIR125B2	-2.30	-1.35	4.09
MIR589	-1.16	0.35	-0.72	42	MIR186	-2.26	-0.66	1.43
MIR7-1	-1.15	1.05	-3.11	43	MIR151A	-2.13	-0.87	1.10
MIR134	-1.13	0.09	-2.03	44	MIR25	-2.07	0.30	1.24
MIR92A2	-1.13	-0.22	-1.17	45	MIR27B	-2.01	-0.77	4.58
MIR381	-1.13	1.50	-3.30	46	MIR23A	-2.01	0.08	3.47
MIR148B	-1.12	1.24	-0.72	47	MIR454	-1.94	0.86	-1.56
MIR584	-1.11	0.10	-2.91	48	MIR190A	-1.93	0.83	-2.51
MIR181A2	-1.10	-0.43	1.90	49	MIR221	-1.89	0.01	0.57
MIR186	-1.07	0.62	-0.80	50	MIR92A2	-1.86	0.27	-0.20

MIR181A1	-1.06	0.40	0.27	51	MIR185	-1.83	-0.83	1.53
MIR98	-1.03	0.31	0.19	52	MIR425	-1.78	0.41	-2.12
MIR320A	-1.03	-0.12	-0.52	53	MIR100	-1.59	1.37	1.80
MIR26A2	-1.03	0.94	-0.50	54	MIR3200	-1.55	-0.95	0.47
MIR296	-1.02	-0.15	0.74	55	MIR181A1	-1.51	-0.84	-0.49
MIR190A	-1.01	1.18	-1.30	56	MIR500A	-1.36	0.14	-0.26
MIR101-1	-0.99	1.17	0.33	57	MIR130B	-1.35	-0.50	2.32
MIR382	-0.98	-0.05	-2.95	58	MIR455	-1.34	-1.24	1.21
MIR146B	-0.96	0.21	-0.53	59	MIR340	-1.34	0.56	0.35
MIR27B	-0.96	0.51	2.42	60	MIR106B	-1.23	-0.24	1.22
MIR30D	-0.95	0.32	-1.45	61	MIR1180	-1.22	0.16	0.95
MIR455	-0.95	1.40	-0.15	62	MIR1301	-1.22	-1.09	-0.75
MIR96	-0.92	1.45	-0.46	63	MIR320A	-1.21	0.45	-1.39
MIR16-2	-0.91	0.43	0.05	64	MIR501	-1.20	0.75	-0.62
MIR21	-0.90	1.07	-0.07	65	MIR181B2	-1.16	-1.20	-1.82
MIR16-1	-0.86	0.34	0.04	66	MIR589	-1.13	-0.97	0.06
MIR182	-0.83	-0.06	-1.30	67	MIR224	-1.06	0.15	0.15
MIR181B2	-0.81	0.35	-1.19	68	MIR182	-0.83	0.74	-0.90
MIR485	-0.79	0.01	-2.93	69	MIR181B1	-0.66	-0.26	-1.61
MIR34A	-0.78	1.20	-1.21	70	MIR708	-0.60	-0.57	1.24
MIR744	-0.70	-0.12	1.22	71	MIR134	-0.55	-0.11	1.41
MIR503	-0.69	-0.80	1.31	72	MIR339	-0.41	-0.99	1.94
MIR454	-0.69	0.75	-0.57	73	MIR34A	-0.31	-0.39	-0.07
MIR181B1	-0.69	0.29	-0.87	74	MIR296	-0.28	-0.18	1.76
MIR135B	-0.69	0.81	-1.60	75	MIR324	-0.24	-1.28	2.24
MIR15A	-0.68	1.42	-0.45	76	MIR485	-0.16	-1.27	2.05
MIR425	-0.63	0.66	-1.28	77	MIR411	-0.02	-0.94	1.44
MIR301A	-0.62	1.80	-0.59	78	MIR744	0.00	-1.34	3.61
MIR708	-0.59	0.75	1.30	79	MIR370	0.07	-1.43	2.51
MIR370	-0.55	-0.20	-1.65	80	MIR382	0.14	0.01	2.46
MIR221	-0.53	0.26	-2.86	81	MIR381	0.14	-0.92	0.50
MIR222	-0.50	0.08	-2.35	82	MIR409	0.26	0.61	1.10
MIR30A	-0.40	0.75	1.82	83	MIR204	0.39	-0.27	1.74
MIR224	-0.31	-0.07	-1.79	84	MIR379	0.48	0.26	0.70
MIR205	-0.29	-0.12	5.04	85	MIR503	0.59	-1.47	0.85
MIR30E	-0.23	1.60	-0.90	86	MIR217	0.90	0.49	-0.41
MIR1301	-0.18	0.33	-1.27	87	MIR7-1	1.46	1.24	-1.55

It is of great significance to examine if any of these highly impacted microRNAs in GSCs regulate stemness- and differentiation-related genes, which is beyond the scope of this thesis research.

SnRNAs and snoRNAs are two class of small RNA molecules of 150 or 60-300 nt, respectively, found within the nucleus. snRNAs have been reported to regulate pro-mRNA processing and maintain telomeres (Matera et al., 2007). SnoRNAs function by guiding the post-transcriptional modification of non-protein coding RNAs, including

rRNAs, tRNAs and snRNAs (Kiss, 2002; Kufel and Grzechnik, 2019). Since our RNA-Seq data were generated from small RNAs of <60 nt that were separated and extracted from acrylamide gel, we did not get much of the full length snRNA and snoRNA. However, we did find that ANG treatment decreased the abundance of their fragments in both GSCs (**Figure 5.12., A-D**), which could be the result of decreased degradation of snRNA and snoRNA or accelerated clean-up of the degradation product of these small RNA species.

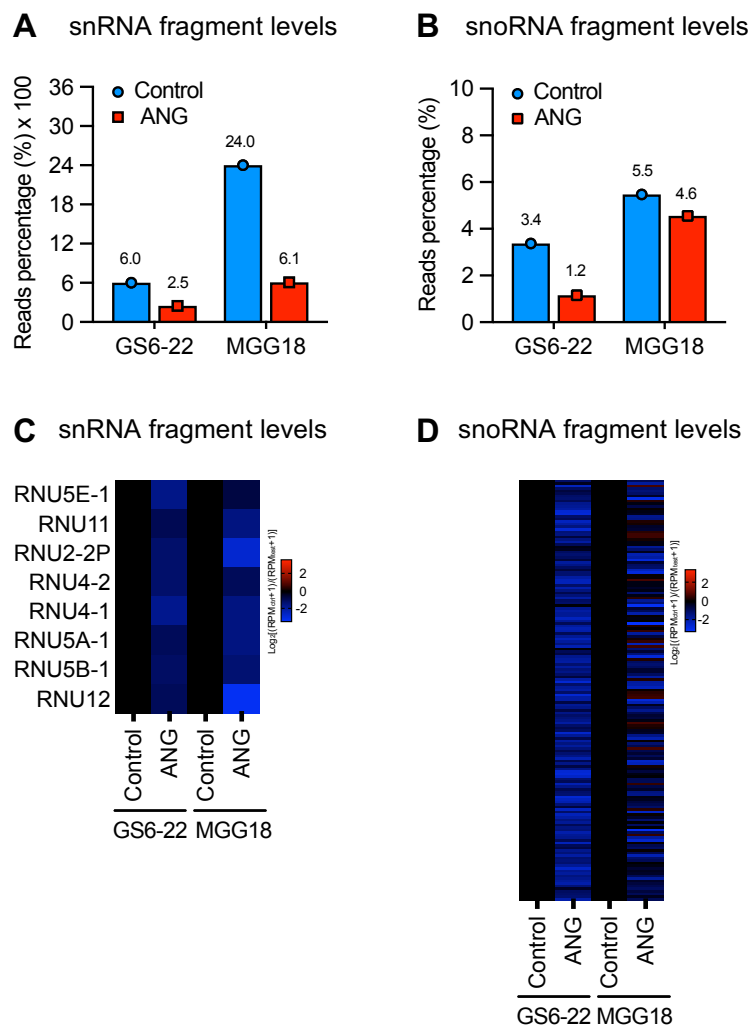


Figure 5.12. The levels of snRNA and snRNA fragments in GSCs in the presence or absence of exogenous ANG.

(A-B) The levels of fragments derived from snRNAs or snoRNAs. The levels of snRNA (A) or snoRNA (B) fragments were calculated by fragment reads divided by total reads of

the respective group. Paired-end clean reads were aligned to reference genome GRCh38.

(C) Heatmaps showing the effect of ANG stimulation on snRNA fragment levels in GS6-22 and MGG18. Each value presents $\text{Log}_2[(\text{RPM}_{\text{control}}+1)/(\text{RPM}_{\text{test}}+1)]$ for the indicated conditions. SnRNAs that have fragment reads less than 10 RPM in PBS-treated vector control group were not included.

(D) Heatmaps showing the effect of ANG stimulation on snoRNA fragment levels in GS6-22 and MGG18. Each value presents $\text{Log}_2[(\text{RPM}_{\text{control}}+1)/(\text{RPM}_{\text{test}}+1)]$ for the indicated conditions. SnoRNAs that have fragment reads less than 10 RPM in PBS-treated vector control group were not included.

In any event, no specific fragments of snRNA and snoRNA were enriched in response to ANG or *PLXNB2* KO in GSCs, and the significance of decreased abundance of the fragments of snRNAs and snoRNAs in GSCs in response to ANG stimulation remain to be determined.

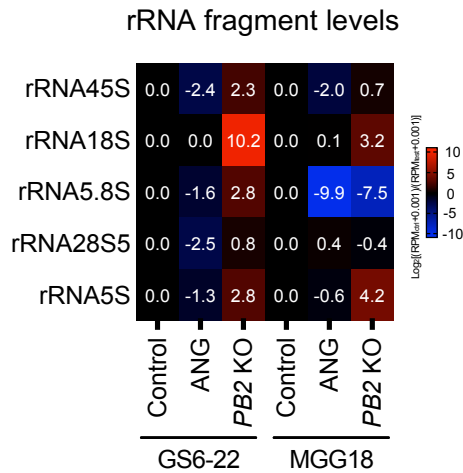


Figure 5.13. The levels of rRNA fragments in GSCs upon ANG stimulation or *PLXNB2* knockout.

Heatmaps showing the effect of ANG stimulation or *PLXNB2* knockout on rRNA fragment levels in GS6-22 and MGG18 cells. Each value presents $\text{Log}_2((\text{RPM}_{\text{test}}+0.001)/(\text{RPM}_{\text{ctrl}}+0.001))$ for the indicated conditions.

ANG has been demonstrated to actively process 5S rRNA to produce a large amount of small RNAs (< 50 nt), later proved to be the 3'-end fragment of 5S rRNA, in prostate CSCs. The bioactive 3'-end fragment of 5S rRNA was shown to regulate

stemness properties of human prostate CSCs and mouse multipotent embryonal carcinoma cells (Li et al., 2020). In the context of GSCs, we found that exogenous ANG decreased while *PLXNB2* ablation increased the abundance of fragments derived from most of the rRNA species (**Figure 5.13.**).

However, no specific changes of rRNA fragments were identified in response to ANG stimulation or upon *PLXNB2* KO in GSCs. Specifically, we did not find any ANG-induced changes of the bioactive 3'-end fragment of 5S rRNA that has been shown to regulate stemness of human prostate CSC (Li et al, 2020), indicating a unique mechanism of ANG-*PLXNB2* in regulating GSCs.

5.6 Chapter contributions

Small RNA sequencing was performed by Genomics, Tufts University Core Facility with technical assistance from Dr. Albert Tai. Other experiments in this chapter were performed by Liang Yuan and supervised by Dr. Guo-fu Hu.

**Chapter 6: Inhibition of PLXNB2 reduces tumorigenicity of GSCs and enhances
chemo-sensitivity**

6.1 *PLXNB2* knockout abolished tumorigenic activity of GSCs

Capacity to initiate orthotopic tumor and ability to pathologically recapitulate the complexity of primary tumor are two functional criteria of CSC stemness (Lathia et al., 2015). To examine the role of *PLXNB2* in regulating GSC stemness *in vivo*, we prepared stable luciferase-expressing vector control and *PLXNB2* KO MGG18 cells and stereotactically implanted them into the SVZ in the brains of athymic nude mice, and monitored tumor growth by Firefly luciferase (Fluc) bioluminescence (BLI) imaging in a schedule shown in Figure 6.1.

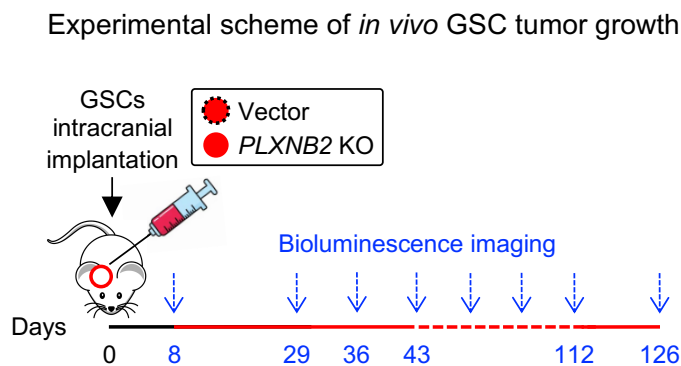


Figure 6.1. Schematic diagram of tumorigenicity experiment.

Luciferase-expressing vector control or *PLXNB2* knockout MGG18 (4×10^5 per mouse) were stereotactically implanted into the brains of athymic nude mice. Tumor growth was monitored by Fluc BLI imaging on the indicated days.

Same number (4×10^5 per mouse) of vector control and *PLXNB2* KO cells were implanted and tumor growth was assessed by BLI (**Figure 6.2.A**) on day 8, 29, 36, 43, 49, 54, 62, 72, 85, 106, 112, and 126 on all live animals. Total flux of each animal was shown in Figure 6.2.B. On day 8, when the first imaging was taken, all animals in both groups gave BLI signals, indicating successful implantation of GSCs. The average total flux in the vector control and *PLXNB2* KO group was $2.8 \pm 0.4 \times 10^5$ and $1.2 \pm 0.2 \times 10^5$ photon/second (p/s), respectively (**Figure 6.2.C**), suggesting that a slower *in vivo* growth of *PLXNB2* KO GSCs or a faster death as compared to the vector controls. On day 29,

the average total flux in the vector control group was $1.0 \pm 0.1 \times 10^{10}$ p/s, representing 35,885-fold increase in tumor mass as compared to that on day 8. In the *PLXNB2* KO group, the average total flux was $1.5 \pm 0.2 \times 10^4$ p/s, indicating a decrease in tumor cell number by 87.5% as compared to that on day 8 (**Figure 6.2.C**). In the vector control group, the first animal died on day 29, the last animal died on day 62, and the median survival was 50 days (**Figure 6.2.D**). All animals in the *PLXNB2* KO group survived until day 126 when they were sacrificed for pathological examination of tumors in the brain. No tumor lesions were found on and around the injection site in all animals of the *PLXNB2* KO group, indicating all the implanted *PLXNB2*-ablated GSCs have died. Serial imaging results show that BLI signals started to be undetectable in all animals in the *PLXNB2* KO group since day 72 (**Figure 6.2., A and B**). Consistent with no tumor growth the *PLXNB2* KO group, the body weight of animals in this group were not changed until the end of experiment, however, the animals in the vector control group suffered a drastic loss of body weight before death (**Figure 6.2.E**). These results demonstrate that *PLXNB2* KO impaired tumor-initiating capability of GSCs *in vivo*. An AACR workshop on cancer stem cells defined CSC as “a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineage of cancer cells that comprise the tumor” (Clarke et al., 2006). In other word, cancer stem cells have three criteria, which are self-renewal ability, differentiation potential, and tumor-initiating capacity. The above results indicate that *PLXNB2* is essential for the tumor-initiating capacity of GSCs. Together with the findings that *PLXNB2* KO diminished self-renewal ability of GSCs (**Figures 4.5. and 4.7.**) and promoted GSC differentiation (**Figures 4.9. - 4.12.**), we tend to conclude that ANG-*PLXNB2* plays an important role in maintaining the stemness of GSCs and that *PLXNB2* inhibition leads to attenuation or loss of the stemness of GSCs.

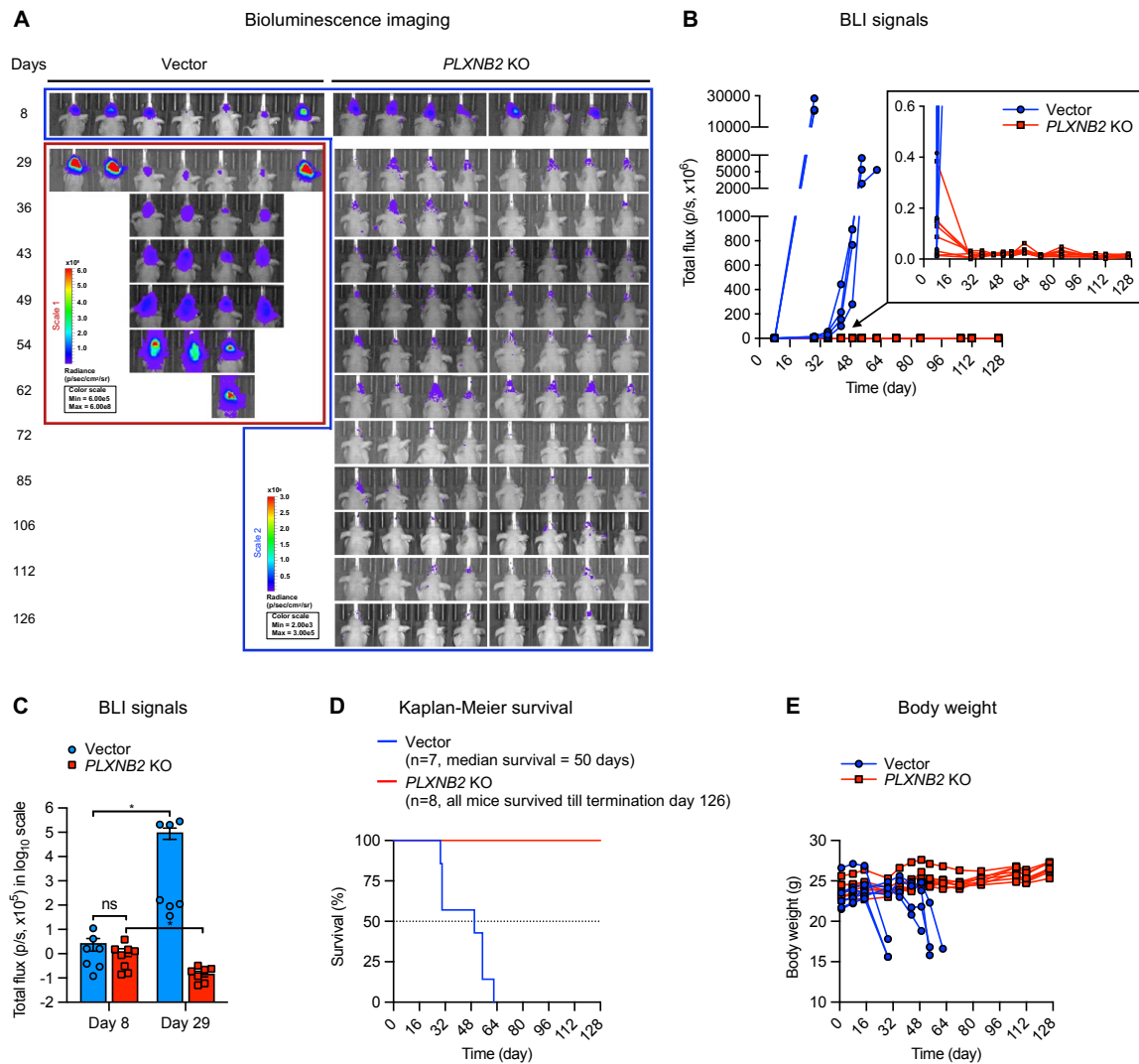


Figure 6.2. *PLXNB2* knockout abolished GSC tumorigenicity.

(A) Bioluminescence live imaging monitoring orthotopic tumor growth. MGG18 vector control group (n = 7), *PLXNB2* knockout group (n = 8).

(B) Quantification of tumor BLI signals (total flux) in mice of vector control or *PLXNB2* knockout group throughout the course of study. Insets (right) shows BLI signals at a lower scale.

(C) Tumor BLI signals on day 8 and 29. * $p < 0.05$, ns = not significant by unpaired two-tailed Student's t test.

(D) Kaplan-Meier survival curves of mice orthotopically implanted with vector control or *PLXNB2* knockout MGG18. The median survival for vector control mice is 50 days. All mice from *PLXNB2* knockout group were alive until the experiment termination day (day 126).

(E) Body weight of experimental mice over time.

To confirm that the xenograft tumors derived from the vector controls of MGG18 recapitulated histological features of the MGG18 primary tumor, a giant cell GBM (Wakimoto et al., 2012), we performed histological analysis of the tumors from the vector control group. These tumors displayed extensive pleomorphism comprising a mixture of cells with various sizes and morphologies and contained bizarre-looking multinucleated giant cells (**Figure 6.3.A**). Consistent with the description of MGG18 intracerebral tumors carried by SCID mice (Wakimoto et al., 2012), we also found that the tumors grown from the control cells were well-circumscribed masses (with distinct tumor borders) and vasculature-enriched with prominent intratumoral hemorrhages (**Figure 6.3., A and B**). No tumors were found in the brain of the mice inoculated with *PLXNB2* KO cells by histological analysis (**Figure 6.3.B**).

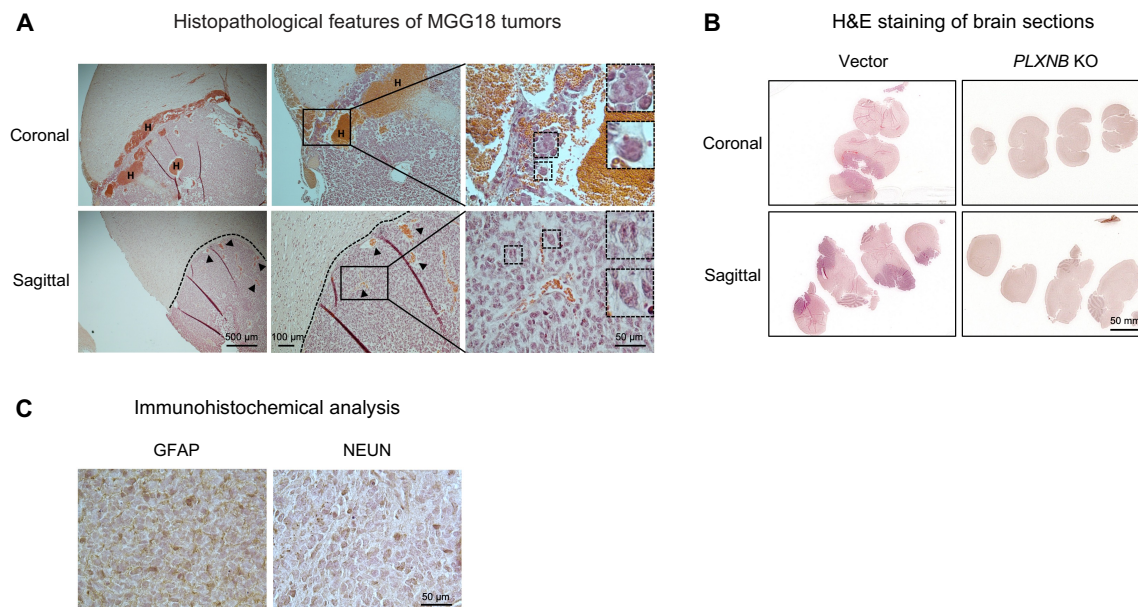


Figure 6.3. Post-mortem histopathological analysis.

(A) Histopathological features of intracranial xenografts of vector control MGG18. Representative H&E staining of coronal (top) and sagittal (bottom) brain sections were shown. Scale bars: 500 (left), 100 (middle) or 50 (right) μ m. MGG18 tumors displayed marked cellular pleomorphism and contain giant and bizarre-looking neoplastic cells with multiple hyperchromatic nuclei. In the right panels, multinucleated giant cells were highlighted by dotted line boxes and magnified in insets. MGG18 tumors are associated

with intratumoral hemorrhages (hemorrhage area labeled as H) and prominent vasculature proliferation (arrowhead). MGG18 tumors were characterized as discrete nodular subtype with distinct borders. Dotted lines delineated brain-tumor borders. (B) Representative H&E staining of brain sections of vector control or *PLXNB2* knockout groups. Scale bar: 5 mm. (C) Representative immunohistochemical staining of GFAP and NEUN in vector control MGG18 xenografts. Scale bar, 50 μ m.

Lastly, the staining of neural lineage-associated proteins, astrocytic marker GFAP and neuronal marker NEUN in xenograft tumors derived from vector control MGG (**Figure 6.3.C**) resembled that found in the primary patient tumors (Wakimoto et al., 2012). Taken together, these data confirmed that the vector control MGG18 cells were able to initiate xenograft tumors resembling the histological and phenotypic features of the original GBM, whereas *PLXNB2* ablation resulted in loss of tumor-initiating capacity.

6.2 PLXNB2 mAbs sensitized GSCs to chemotherapeutic agent TMZ *in vivo*

TMZ is an oral DNA alkylating drug that produces cytotoxic lesions by methylating guanines at O⁶ position. O⁶-MG incorrectly pairs with thymine that triggers mismatch repair and leads to apoptosis (Mrugala and Chamberlain, 2008). While TMZ remains the most effective chemotherapeutic agent for treating GBM to date (Stupp et al., 2005), chemoresistance to TMZ occurs in most GBMs and is the leading cause for disease recurrence and treatment failure (Lee, 2016; Mrugala and Chamberlain, 2008). GBM evades TMZ-mediated cytotoxic effect via various mechanisms (Jiapaer et al., 2018). Overexpression of MGMT, a methyltransferase that directly repairs TMZ-induced O⁶-MG lesions, is one of the major contributors to TMZ resistance (Mrugala and Chamberlain, 2008). Much effort has been made to tackle MGMT-positive GBMs. Compounds that aim to down-regulate *MGMT* (promoter methylation and transcriptional suppression) or enzyme efficacy (use of irreversible false MGMT substrates) are being intensively investigated in clinical studies (Jiapaer et al., 2018; Nakada et al., 2012). Yet,

TMZ resistance also arise from other mechanisms independent of MGMT, such as inactivation of mismatch repair (Atkins et al., 2015), elevated base excision repair (Erasmus et al., 2016), intrinsic characteristics of GSCs (Jiapaer et al., 2018), and etc.

Because inhibition of ANG/PLXNB2 axis attenuated GSC stemness (**Chapter 3 and Chapter 4**) and intracranial tumorigenicity (**Figure 6.2.**), we sought to examine if PLXNB2 inhibitors could sensitize GSCs to TMZ and delay disease recurrence. MGG18 was used for this purpose. As described before, MGG18 harbors *PTEN* and *CDKN2A* deletion (Wakimoto et al., 2012). MGG18 does not express MGMT but is highly resistant to TMZ with an *in vitro* IC₅₀ of 445.8 μ M (Jahan et al., 2017). MGG18 was also found to be insensitive to PARP inhibitors (Ning et al., 2019). PARP is known to regulate MGMT-independent chemoresistance and is one of the most investigated DNA repair enzymes for chemo-sensitization therapy (Gupta et al., 2018; Tentori et al., 2014). Thus, the TMZ-resistant, MGMT-negative, and PARPi-insensitive MGG18-propagated GBM, is suitable for us to examine the effect of PLXNB2 mAbs on chemo-resistance and disease recurrence.

Stable luciferase-expressing MGG18 cells (4×10^5 cells per mouse) were stereotactically implanted into the SVZ in the brains of athymic mice, and tumor growth was monitored by BLI starting from post-implantation day 5 in a schedule shown in Figure 6.4. On day 28, when the tumors grew to a certain size and when the blood-brain barrier was projected to become compromised, the animals were separated into two groups and treated with TMZ or TMZ + PLXNB2 mAb, respectively. Table 6.1 lists the total flux of each mouse in both groups on day 28. There was considerable size distribution of the initial tumors among the animals, probably due to inter-animal heterogeneity. These animals were first categorized into two groups based on the initial tumor size. The first group consisted of 10 animals that had the flux signal ranging from 2.3×10^5 to 1.9×10^6 p/s, and the second group had 5 mice with the flux value between

5×10^7 and 1.2×10^{10} p/s. These two groups of mice were then further separated into two subgroups designated as group TMZ and group TMZ + PB2 mAb. As shown in Table 6.1., group TMZ had 5 mice bearing smaller tumor size of average flux 8.5×10^5 p/s and 2 mice bearing larger tumor size of average flux of 3.7×10^9 p/s. Group TMZ + PB2 mAb had 5 mice of average flux 9.4×10^5 p/s and 3 mice of average flux of 4.0×10^9 p/s. The average flux signals, before starting the treatment, were 1.1×10^9 (n = 7) and 1.5×10^9 (n = 8) p/s, respectively, in the animals of TMZ and TMZ + PB2 mAb groups, respectively (Table 6.1 and Figure 6.5.A).

Experimental scheme of *in vivo* experiment

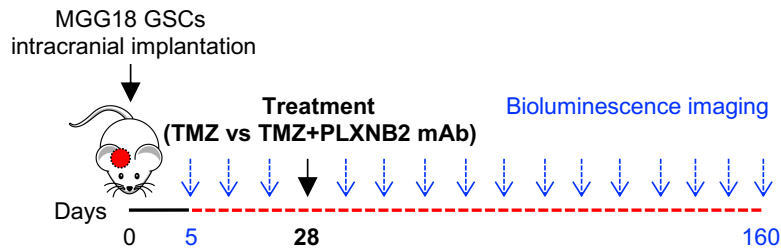


Figure 6.4. Schematic diagram of *in vivo* chemosensitization experiment.

Luciferase-expressing MGG18 vector control GSCs (4×10^5) were stereotactically implanted into the brains of athymic nude mice. Based on the size of tumors, mice were randomized into two treatment groups on day 28: TMZ group (25 mg/kg TMZ, n = 7) or TMZ + PB2 mAb group (25 mg/kg TMZ + 30 mg/kg PB2 mAb, n = 8). Tumor growth was monitored by Fluc BLI imaging on indicated days.

Table 6.1. BLI signals of orthotopic tumors on day 28

Day 28				
	TMZ	Average	TMZ + PB2mAb	Average
Total flux (p/s)	2.3E+05	8.5E+05	4.1E+05	9.4E+05
	9.6E+05		6.5E+05	
	9.7E+05		7.6E+05	
	1.0E+06		1.0E+06	
	1.1E+06		1.9E+06	
	2.6E+09	3.7E+09	5.0E+07	4.0E+09
	4.8E+09		2.8E+08	
			1.2E+10	
Average	1.1E+09		1.5E+09	
T-test	0.77			

A single injection of TMZ (25 mg/kg, i.p. or TMZ + PLXNB2 mAb (25 mg/kg TMZ + 30 mg/kg PLXNB2 mAb, i.p.) on day 28 resulted in a significant shrinkage of tumor size in both groups as indicated by substantial decrease of BLI signals on day 36 (**Figure 6.5.B**). The degree of reduction in BLI signals were similar in both groups, indicating that TMZ treatment was the cause of tumor shrinkage and that PLXNB2 mAb did not contribute to TMZ-induced death of MGG18-derived GBM cells. On day 54, BLI signals started to increase in the TMZ group but not in the TMZ + PB2 mAb group (**Figure 6.5.B**), indicating tumor recurrence only in the TMZ group. Weekly BLI showed that by day 74, all 7 animals in the TMZ group had relapsed tumors while all 8 animals in the TMZ + PB2 mAb group remained in the remission state (**Figure 6.5.C**).

The remission and recurrence of MGG18 tumors resembled that of the clinical course of most GBM patients. Mice that received TMZ alone responded to the chemotherapy, but uniformly underwent disease relapse starting from day 54 (**Figure 6.5., B, C, and D**), experienced progressive weight loss as tumors propagated (**Figure 6.5.E**) and inevitably succumbed to orthotopic tumors with the median survival of 83 days (**Figure 6.5.F**). Mice treated with TMZ together with PLXNB2 mAb had a similar initial response but remained in remission much longer and delayed the recurrence (**Figure 6.5, B-D**), and substantially extend the median survival to 106 days (**Figure 6.5.F**), representing a 28% extension in survival. Of note, two of the eight mice in the TMZ + PB2 mAb group remained disease free until day 160, the end of the experiment (**Figure 6.5.F**). These two mice, one (mouse No. 3009) from the initially large tumor subgroup and one (mouse No. 3017) from the small tumor subgroup, did not experience any weight loss (**Figure 6.5.E**) and gain in BLI signals (**Figure 6.5.G**) during the course. Actually, BLI signals were undetectable (**Figure 6.5.H**) on the day they were sacrificed for post-mortem examinations (**Figure 6.5.I and Figure 6.6.B**), which revealed no tumor lesions in and around the injection site.

These results demonstrate that combinatorial treatment of TMZ and PLXNB2 mAb prevented MGG18 tumor relapse *in vivo*, suggesting a chemo-sensitization function of PLXNB2 against TMZ-resistant GBM.

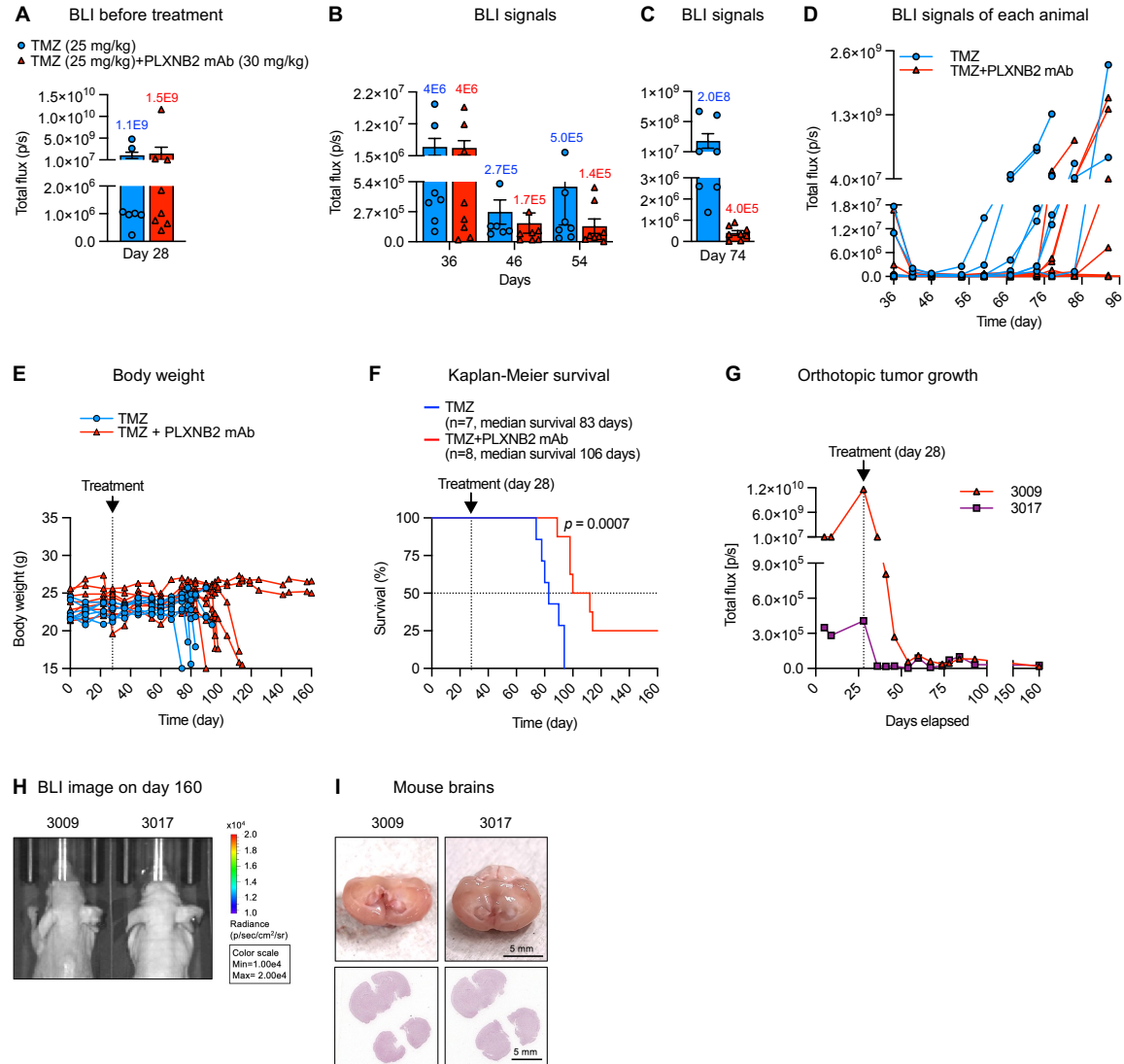


Figure 6.5. PLXNB2 mAb sensitized GSCs to TMZ therapy and prolonged the survival.

(A) Orthotopic tumor growth on day 28, prior to treatments. Tumor BLI signal was presented by the total flux (p/s). TMZ group (n = 7), TMZ + PB2 mAb group (n = 8).
 (B) Tumor total flux on day 36, 46, and 54 (treatment on day 28).
 (C) Tumor total flux on day 74.
 (D) Orthotopic tumor growth in mice treated with TMZ or TMZ + PB2 mAb.
 (E) Body weight of mice treated with TMZ or TMZ + PB2 mAb.

(F) Kaplan-Meier survival curves of mice treated with TMZ or TMZ + PB2 mAb. The median survival time was 83 and 106 days for TMZ and TMZ + PB2 mAb groups, respectively. Log-rank test was used to calculate statistical significances.

(G) Orthotopic tumor growth in mouse 3009 and 3017 that were treated with TMZ + PB2 mAb.

(H) BLI images of mouse 3009 and 3017 that were treated with TMZ + PB2 mAb on day 160.

(I) Images of coronal section of the brain of mouse 3009 and 3017. Brains were harvested immediately after animal were euthanized on day 160. Scale bar: 5 mm.

Brain images and H&E staining of mouse brains or tumor lesions are shown in

Figure 6.6.

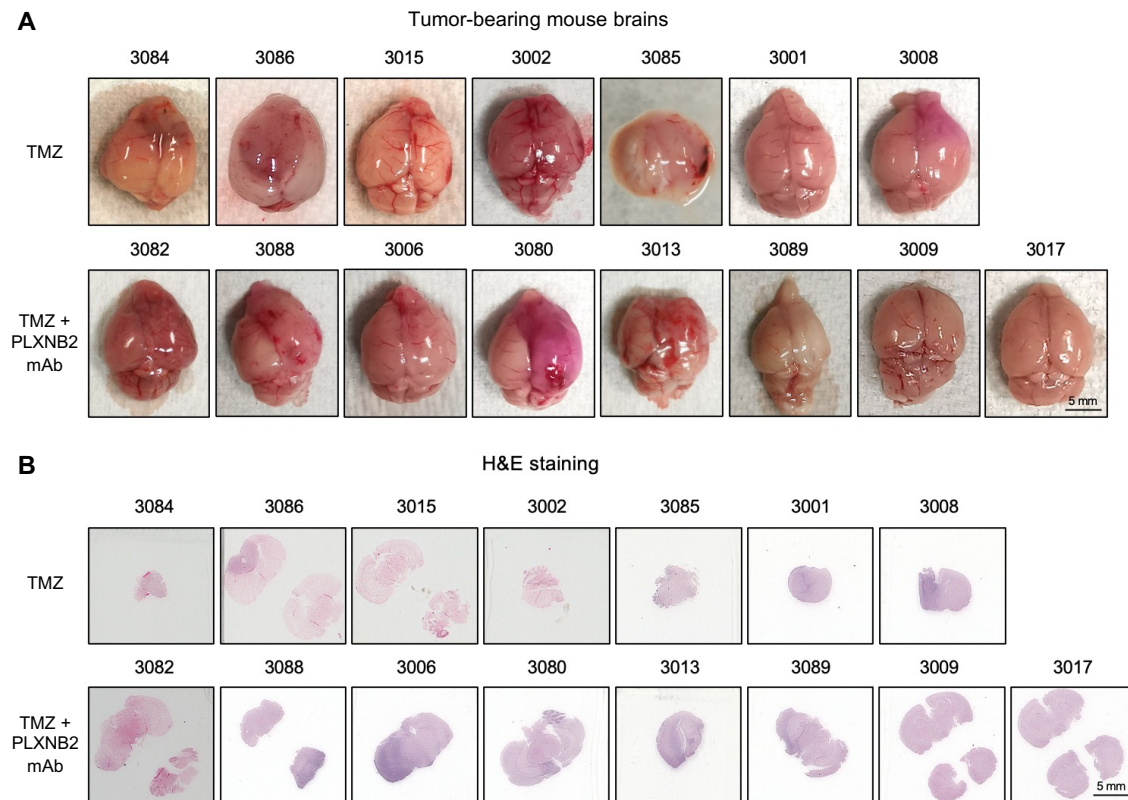


Figure 6.6. Post-mortem examination of mouse brains.

(A) Mouse brains of TMZ group (n = 7) or TMZ + PB2 mAb group (n = 8). Scale bar: 5 mm.

(B) H&E staining of brain or tumor sections. Scale bar: 5 mm.

To demonstrate that the PLXNB2 mAb indeed crossed the BBB of tumor bearing mice, we examined the abundance of mouse IgG in the tumor lesions from the TMZ

group and from the TMZ + PB2 mAb group. Immunoblotting analysis of tumor lysates (25 μ g total protein) with an antibody against mouse IgG revealed two strong bands corresponding to the molecular weight of the light and heavy chains of IgG in samples from the TMZ + PB2 mAb group but not from the TMZ group (**Figure 6.7.A**).

Immunofluorescence staining with an Alexa 488 conjugated antibody against mouse IgG showed punctate staining in the section from the TMZ + PB2 mAb group but not in those from the TMZ group (**Figure 6.7.B**). These results confirmed that i.p.-injected PLXNB2 mAb indeed crossed the BBB of the GBM-bearing mice and penetrated into the tumor tissues.

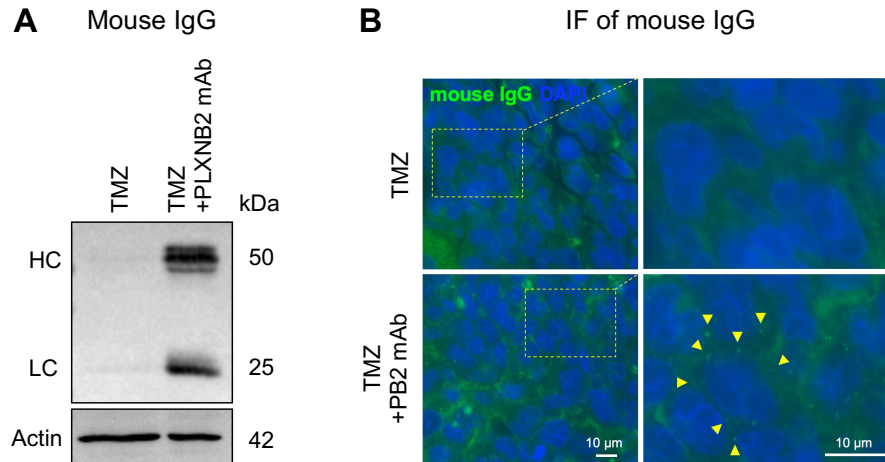


Figure 6.7. A successful delivery of PLXNB2 antibody to intracranial xenografts.

(A) Immunoblotting of mouse IgG heavy and light chains using 25 μ g total protein extracted from xenograft tumor tissues (n = 3). Actin was used as the loading control. Relative levels were determined by ImageJ and normalized to TMZ group.

(B) Immunofluorescence staining of mouse IgG on xenograft tumor sections (n = 3). Alexa Fluor-488 conjugated anti-mouse IgG (green) was employed to stain mouse IgG. Highlighted regions by dotted lines were magnified on the right. Green puncta were labeled by yellow arrowheads. Nuclei were stained by DAPI. Scale bar, 10 μ m.

There was no difference in end tumor size (**Table 6.2**) between two groups and no difference in tumor invasions (data not shown).

Table 6.2. Tumor weight analysis

TMZ		TMZ + PB2 mAb	
Mouse	Tumor weight (g)	Mouse	Tumor weight (g)
3084	0.10	3082	0.22
3086	0.10	3006	0.09
3015	0.10	3088	0.09
3085	0.13	3080	0.26
3002	0.14	3013	0.10
3001	0.08	3089	0.12
3008	0.10		
Average	0.11	Average	0.15
SD	0.02	SD	0.08

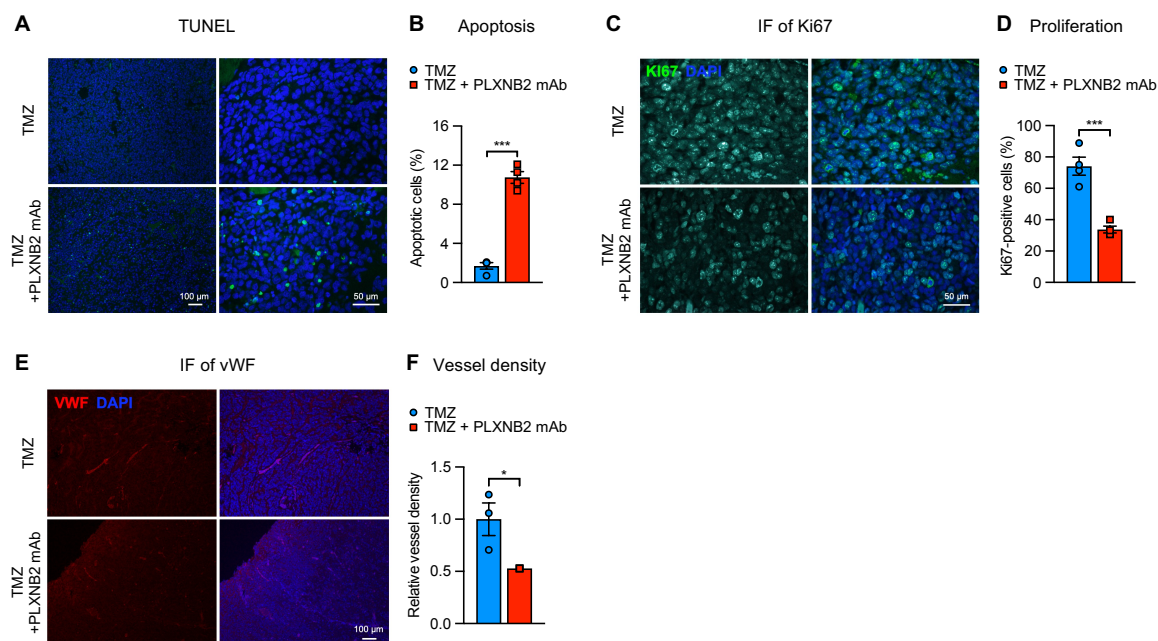


Figure 6.8. PLXNB2 mAb enhanced apoptosis, suppressed proliferation, and attenuated angiogenesis of orthotopic MGG18 tumors.

(A-B) Apoptotic cells detected by TUNEL assay in xenograft tumor sections of mice treated with TMZ or TMZ + PB2 mAb (n = 4). The rates of TUNEL positive cells were analyzed using 40x images (250-350 cells per image). Scale bar: 50 or 100 μ m. A, representative images; B, quantification of apoptotic cells.

(C-D) Immunostaining of Ki67 in xenograft tumor sections of mice treated with TMZ or TMZ + PB2 mAb (n = 4). Scale bar: 50 μ m. C, representative images; D, quantification of Ki67-positive cell rates.

(E-G) Immunostaining of VWF in xenograft tumor sections of mice treated with TMZ or TMZ + PB2 mAb (n = 3 - 6). Scale bar: 100 μ m. E, representative images; F, relative vessel density normalized to TMZ group by quantifying numbers of vessels per field.

* $p < 0.05$, *** $p < 0.001$ by unpaired two-tailed Student's t test.

However, TUNEL staining showed that apoptosis was robustly enhanced in the tumor lesions from TMZ + PB2 mAb group as compared to the TMZ group (**Figure 6.8.A**). The percentage of apoptotic cells was 1.7% in the TMZ-treated lesions and was 10.7% in the lesions treated with TMZ + PB2 mAb (**Figure 6.8.B**), representing 6.4-fold increase by PLXNB2 mAb. Ki67 staining showed that cell proliferation was lower in the tumor section from TMZ + PB2 mAb group as compared to that from the TMZ group (**Figure 6.8.C**). Ki67-positive cells were 74.1 and 33.7%, respectively, in the TMZ and TMZ + PB2 mAb groups, indicating that PLXNB2 mAb decreased cell proliferation by 54.5% (**Figure 6.8.D**). Moreover, tumor angiogenesis was also lower in the TMZ + PB2 mAb group than in the TMZ group as shown by VWF staining (**Figure 6.8, E-F**). These results are consistent with the *in vitro* findings that PLXNB2 inhibition either by gene ablation or by mAbs treatment impaired sphere formation (**Figures 4.2 and 4.5**), decreased cell proliferation (**Figures 4.2 and 4.5**), and induced apoptosis (**Figure 4.9, C-E**) of GSCs *in vitro*.

We have demonstrated that ANG-PLXNB2 promoted GSC stemness through the production of bioactive tiRNAs, primary 5'tiRNA-Gly-GCC (**Figures 5.7., 5.8., and 5.9.B**). To understand if PLXNB2 mAb delayed recurrence of TMZ-treated MGG18 tumors through 5'tiRNA-Gly-GCC, we first performed small RNA gel electrophoresis to examine the level of small RNAs extracted from tumor tissues dissected from all the tumor-bearing animals of the TMZ group (7 mice) and the TMZ + PB2 mAb group (6 mice, 2 mice were tumor free). Figure 6.9.A showed the abundance of small RNAs were in general lower in the TMZ + PB2 mAb group than in the TMZ group.

The area of 35-40 nt, corresponding to size of tiRNA, was quantified and showed that the average intensity of the 6 tumors from TMZ + PB2 mAb group was 50.3% of that of the 7 tumors from the TMZ group (**Figure 6.9.B**). Interestingly, mice bearing the tumors of higher small RNA levels seem to have had a worse survival than that of lower

small RNA levels across both groups. There is a significant inverse correlation between animal survival and the intra-tumor level of small RNA (**Figure 6.9.C**), the meaning of which remains to be investigated.

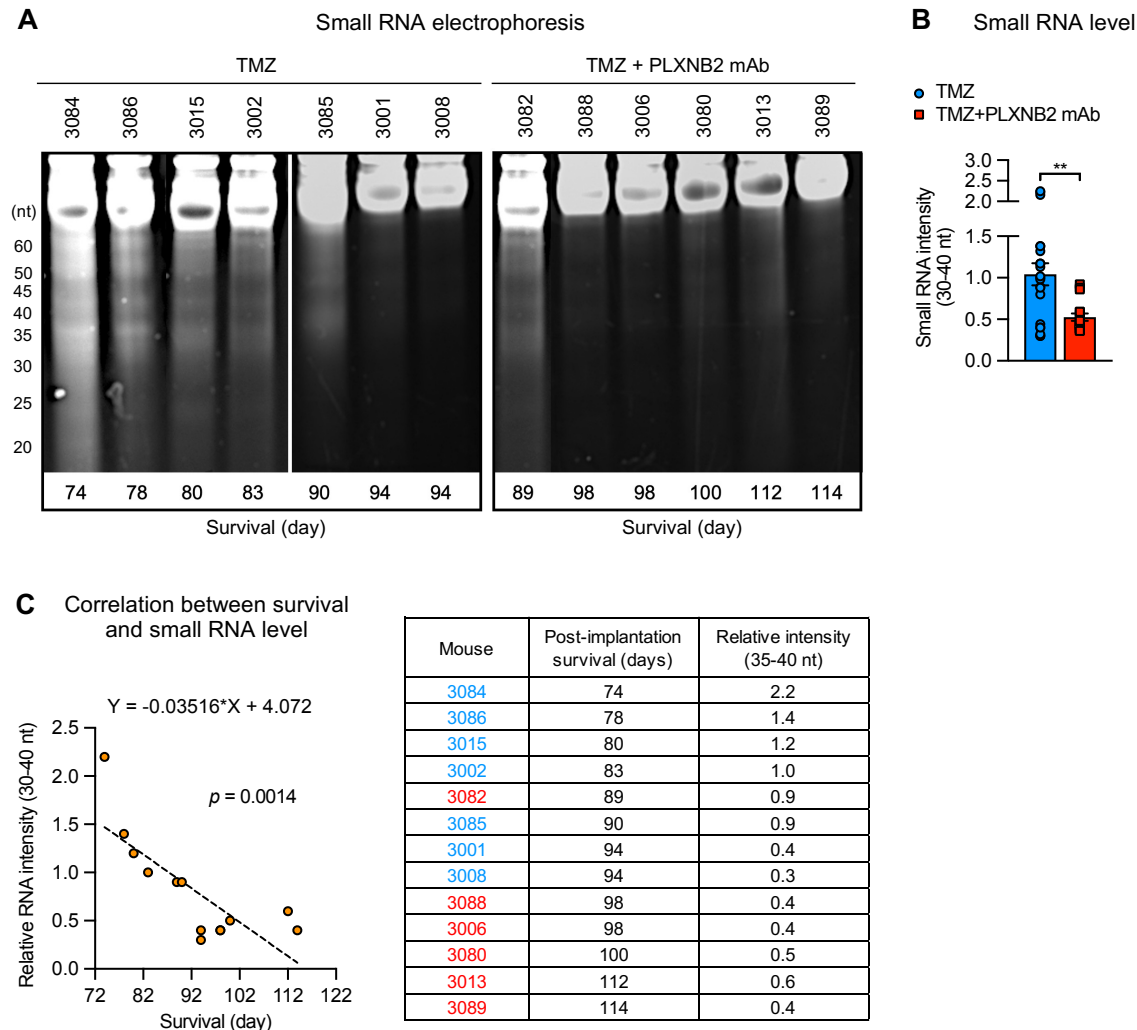


Figure 6.9. PLXNB2 mAb attenuated small RNA levels in xenograft tumors of TMZ-treated mice.

(A-B) Small RNA levels in xenograft tumors from TMZ or TMZ + PB2 mAb group (n = 6 - 7). A, 20 µg total RNA analyzed by polyacrylamide gel electrophoresis and SYBR Gold staining. Survival time was indicated at the bottom. B, band intensities (35-40 nt) were determined by Image J and presented as relative values normalized to the value of sample 3002 (TMZ group). ** $p < 0.01$ by unpaired two-tailed Student's t test.

(C) Correlation analysis of survival time (x-axis) and RNA band intensity at 35-40 nt (y-axis). Pearson correlation analysis was performed, $r^2 = 0.62$ and $p = 0.0014$. Detailed information was presented as a table panel, TMZ group labeled in blue and TMZ + PB2 mAb in red.

Next, we performed TaqMan small RNA assay to quantify tiRNA-Gly-GCC in each tumor and found that tumors treated with TMZ + PB2 mAb had significantly lower tiRNA-Gly-GCC levels than those treated with TMZ alone (**Figure 6.10.A**). The average level of tiRNA-Gly-GCC in the TMZ + PB2 mAb group is 4.3-fold lower than (or 23% of) that in the TMZ group (**Figure 6.10.B**). Again, we noticed a positive correlation between the level of tiRNA-Gly-GCC and animal survival (**Figure 6.10.C**).

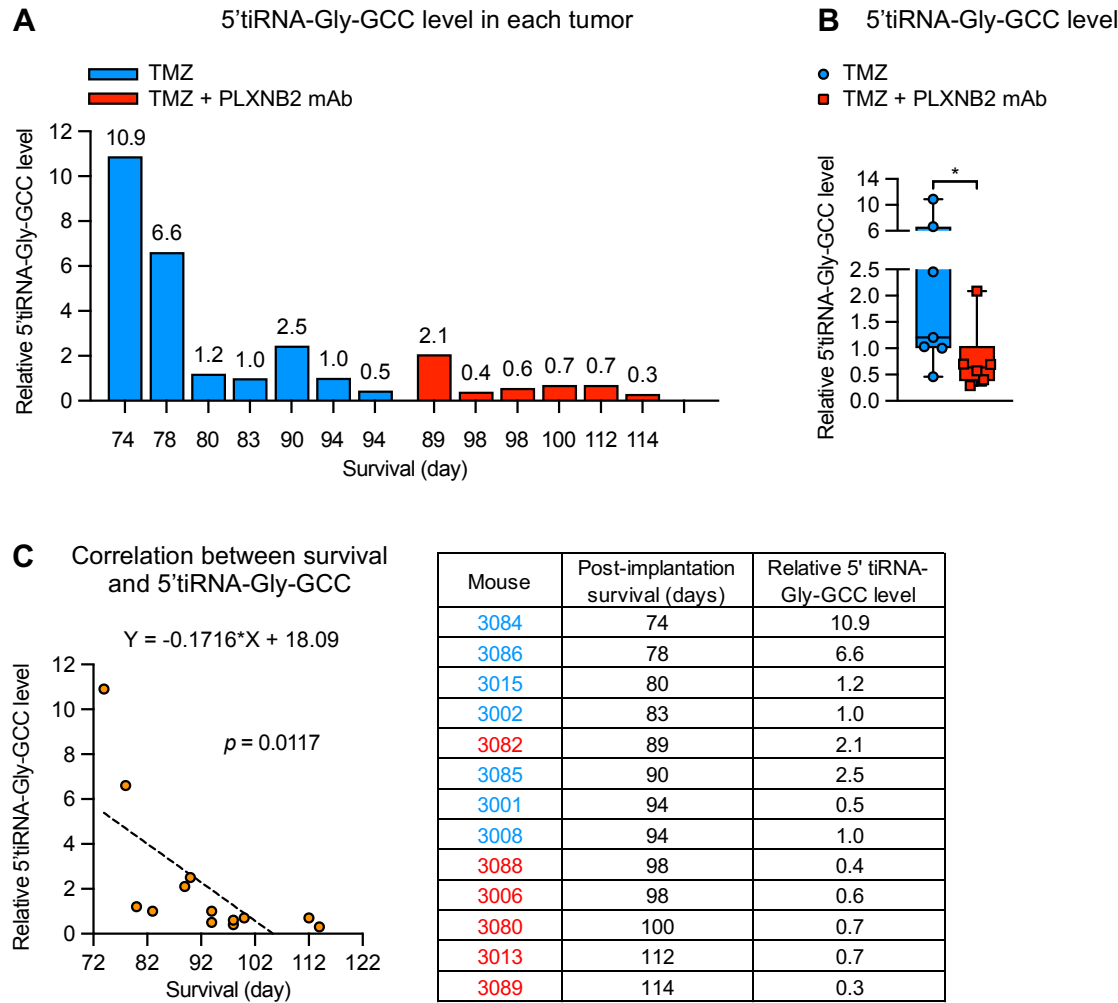


Figure 6.10. PLXNB2 mAb decreased the level of 5'tiRNA-Gly-GCC in xenograft tumors of TMZ-treated mice.

(A) The relative 5'tiRNA-Gly-GCC level of each tumor. Survival time for matched tumor-bearing mice is indicated at the bottom. 5'tiRNA-Gly-GCC levels were determined by TaqMan™ small RNA assay and normalized to sample 3002 (TMZ group).

(B) Relative 5'tiRNA-Gly-GCC levels in xenograft tumors of TMZ or TMZ + PB2 mAb treated mice (n = 6 - 7). * $p < 0.05$ by unpaired two-tailed Student's t test.
(C) Correlation analysis of tumor-bearing mice post-implantation survival (x-axis) and relative 5'tiRNA-Gly-GCC level (y-axis). Pearson correlation analysis was performed, $r^2 = 0.45$ and $p = 0.0117$. Detailed information was presented as a table panel, TMZ group labeled in blue and TMZ + PB2 mAb in red.

All above results demonstrate that PLXNB2 mAb could be successfully delivered to GBM tissues in the brain, probably due to a compromised BBB of the tumor-bearing mice, and significantly delayed the recurrence of GBM upon TMZ therapy. Delay of disease relapse by PLXNB2 mAb was associated with a decrease in the level of tiRNA-Gly-GCC, probably owing to the inhibition of ANG activity, an indication of target engagement of PLXNB2 mAb for its therapeutic effect. Together with the finding that *PLXNB2* KO decreased self-renew (**Figures 4.5 and 4.7**), promoted exhaustion (**Figure 4.8.**), induced differentiation (**Figures 4.9. - 4.12.**), and abolished intracranial tumorigenicity of GSCs (**Figure 6.2.**), these results suggest a feasible strategy to target ANG-PLXNB2 pathway to reduce chemo-resistance and to prevent or delay GBM recurrence.

6.3 Chapter contributions

Experiments were performed by Liang Yuan and supervised by Dr. Guo-fu Hu.

Chapter 7: Discussion and Future Directions

7.1. ANG and PLXNB2 as a pair of novel regulators of GSCs

Previous studies from our laboratory and others have shown that ANG (Eberle et al., 2000; Hu et al., 2019; Yang et al., 2022) and PLXNB2 (Le et al., 2015; Shinoura et al., 1995) are upregulated in GBM, and that their levels are inversely correlated to overall survival of GBM patients. ANG, in particular, has been heavily implicated in GBM malignancy as it was demonstrated to be the most enriched angiogenic protein in glioblastoma-derived microvesicles (Skog et al., 2008) and the most up-regulated proangiogenic factor in GBM following anti-VEGF therapy (Lucio-Eterovic et al., 2009). Yet, experimental investigation of its roles in GBM biology has just begun.

The *Nestin-tva;Ink4a/Arf^{-/-};Pten^{-/-}* mice have been shown to develop pro-neural GBM upon *PDGFB* overexpression in neuronal precursor cells. With the help of RCAS/*tva* system, intracranial injection of DF-1 chicken fibroblasts that were infected with RCAS-PDGFB-HA vector enabled the cell-specific *PDGFB* expression and thereby triggered tumorigenesis in the mouse brain (Hambardzumyan et al., 2009). Of the six murine *Ang* genes, *Ang1* is the human ortholog that shares the same gene structure as human ANG (Dyer and Rosenberg, 2005) and shows high similarity in terms of amino acid sequence (Bond and Vallee, 1990), crystal structure (Holloway et al., 2005), and, most importantly, RNase and angiogenic activities (Fu et al., 1999; Nobile et al., 1996). By crossing *Ang1^{-/-}* mice with *Nestin-tva;Ink4a/Arf^{-/-};Pten^{-/-}* mice, the role of ANG in GBM pathogenesis has been first described. *Ang1* deficiency was found to slow down disease progression and render a phenotype of reduced GBM cell proliferation, tumor angiogenesis, vascular association, and invasion. *Ang1* deficiency was also demonstrated to significantly prolong the survival PDGF-induced pro-neural GBM mice, and a dose-dependent effect of Ang1 in the survival has been implicated (Yang et al., 2022). While the role of ANG to exacerbate malignancy was well-illustrated in GBM, it

remains unknown if ANG regulates the stemness of GSCs that initiate tumors and constantly fuel the resilience of GBM.

In Chapter 3, we demonstrated that ANG promoted self-renewal of patient-derived GSCs (**Figure 3.1., A, B, and D**) and that its effect occurred in a dose-dependent manner (**Figure 3.1.C**). We also found that ANG was capable of sustaining GSCs to propagate as highly organized spheroids when stem cell growth factors were largely deprived (**Figure 3.2.A**). This finding adds to a growing body of research efforts to reveal how cancer stem cells cope with suboptimal environments and retain their self-renewing capacity during disease progression (Li and Neaves, 2006; Plaks et al., 2015; Shingu et al., 2017). Normal human plasma has ANG circulating at a concentration of ~250 mg/L (Bläser et al., 1993) and ANG is highly upregulated in numerous human cancers including GBM (Barton et al., 1997; Eberle et al., 2000; Hartmann et al., 1999; Li et al., 1994; Miyake et al., 1999; Montero et al., 1998; Shimoyama et al., 1996; Verstovsek et al., 2001b; Yoshioka et al., 2006). Our results suggest that ANG is one of the factors that regulates self-renewal, differentiation, and exhaustion of GSCs *in vitro*. We also presented strong evidence that the ANG-PLXNB2 axis regulates tumor-initiating capacity of GSCs, drug resistance, and disease recurrence of GSC tumors *in vivo*. Future study should focus on if ANG sustains the stemness of GSCs *in vivo*, especially when they migrate out of their niches. For example, *Ang1^{-/-}* mice may be crossed with tumor suppressor floxed strains, such as *Nf1^{flox/flox};p53^{flox/flox}*, *Nf1^{flox/flox};p53^{flox/-}*, and *Nf1^{flox/+};p53^{flox/flox};Pten^{flox/+}* (Alcantara Llaguno et al., 2009; Chen et al., 2012b). Stereotactic viral cre injection inactivates tumor suppressors in the cells of the SVZ and the resultant tumorigenic neural stem/progenitor cells will allow us to uncover if ANG supports GSCs to escape niche constraints for long-term clonal growth and intraparenchymal invasion.

ANG has three functional domains (Hallahan et al., 1991; Hu et al., 1991; Moroianu and Riordan, 1994; Shapiro et al., 1986) and has been shown to employ different domains to regulate various cell types under different circumstances. Human HSPCs have been shown to rely on receptor binding and ribonucleolytic functions of ANG, but not nuclear localization, to sustain their self-renewal abilities (Goncalves et al., 2016). In response to amyloidosis, ANG has been demonstrated to render liver hepatocellular carcinoma cells full cytoprotective activity independent of both ribonucleolytic activities and nuclear functions (Del Giudice et al., 2014). We demonstrated that all three domains of ANG are required for regulation of GSC properties. In Chapter 3, we utilized ANG variants that are defective in receptor binding (R66A), ribonucleolytic activity (K40Q), or nuclear translocation (R33A) (**Figure 3.4.A**) as well as neamine, a small molecule that blocks nuclear translocation of ANG (**Figure 3.4., C and D**) to analyze sphere formation and found that the three domains of ANG are indispensable for GSC regulation. ANG has been demonstrated to regulate the primitive cell state of HSPCs (Goncalves et al., 2016) and prostate CSCs (Li et al., 2020) through different RNA metabolisms, of which cytoplasmic functions of ANG were stressed. Prior to our research, nuclear functions of ANG were highly unanticipated/underestimated in primitive cell types. In this work, we demonstrated nuclear and cytoplasmic presence of endogenous ANG in GSCs (**Figure 4.6., A and C**) and, most importantly, revealed the fact that exogenous ANG was distributed to these two subcellular locations proportionally according to the same ratio of the endogenous ANG (**Figure 5.1., A and B**). As ANG is abundantly present in GBM tumor mass (Eberle et al., 2000; Hu et al., 2019; Skog et al., 2008) and circulating in human plasma (Bläser et al., 1993), it is conceivable that these tumor-secreted and circulating ANG will enhance the level of ANG in the cytoplasm and in the nucleus of GSCs. Our study has shown that ANG enhances production of tiRNA in GSCs (**Figure 5.7.**) and that tiRNA is an effector of

ANG in regulating GSC properties (**Figure 5.8.**). However, it remains to be determined if tRNA biogenesis in GSCs is attributed to nuclear ANG or cytoplasmic ANG. Future studies to differentiate the respective function of nuclear and cytoplasmic ANG in GSC regulation is warranted.

PLXNB2 is an identified ANG receptor for its roles in mediating diverse biological functions of ANG in different cell types (Yu et al., 2017). We showed that ANG and PLXNB2 constitute a ligand-receptor pair to regulate GSC stemness, a function that has not been investigated before. In chapter 4, we demonstrated a vital role of PLXNB2 in GSCs through antibody neutralization (**Figure 4.2.**) as well as gene inactivation (**Figure 4.5., 4.7., 4.8., 4.9.**) that PLXNB2 inhibition decreased GSC self-renewal, accelerated stem cell exhaustion, and promoted differentiation. We also confirmed that ANG and PLXNB2 are indeed a regulatory pair in GSCs as shown by the findings that *PLXNB2* ablation abolished cellular uptake of ANG (**Figure 4.6., A-D**) as well as ANG-stimulated tumorsphere formation (**Figure 4.6., E-J**).

PLXNB2 upregulation is commonly found in GBM and is associated with an adverse clinical outcome (Le et al., 2015; Shinoura et al., 1995; Towner et al., 2013). Prior to our research, PLXNB2-mediated GBM malignancy was mainly about regulating its invasive growth. PLXNB2 has been demonstrated to modulate the biomechanical properties of GSCs and facilitate their migrations along the perivascular and axon fiber tracts. These processes were found to be mediated by the intracellular GAP domain and were activated by SEMA4C and SEMA4D, the other two ligands of PLXNB2 (Huang et al., 2021; Le et al., 2015).

A link between PLXNB2 and stem cell differentiation has been implicated by pathway enrichment analyses of *PLXNB2*-deficient GSCs (Huang et al., 2021) and human embryonic stem cells (Junqueira Alves et al., 2021). Our data provides direct experimental evidence for the first time of the role of PLXNB2 in GSC differentiation. By

gene expression profiling, we confirmed that *PLXNB2* knockout elevated neuronal differentiation-associated transcripts in GSCs (**Figure 4.9.A**). Further, *PLXNB2* KO GSCs were shown to be more susceptible to differentiation induction (**Figures 4.10., 4.11., and 4.12.**). It should be noted that the resulted changes in GSC properties upon *PLXNB2* ablation might not be solely the consequence of the loss of ANG activity and could be also contributed by disruptions of SEMA4C signaling.

ANG and SEMA4C are known to trigger different signaling pathways upon binding to PLXNB2 (Yu et al., 2017). SEMA4C has been shown to activate ErbB-2 and RhoA/RAS via PLXNB2 and thereby promotes proliferation and migration of GSCs (Huang et al., 2021; Le et al., 2015) and normal CNS cell precursors (Deng et al., 2007). SEMA4C/PLXNB2 signaling has also been shown to upregulate stemness regulators, *SOX2* and *ALDH3A1*, and promote the formation of mammospheres of breast CSCs (Gurrapu et al., 2018). However, SEMA4C has been found to be unable to elevate pro-self-renewal transcript levels in PLXNB2-expressing HSPCs (Goncalves et al., 2016). A decent SEMA4C expression has been documented for GSCs (Huang et al., 2021). At present, the role of SEMA4C/PLXNB2 signaling in the stemness of GSCs is unclear. *SEMA4C* is un-differentially expressed between GBM and normal brain tissues (**Figure 1.5.A**) and its levels are found not to be correlated with the survival of GBM patients (**Figure 1.5.B**). Future studies using recombinant SEMA4C as well as *SEMA4C* KO GSCs may provide more insights into the role of SEMA4C-PLXNB2 in the self-renewal and tumorigenicity of GSCs. It will also be interesting to study the role of SEMA4D, another suspected ligand for PLXNB2 (Masuda et al., 2004; Witherden et al., 2012), in GSCs. Those will be the subject of future studies.

Our current study provides significant evidence that ANG and PLXNB2 constitute a ligand-receptor pair to regulate GSC stemness as well as *in vivo* tumorigenicity and

therapeutic response (**Chapter 6**) by not only *PLXNB2* ablation analysis but also employment of antibodies that specifically target the ANG-binding site on *PLXNB2*.

It will also be interesting to investigate the role of *RNASE4*, an ANG homolog that shares the same promoters (Sheng et al., 2014a) and is often co-expressed with ANG (Dyer and Rosenberg, 2005; Li et al., 2013). The functional similarities between ANG and *RNASE4* have been validated in terms of angiogenic and neurogenic activity as well as ribonucleolytic and nuclear translocation capacity (Li et al., 2013). In the context of GBM, *RNASE4* is significantly upregulated (unpublished data, normal: 4.29 versus tumor: 19.8 in TPM) but, unlike ANG, its levels are not correlated to patient survival (UALCAN online software). Since *RNASE4* does not bind to *PLXNB2* (Yu et al., 2017), it will be of therapeutic significance to assess its roles in GSCs as it may provide a mechanism of overcoming resistance to anti-*PLXNB2* therapy in GBM. Therefore, the role of *RNASE4* and its receptors, yet to be identified, will be of considerable interest for continued therapeutic studies.

Overall, our study demonstrates that ANG pairs with its functional receptor *PLXNB2* to regulate GSC stem cell properties. *PLXNB2* mediates ANG cellular uptake and ANG relies on both ribonucleolytic activity and nuclear functions to enable the stemness-promoting effects in GSCs. This part of work also demonstrates the therapeutic potential of *PLXNB2* inhibition as it was found to abolish ANG-stimulated self-renewal, accelerate stem cell exhaustion, and promote neuronal differentiation of GSCs. The mechanistic and therapeutic implications of the ANG-*PLXNB2* regulatory pair in GSCs are discussed in more details below.

7.2. ANG/PLXNB2 regulates biogenesis of small RNAs, including 5'-tiRNA-Gly-GCC that enhances stemness of GSCs

The work presented in Chapter 5 mechanistically characterized the regulatory function of ANG-PLXNB2 in GSCs. We elucidated that ANG promoted GSC stemness through an elevated production of bioactive tiRNA, in particular 5'tiRNA-Gly-GCC. We also revealed that ANG took part in the metabolism of microRNAs, snRNAs, and snoRNAs in GSCs. All these actions of ANG were mediated by PLXNB2.

Previous studies have identified several ANG-mediated RNA processing events that regulate stem cell properties under physiological or pathological conditions. For instance, in HSPCs, ANG was found to accumulate in SGs and mediate tiRNA biogenesis. tiRNAs function to restrict global protein synthesis and upregulate pro-self-renewal transcripts, thereby preserving the regenerative potentials of HSPCs (Goncalves et al., 2016). In prostate CSCs, ANG was found to promote self-renewal through actively producing another class of small RNA, the 3'-end fragments of 5S rRNA, which promotes prostate tumorsphere propagation, a stemness-related transcriptional profile, and a chemo-resistant phenotype (Li et al., 2020). Further, the self-renewal regulatory roles have been well documented in microRNA biology in pluripotent stem cells and during somatic cell reprogramming (Lee et al., 2016b). Recently, ANG-mediated microRNA turnover or decay has been implicated as a regulator of cellular function, including angiogenesis and cell survival (Thomas et al., 2018; Weng et al., 2022).

Our mechanistic investigation started off from determining the subcellular localization of ANG in GSCs. ANG was predominantly present in the cytoplasm with a lower but decent level found in the nucleus where it is mainly located in the nucleolus (**Figure 5.1., A and B**). Addition of exogenous ANG proportionally elevated the levels in both nucleus and cytoplasm (**Figure 5.1., B and C**), a phenomenon that is in agreement

with the functionality assay using ANG variants (**Figure 3.4.**) that suggested that both nuclear and cytoplasmic functions of ANG are necessary for regulation of GSC properties.

In the context of stem cells, rRNA synthesis has been far less studied for the notion that stem cells are quiescent with a reduced cell cycling (Bastide and David, 2018). Yet, stemness is first about a cell state free of any fate decisions. *Drosophila* studies showed that ovarian germline stem cells maintain high levels of rRNA transcription to delay differentiation whereas reduced rRNA production promotes morphological changes that accompany the early steps of germ cell differentiation (Zhang et al., 2014). It will be important to assess whether inhibition of rRNA transcription or blockade of nuclear translocation of ANG induces GSC differentiation. The findings shown in Figure 3.4.C and D that neamine treatment reduced spherical growth of GSCs and led to a pro-differentiating cell morphology implicate a role of ANG-mediated rRNA transcription in stemness regulation. However, more molecular analyses will be required to solidly answer this question.

In addition, fluctuation of snoRNAs, key players in rRNA modification and maturation, also influences self-renewal ability of CSCs (Mannoor et al., 2014). Whether ANG is involved in snoRNA processing is an important, yet underexplored, question that has begun to be studied in this work (**Figure 5.12.**). Detailed experiments to elucidate the role of nuclear ANG are important both scientifically and clinically for elucidating the mechanism of ANG-PLXNB2 mediated regulation and for developing GSC-targeted therapies.

Analyses of cytoplasmic ANG also raised a number of intriguing questions. First, unlike other primitive cell types (Goncalves et al., 2016; Li et al., 2020), co-localization of ANG with SG marker PABP is less evident in GSCs, even, in the presence of exogenous ANG (**Figure 5.1.D**). This suggests that, in GSCs, cytoplasmic ANG functions at least

partially independent of SG-mediated pathways (Pizzo et al., 2013). In GSCs, PABP tends to accumulate in the nucleus or perinuclear regions, a phenomenon often linked to protein expression restriction (Gray et al., 2015; Kumar and Glaunsinger, 2010).

Discerning PABP nucleocytoplasmic distribution and its respective functional consequences, as well as the involvement of ANG, is the subject of future studies.

Through RNA gel electrophoresis analysis, we found that ANG/PLXNB2 regulates small RNA production in patient-derived GSCs. We observed that addition of exogenous ANG drastically elevated the levels of small RNAs in the size range of 35 to 40 nt in PLXNB2-expressing GSCs, whereas *PLXNB2* ablation voided the effect of ANG (**Figure 5.2.**). Next, we sequenced small RNAs (≤ 60 nt) for the purpose to determine (1) the RNA substrate(s) of the ribonuclease ANG in GSCs, (2) if this process is mediated by PLXNB2, (3) whether the ribonucleolytic cleavage enriches specific small RNA species and if those are responsible for ANG-induced self-renewal functions in GSCs, and (4) how ANG affects small RNA metabolisms in GSCs, such as microRNAs.

We first examined tRNA fragment levels and found that ANG remarkably increased tRF production and *PLXNB2*-null cells failed to respond to ANG (**Figure 5.3. and Table 5.1.**). Further, we found that ANG treatment preferentially enriched tRFs derived from tRNA-Gly-GCC (2,3,5), tRNA-Val-CAC-2, and tRNA-Lys-CTT-2 and they were on the contrary, the most diminished species in *PLXNB2* knockout cells (**Figure 5.4. and Tables 5.2. and 5.3.**). We also identified tRNA-Gly-GCC, tRNA-Val-CAC, and tRNA-Lys-CTT as the top affected tRF species whose abundance was mostly regulated by the ANG/PLXNB2 axis in both GSC lines (**Figure 5.5.**).

Intriguing, we found that ANG did not universally enhance the production of all tRFs, rather, it displayed certain specificities. For instance, tRF-Lys-TTT was the third most abundant tRF found in both GSC lines, which was not regulated by ANG as

indicated by the finding that ANG treatment did not increase its level but rather resulted in a decrease of its levels in both GSC lines (**Figure 5.5., A-B**).

As tRF-Gly-GCC was the most abundant and most upregulated small RNA by ANG, we analyzed its composition and found that 5'tiRNA-Gly-GCC was the predominant component (**Figure 5.6. and Table 5.6.**). tiRNAs have been demonstrated to play roles in promoting cellular adaptation (Ivanov et al., 2011), yet characterization of context-specific tiRNA and its functions has just begun. For examples, 5' tiRNA-His-GTG was found to be the most upregulated tiRNA in human colorectal cancer tissues. It played oncogenic roles in disease progression and its production was regulated through the hypoxia/HIF1 α /ANG axis (Tao et al., 2021). 5' tiRNA-Pro-CGG-1 was preferentially enriched in osteoblast-derived extracellular vesicles (EVs) that played roles to enhance protein translation, cell proliferation, and differentiation of the recipient granulocyte-monocyte progenitors (GMPs) (Kfoury et al., 2021). Findings from our work allowed us to propose that 5'tiRNA-Gly-GCC was a downstream effector of ANG-PLXNB2 axis that mediated the regulatory function of ANG in GSCs. We validated this hypothesis by *in vitro* self-renewal assay (**Figure 5.8.**), gene expression profiling (**Figure 5.9.**), and *in vivo* studies (**Figures 6.9. and 6.10.**). Specifically, we showed that transfection of 5' P-tiRNA-Gly-GCC enhanced the sphere-forming activity in *PLXNB2*-expressing GSCs, even though this effect was not entirely comparable to ANG stimulation (**Figure 5.8.D**). We also showed that the combination treatment by ANG and tiRNA-Gly-GCC expression had no additive or synergistic effect (**Figure 5.8.D**). These observations indicate that ANG regulates GSC self-renewal through the actions of bioactive tiRNA-Gly-GCC. Further, we showed that transfection of 5'-P-tiRNA-Gly-GCC in *PLXNB2*-null GSCs were able to enhance sphere formation, although to a much decreased extent as compared to that of *PLXNB2*-expressing cells (**Figure 5.8., A-C**).

The observation that transfection of tiRNA-Gly-GCC in *PLXNB2*-null GSCs did not fully phenocopy *PLXNB2*-expressing cells could be explained by several reasons. 1) ANG-induced other tRFs, such as tRNA-Val-CAC and tRNA-Lys-CTT, or ANG-mediated metabolism of other RNAs had functional roles in promoting GSC self-renewal. Some early reports support this hypothesis. For examples, in oral squamous cell carcinoma, 5' tiRNA-Val-CAC-2 was found to act as the essential member of oncogenic small RNA regulatory module that negatively regulates G1/S transition of cell cycle as well as cell differentiation (Dhahbi et al., 2019). In bladder cancer, elevated 5' tRF-Lys-CTT levels were found to be significantly associated with more aggressive phenotype, sooner progression, and poorer clinical outcome (Papadimitriou et al., 2020). It will be of significance to elucidate if these tRFs (or tiRNAs) also regulate stemness properties in GSCs. In this work, we reported that ANG also down-regulated some tRFs in GSCs, such as tRF-Lys-TTT (**Figure 5.5., A and B**). Downregulation of certain tRFs have been described in other cancer types (Chen et al., 2021; Olvedy et al., 2016). In particular, tRF-Lys-TTT-12 and tRF-Lys-TTT-14 were found to be the top downregulated small RNAs in high-grade serous ovarian cancer (Chen et al., 2021). Future work will need to functionally assess the roles of these down-regulated tiRNAs in GSCs. Defining mechanistic basis for the differential regulation of distinct tRFs by ANG/*PLXNB2* axis is beyond the scope of this work, but will be of particular interest for elucidating how ANG employs RNA processing machineries to target tRNAs with specificity and to promote context-specific functions of tiRNA.

Moreover, ANG/*PLXNB2* has been shown to regulate the stemness of prostate CSCs by producing 3'-end fragment of 5S rRNA (Li et al., 2020). This finding further underlined the diverse substrates of ANG as well as the important roles of small RNA biogenesis in CSC biology.

In addition to PLXNB2-mediated ANG internalization, *PLXNB2* knockout will likely also affect stemness via other PLXNB2-mediated pathways, including the aforementioned ErbB-2 and RhoA/RAS signaling (Gurrapu and Tamagnone, 2019). Further, *PLXNB2* knockout will also impair the SEMA4C/PLXNB2 axis that has been shown to downregulate *SOX2* and *ALDH3A1* and inhibits mammosphere formation (Gurrapu et al., 2018).

To our knowledge, this work is the first functional characterization of specific tiRNA (5'tiRNA-Gly-GCC) in GSCs and shows clinical promise in GBM therapeutics. A recent study by Gyuris et al. demonstrated that 5'tiRNA-Gly-GCC was preferentially enriched in the ribonucleoprotein (RNP) complex of mouse primary GBM cultures (Gyuris et al., 2019), suggesting a role of this particular tiRNA in GBM malignancy. This finding further underlined the usefulness of blocking ANG endocytosis in GBM therapy because it will abolish tiRNA production in the source cells.

Moreover, 5' tiRNAs are known to function through displacing eIF4F from capped mRNA, which inhibits cap-dependent translation initiation but specifically permits IRES-mediated translation (Ivanov et al., 2011; Ivanov et al., 2014). 5' tiRNAs were also shown to promote the assembly of stress granules that protects mRNAs under stress and has roles in the acquisition of chemoresistance (Zhang et al., 2020b). Recent work of Mo et al. revealed a novel mechanistic model that 5' tiRNA-Val inhibited the translation of *FZD3* mRNA by directly targeting its 3'-UTR (Mo et al., 2019). Future work will need to examine which mechanism(s) indeed takes part in enabling the regulatory functions of tiRNA in GSCs.

Besides demonstrating that tiRNA is an effector of ANG-PLXNB2 axis and a mediator of GSC stemness, our current work also characterized several molecular events underpinning the regulatory function of ANG-PLXNB2 in GSCs. We profiled stemness-related gene expression and found an evidently elevated expression of

several key stemness regulators, such as *SOX2* (Hemmati et al., 2003), *OCT4* (Schoenhals et al., 2009), *L1CAM* (Bao et al., 2008), and *CDCP1* (Bühning et al., 2004; Kimura et al., 2006) in GSCs at the first 10-30 min post ANG stimulation (**Figure 5.9.A**). As incubation time proceeded (7 days), another group of stemness-related genes led the latter wave of the gene expression. They are *SOX9* (Scott et al., 2010), *PROM1* (Singh et al., 2003), *FOXO3* (Sunayama et al., 2011), and *MUSASHI1* (Anido et al., 2010) (**Figure 5.9.B**). *SOX2* (Lopez-Bertoni et al., 2022) and *SOX9* (Sabelström et al., 2019; Swartling et al., 2012) are both transcriptional factors that have essential roles in regulating GSC self-renewal. *SOX9* expression can be directly induced by *SOX2* at the transcriptional level via a specific binding on the promoter (Domenici et al., 2019). *SOX2* has been shown to activate oncogenic miR-10b-5p that represses *TET2* and thus promotes stemness in GSCs, presumably by transcriptional reprogramming (Lopez-Bertoni et al., 2022). Co-expression of *SOX2* and *OCT4* has been also shown to induce global downstream methylation events, thereby promoting the stemness of GSCs (Lopez-Bertoni et al., 2015). While cell surface CD133 has been widely used to identify and enrich CSCs, a recent study revealed the stemness regulatory role of cytoplasmic CD133 as it maintains the undifferentiated status of cells through capturing GABARAP at centrosomes and inhibiting autophagic activity (Izumi et al., 2019). Epigenetic regulation of the promoter CpG island of *PROM1* has been shown to critically regulate CD133 expression in GSCs (Gopisetty et al., 2013). In addition, FOXO transcription factors are known to maintain the self-renewal of HSCs, and recent studies indicate that FOXO-dependent signaling is required for long-term regenerative potentials of normal and malignant stem cells (Tothova and Gilliland, 2007). Moreover, as a post-transcriptional regulator, *MUSASHI1* promotes stemness properties of GBM cells by controlling mRNA turnover and cell cycle regulation (Battelli et al., 2006; Chen et al., 2020). The relationship of these ANG-regulated stemness genes will be an interesting topic for

future study. Also, it will be interesting to examine the potential role of tiRNA in the expression of these stemness genes in GSCs. We did find that 5'tiRNA-Gly-GCC rendered GSCs a comparable transcriptional profile as ANG after 7-day incubation, suggesting a possible role of 5'tiRNA-Gly-GCC in mediating the stemness transcriptional profile upon ANG stimulation (**Figure 5.9.B**). It is conceivable that several programs or pathways coordinate the stemness properties of GSCs upon ANG treatment. It will be of particular interest to elucidate the regulatory tempo of different molecular determinants in response to ANG in terms of GSC stemness, but it is beyond the scope of this study.

In Chapter 5, we assessed the effect of ANG/PLXNB2 on the biogenesis or metabolism of other RNAs. ANG/PLXNB2 seems to universally downregulate microRNAs, suggesting a role in microRNA decay (**Figure 5.11.**), a phenomenon that has been implicated in two recent studies (Thomas et al., 2018; Weng et al., 2022). We have listed the most-affected microRNA species (Log_2 (fold-change) ≤ -2) in ANG-treated PLXNB2-expressing GSCs. Among them, MIR598, MIR25 and MIR29A were drastically downregulated in both GS6-22 and MGG18 cells (**Table 5.8.**). MIR598 has been demonstrated to directly target 3'UTR of unconventional prefoldin RPB5 interactor (URI), a scaffolding protein playing roles in ubiquitination and transcription. By this function, MIR598 inhibits the proliferation, invasion, and migration of ovarian cancer cells (Xing et al., 2019). MIR598 was also found to target JAG1 to inactivate Notch signaling pathway. By doing this, MIR598 suppresses metastasis of colorectal cancer (Chen et al., 2017). A role of MIR598 in stemness has also been described in gastric cancer stem-like cells in which MIR598 attenuated cell self-renewal by directly downregulating regulator of ribosome synthesis1 (RRS1) and by suppressing the expression of *OCT4* and *SOX2* (Ma et al., 2019). In opposite, MIR25, a microRNA that was also downregulated by ANG, was actually shown to promote the stemness of neuroblastoma cells by activating WNT signaling via a direct binding to GSK3 β 3' UTR (Chen et al., 2019a). Moreover, a role of

MIR29A in maintaining the stemness of NSCs while promoting neurite outgrowth has also been reported recently (Ma et al., 2020). These previously identified roles of microRNA species in stemness, together with our findings that their levels were regulated by ANG, suggest that ANG-mediated microRNA biogenesis and/or metabolism may play a role in GSC stemness regulation. Over-expression of the most-affected microRNAs in GSCs may provide a good glimpse of their functions.

In addition to tiRNA and microRNA, ANG/PLXNB2 was also found to decrease the levels of fragments derived from snRNAs and snoRNAs (**Figure 5.12.**). This finding was highly unanticipated as ANG is projected to process RNAs into small fragments. We raise two hypotheses: (1) ANG lacks the endoribonuclease capacity for snRNAs or snoRNAs. Indeed, whether ANG has endoribonuclease activity on snRNA or snoRNA remains to be determined to date. (2) ANG accelerates the degradation of the fragments from snRNAs or snoRNAs but has no effect on the intact snRNAs and snoRNAs. However, since we were focusing on sequencing RNAs of <60 nt, the abundance of the intact snRNAs and snoRNAs, the majority of them having a size of over 60 nt, was unknown. The role of ANG in snRNA and snoRNA biogenesis, metabolism, and function is unclear at present.

Lastly, we examined the role of ANG/PLXNB2 on rRNA fragments as ANG has been demonstrated to actively process 5S rRNAs to produce the 3'-end fragment of 5S rRNA that regulates stemness properties of prostate CSCs (Li et al., 2020). In GSCs, ANG treatment did not specifically enrich any rRNA fragments (data not shown), indicating context cell type-specific role of ANG.

Collectively, data described in Chapter 5 demonstrate that ANG/PLXNB2 regulates small RNA biogenesis in GSCs, in particular the production of tiRNA, such as 5'tiRNA-Gly-GCC that promotes the stemness of GSCs. Further interrogation of the role of ANG/PLXNB2-mediated microRNA metabolism (decay), particularly as a stemness

regulator is warranted. It is of practical significance to assess the effect of PLXNB2 inhibitors, especially the mAbs, in small RNA biogenesis in GSCs *in vitro* and *in vivo*.

7.3. PLXNB2 as a therapeutic target of GBM by reducing chemoresistance and delaying disease relapse

Given the high concentration of ANG in circulating human plasma (Bläser et al., 1993), targeting the receptor PLXNB2 is a more feasible and practical approach for clinical translation. In Chapter 6, we comprehensively examined the effect of PLXNB2 inhibition in GSC tumorigenicity and chemosensitization via monitoring orthotropic GSC propagation upon *PLXNB2* genetic ablation or treating with specific antibodies.

In a healthy CNS, PLXNB2 is restrictedly expressed in SVZ-neuroblasts (Saha et al., 2012) and cerebellar granule neuron precursors (Van Battum et al., 2021) where PLXNB2 modulates cell propagation, migration, and differentiation. Previous studies that assessed the physiological functions of PLXNB2 in the CNS have demonstrated its roles in neurodevelopment (Deng et al., 2007; Friedel et al., 2007) and adult neurogenesis (Daviaud et al., 2016; Saha et al., 2012). In GBM, increased PLXNB2 expression has been well documented (Huang et al., 2021; Le et al., 2015; Shinoura et al., 1995). Actually, PLXNB2 was originally identified from GBM by differential expression screening as compared to low grade astrocytoma (Shinoura et al., 1995). A role of PLXNB2 in facilitating the invasive growth has been demonstrated in both primitive (Huang et al., 2021) and differentiated (Le et al., 2015) GBM cell compartments. To our knowledge, however, no studies have *in vivo* demonstrated a role of PLXNB2 in GSC stemness with the essential functional criteria: to orthotopically reproduce heterogeneous parental gliomas, nor have PLXNB2 inhibitors been assessed for the chemo-sensitizing efficacy in GBM animal models.

Several keynote findings arose from the present study. First, we demonstrated that *PLXNB2* inhibition by gene ablation drastically suppressed intracranial tumorigenicity of patient-derived GSC MGG18 (**Figure 6.2., A and B**). *PLXNB2* knockout largely prolonged GSC-bearing mice survival. The median survival of mice inoculated with *PLXNB2*-expressing vector control MGG18 cells was 50 days whereas none of the mice inoculated with *PLXNB2*-null MGG18 succumbed to orthotopic tumors until the experiment was terminated on day 126 (**Figure 6.2.D**). As a matter of fact, none of the *PLXNB2*-null MGG18 cells survived in the orthotopic region of the mouse brain (**Figure 6.2., A-C**). This finding is largely consistent with the observation of a GBM infiltration study that also employs *PLXNB2* knockout GSCs as the intracranial transplants. Huang et al. reported that *PLXNB2* deletion remarkably decreased intracranial expansion of GSCs (SD2 and SD3) and significantly prolonged animal survival, with the median survival increased from 50 days in control mice to 74 days in KO mice. Despite that we utilized completely different GSC lines, the control mice in our experiments and in the experiment reported by Huang et al had the same median survival (50 days). However, *PLXNB2* deletion was found to provide much stronger efficiency in suppressing intracranial cell growth in our study. This difference could be the result of different sensitivity to *PLXNB2* knockout between the two GSC lines used in the two studies. It could also be because the incomplete deletion of *PLXNB2* in study reported by Huang et al. It is noted that *PB2* KO SD2 and SD3 cells in the Huang study still expressed a small amount of *PLXNB2* as indicated by the immunoblot bands of the *PLXNB2* full length at 240 kDa in both lines and the α -chain at 175 kDa in *PB2* KO SD3 cells. The remnant *PLXNB2*-expressing cells in supposed *PB2* KO SD2/3 populations may also explain why no apparent differences was found when they were subjected to sphere formation analysis. (Huang et al., 2021). Additionally, a quite similar study that examined hESCs colony formation showed that *PLXNB2* knockout decreased the colony

size by ~83% on day 6. Of note, a trace of PLXNB2 expression was still found in these *PB2* KO hESCs (Junqueira Alves et al., 2021). Lastly, we confirmed that xenografts of vector control GSCs highly recapitulated histopathological features of MGG18 primary tumor, a giant cell GBM (Wakimoto et al., 2012) (**Figure 6.3.**). To our knowledge, this is the first report of a role of PLXNB2 in tumor-initiating capacity of GSCs. Given that PLXNB2 mediates multiple signaling pathways, a more extensive *in vivo* study using inhibitors as well as specific antibodies are warranted.

Second, we demonstrated a chemo-sensitizing function of PLXNB2 mAbs in orthoptic GSC tumors. PLXNB2 mAbs have been shown to sensitize prostate CSC tumors to DTX, the first line chemotherapy drug for advanced prostate cancer, and significantly delayed disease recurrence in a murine model (Li et al., 2020). We, thus, sought to examine if PLXNB2 mAbs could similarly sensitize GSC tumors to TMZ. We employed MGG18 GSCs for this purpose for several reasons. (1) As described in the above chapters, MGG18 is MGMT-negative, so MGMT inhibitors will not sensitize MGG18 to TMZ. (2) PARP is known to regulate MGMT-independent chemoresistance (Gupta et al., 2018; Tentori et al., 2014), yet MGG18 was found to be insensitive to PARP inhibitors (Ning et al., 2019). (3) MGG18 is non-MYC GSCs with elevated homologous recombination activity (Ning et al., 2019). An enhanced DNA repair is believed to be responsible for PARPi insensitivity (Rundle et al., 2017), and Ataxia Telangiectasia mutated and Rad3-related (ATR) kinase is a major regulator for DNA damage response (Barnieh et al., 2021). Thus, olaparib (PARP inhibitor) and VE822 (ATR inhibitor) have been combined to treat MGG18-propagated xenografts but they prolonged the mouse survival by only 14% (Ning et al., 2019). MGG18 tumors represent a disease lack of effective therapeutics and was suitable for our purpose.

Strategically, we allowed MGG18 tumors to propagate to a certain extent to better mimic the compromised BBB of GBM patients and, more importantly, to permit

antibody penetration. Then, mice were treated by PB2 mAb (30 mg/kg) or control (PBS) in the presence of TMZ (25 mg/kg) on post-implantation day 28. Even though that MGG18 has been reported to be resistant to TMZ *in vitro* (Jahan et al., 2017; Ning et al., 2019), which we confirmed in our own experiments that the IC₅₀ was 200 μ M, MGG18 tumors in the orthotopic site of the mouse brain was sensitive to treatment with 25 mg/kg TMZ. At present, we don't know the reason of the discrepancy between *in vitro* and *in vivo* TMZ sensitivity of MGG18 cells. One of the reasons could be that the *in vivo* tumors derived from MGG18 cells were mainly differentiated heterogeneous GBM cells that have reduced stemness as compared to the original MGG18 cells. It could also be that the local TMZ level had reached a threshold for its cytotoxic activity to MGG18. No matter what the reasons were, we found that the reduction of tumor size by TMZ alone and by TMZ + PB2 mAb was comparable until day 46 (**Figure 6.5.B**). Tumors treated with TMZ alone started to rebound by day 54, indicating disease relapse, an inevitable phenomenon of GBM patients under TMZ therapy (**Figure 6.5.B**). However, there was no sign of disease relapse in the group treated with TMZ + PB2 mAb until day 74 (**Figure 6.5.C**), indicating a therapeutic benefit of PLXNB2 mAb in a setting of combinatorial treatment with TMZ. PLXNB2 mAb extended the median survival of tumor-bearing mice from 83 to 106 days, representing a 28% increase in survival (**Figure 6.5.F**), double the survival benefit of combinatorial treatment with PARP inhibitor olaparib and ATR inhibitor VE822. It is also encouraging that two of the eight TMZ + PB2 mAb treated mice (25%) did not show any sign of disease recurrence and maintained healthy till the last day of the experiment (day 160) (**Figure 6.5., E-I**). These results provide proof-of-concept data that PLXNB2 mAbs are able to delay or prevent the relapse of TMZ-treated GSC tumors. Future work would need to examine the toxicity of PLXNB2 mAb, document its safety profile and therapeutic window, as well as optimize its dose and dosing regimen. Our work also suggest that it is feasible to develop small

molecule inhibitors of the ANG-PLXNB2 axis to sensitize GBM to chemotherapy. Small molecule inhibitors are more amendable to cross the BBB than antibodies.

Third, we confirmed by immunoblotting and immunofluorescence that PLXNB2 mAb, delivered through i.p. injection, indeed crossed the BBB of tumor-bearing mice and reached to the tumor tissues. The PLXNB2 mAb used in this experiment was mouse IgG. Though IgG-like immunoreactivity has been reported in the brain of several immune-competent mouse strains (ICR, Balb/c, C57BL/6, and AKR/J), it was later found to come from mouse microglial that uptake serum IgG (Hazama et al., 2005). We assessed the protein extracted from the xenograft GBM tumors and showed an abundant presence of mouse IgG reactivity in PB2 mAb-treated tumors but not in TMZ-treated tumors (**Figure 6.7.A**), indicating that 1) there was a very low level of endogenous IgG in the tumor tissues suggesting infiltration of few immune cells into the tumor tissue from the immunodeficient host, and 2) i.p.-injected IgG indeed penetrated into the brain, probably due to a compromised BBB in the tumor-bearing mice. Immunofluorescence staining confirmed the existence mouse IgG in the tumor section derived from PB2 mAb-treated mice (**Figure 6.7.B**). These data provide supporting evidence that delayed disease relapse in the animals treated with PB2 mAb + TMZ was indeed due to the activity of PLXNB2 mAb in the brain.

Fourth, we confirmed by IHC and IF that treatment with PB2 mAb resulted in an increase in apoptosis (**Figure 6.8. A and B**) and a decrease in proliferation (**Figure 6.8. C and D**) and angiogenesis (**Figure 6.8. E and F**). These *in vivo* results are consistent with the *in vitro* finding that PLXNB2 inhibition either by gene ablation or by mAb treatment impaired sphere formation (**Figures 4.2., 4.5., and 4.7.**) and promoted apoptosis (**Figure 4.9.**). They are also consistent with the known function of PLXNB2 in mediating ANG-induced cell survival and angiogenesis (Yu et al., 2017), suggesting an on-target effect of PLXNB2 mAb. Recently, SEMA4C-PLXNB2 signaling has been

demonstrated as a modulator of cell biomechanics that influences cell-cell adhesiveness and cell durotaxis behaviors of GSCs (Huang et al., 2021; Junqueira Alves et al., 2021). At present, we don't know if cell-cell/matrix biomechanic interactions were altered upon PLXNB2 mAb treatment *in vivo* but this will be an interesting aspect of future investigation. It will also be very interesting to study whether and how PLXNB2 mAb treatment affects the stemness of GSC tumors and the lineage preference upon differentiation by examining the *in vivo* expression profile of stemness and differentiation genes and the changes upon PLXNB2 mAb treatment.

Lastly, we demonstrated that PLXNB2 mAb treatment *in vivo* resulted in a decrease in the level of small RNA in the tumor tissues. These small RNA include 5'tiRNA-Gly-GCC that has been shown to be regulated by ANG-PLXNB2. The significance of this finding is two-fold. First, it demonstrated an on-target effect of PLXNB2 mAb *in vivo* because ANG is so far the only ribonuclease that is responsible for tiRNA production. No other ribonucleases have been shown to produce specific tiRNAs. Second, tiRNAs have been reported in the pathology of many types of human cancers, including breast cancer (Cui et al., 2019; Farina et al., 2020; Feng et al., 2018; Honda et al., 2015; Santarpia et al., 2016; Sun et al., 2018), prostate cancer (Honda et al., 2015; Olvedy et al., 2016), lung cancer (Shao et al., 2017), colorectal cancer (Li et al., 2019; Tao et al., 2021; Wu et al., 2021), ovarian cancer (Zhang et al., 2019b), clear cell renal cell carcinoma (Nientiedt et al., 2016), chronic lymphocytic leukemia (Katsaraki et al., 2019), head and neck squamous cell carcinoma (Victoria Martinez et al., 2015), and gastric cancer (Zhang et al., 2020a; Zhu et al., 2019). tiRNAs have been shown to exacerbate human malignancies via promoting cell proliferation (Honda et al., 2015; Shao et al., 2017), metastasis (Li et al., 2019; Zhang et al., 2019b), and drug resistance (Cui et al., 2019; Zhang et al., 2020b), and their levels have been demonstrated with prognostic values (Feng et al., 2018; Jin et al., 2019; Karousi et al., 2019; Katsaraki et

al., 2019; Olvedy et al., 2016; Sun et al., 2018). We have shown in the previous chapters that a specific subgroup of tRNAs was regulated in GSCs through ANG-PLXNB2 axis, which includes tRNA-Gly-GCC. Thus, a feasible mechanism of action of PLXNB2 mAb in sensitizing MGG18 tumors to TMZ *in vivo* and in delaying disease relapse would be that PLXNB2 mAb blocked the binding of ANG to PLXNB2 to inhibit cellular uptake of ANG, reduced the level of tRNA-Gly-GCC, and resulted in a decrease in GSC stemness. Prior to our work, to our knowledge, no studies have demonstrated a functional role of PLXNB2 in regulating tRNA levels in GBM tumors, nor has a role of a specific tRNA (5'tRNA-Gly-GCC) been proposed in GSC-populated tumors. 5'tRF-Gly-GCC has been found to be drastically elevated in the plasma of CRC patients and its role as a diagnostic biomarker has been proposed (Wu et al., 2021). Yet, pathological implications of this phenomenon remain unexplored. A preferential enrichment of tRFs, including 5'tRNA-Gly-GCC, has also been demonstrated in mouse glioma EVs (Gyuris et al., 2019). Elevated levels of ANG are widely found in GBM tumor mass (Eberle et al., 2000; Hu et al., 2019; Lucio-Eterovic et al., 2009; Skog et al., 2008) and in patient-derived GSCs (**Figure 3.3.A**). Our study provides the first mechanistic link between elevated tRNA levels and their roles in GBM pathology. ANG/PLXNB2 regulated 5'tRNA-Gly-GCC production in GSCs (**Figure 5.7.**) and 5'tRNA-Gly-GCC mediates ANG-induced self-renewal functions of GSCs (**Figure 5.8.**). PLXNB2 mAb decreased the levels of 5'tRNA-Gly-GCC in GSC-propagated orthotropic tumors in mice (**Figure 6.10.**) and sensitized tumors to chemotherapy (**Figure 6.5.**). We also propose for the first time a prognostic role of 5'tRNA-Gly-GCC in GBM (**Figure 6.10.C**), a function has been implicated in a study of GSC-released extracellular RNAs (Wei et al., 2017). A clinical correlation between 5'tRNA-Gly-GCC levels and patient survival remains to be determined. Further studies on the functions of 5'tRNA-Gly-GCC on tumor growth,

progression, stemness, and resistance will provide an exciting avenue in therapeutics of GBM and beyond.

7.4. Conclusions

Overall, we demonstrate that ANG and PLXNB2 regulate GSCs, a major driving force contributing to GBM resilience. The ANG-PLXNB2 axis regulates GSC self-renewal, differentiation, tumorigenicity, and chemotherapeutic response, all of which are pathologically- and therapeutically-relevant. Mechanistically, we demonstrate that ANG/PLXNB2 regulates small RNA metabolism in GSCs, in particular, we demonstrated that ANG-PLXNB2 elevates 5'tiRNA-Gly-GCC that enhances GSC stemness. Further, we demonstrate that PLXNB2 is a feasible therapeutic target that suffices the modulation of ANG/PLXNB2 signals in a pre-clinical setting and may be therapeutically harnessed for extinguishing GSC-mediated relapse. Lastly, we provide proof-of-concept data that PLXNB2 mAb is a promising pharmacologic agent to sensitize GBM to the first-line chemo drug, TMZ. It will be important to continue with further *in vivo* studies to assess the long-lasting effect of PLXNB2 inhibition in disease progression and in pathophysiology of other CSC-driven malignancies.

Chapter 8: Bibliography

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