

Premature senescence in primary glioblastoma cells

by

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ABSTRACT

Glioblastoma is the most common and fatal adult primary brain tumour. Despite maximum therapy, median survival time is 14 months after diagnosis. Senescence is a cellular stress response that results in irreversible growth arrest with continued metabolic activity. It has been shown to be a novel mechanism to inhibit tumorigenesis and tumor progression. However, the role of senescence in glioblastoma is poorly understood. Furthermore, resistance to therapy is believed to be in large part due to extensive heterogeneity in glioblastoma at the molecular level. While this has shed light on the biological understanding of glioblastoma, the impact of such heterogeneity in glioblastoma with respect to therapeutic mechanisms such as senescence induction is largely unknown and warrants further investigation.

Primary glioblastoma cells constitute an important model of study as they are a closer representation of the parent disease. In the present study, previously isolated primary glioblastoma cells from human patients were characterized according to their molecular subtype by microarray expression analysis. PriGO7A, PriGO8A and PriGO9A cells were predominantly of the classical subtype whereas PriGO17A were predominantly mesenchymal. I investigated the response of these PriGO cells towards various stress inducing agents to determine their capability to undergo senescence.

PriGO8A and PriGO9A cells underwent senescence in response to serum characterized by increased SA β Gal activity, PML bodies, p21 and morphological changes characteristic of senescence. This occurred in the absence of a detectable DNA damage response as seen without an increase in γ H2AX foci. There was also a lack of a senescence-associated secretory phenotype. Ionizing radiation, known to induce senescence in fibroblasts by inducing double stranded DNA damage, caused cell death but not senescence in PriGO8A and PriGO9A cells. Similarly, exposure of PriGO8A and PriGO9A cells to Triapine (an agent known to cause single stranded DNA damage) induced cell death without senescence. PriGO17A cells did not show evidence of cell death or senescence upon exposure to any of the above agents.

In subsequent studies, I investigated the molecular mechanism responsible for induction of senescence in PriGO cells. Microarray expression analysis revealed that serum exposure in PriGO8A cells increased the expression of genes associated with the Transforming growth factor- β (TGF β) pathway. The response of PriGO8A cells to serum was attributed at least in part to TGF β that was dependent on basal expression of the TGF β activator protein thrombospondin. PriGO7A cells lacked basal thrombospondin expression and did not undergo senescence in response to serum, but exhibited senescence in response to TGF β . PriGO17A cells, on the other hand, exhibited senescence in response to TGF β only when Ras activity was blocked.

In conclusion, primary glioblastoma cells retain a functional senescence program capable of undergoing senescence in response to TGF β , which suggests senescence can potentially be exploited therapeutically in glioblastoma. In addition, the response to therapeutic agents in glioblastoma is influenced by the molecular heterogeneity present in primary glioblastoma cells not only of different subtypes but also within the same subtype.

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ABBREVIATIONS

AMH: anti-müllerian hormone
AML: Acute myeloid leukemia
ARF: Alternate reading frame
ATCC: American Type Culture Collection
ATF3: Activating transcription factor 3
ATM: ataxia-telangiectasia mutated
ATR: ATM and Rad3-related
ATRX: Alpha-Thalassemia/Mental Retardation

BMP4: Bone Morphogenic Proteins
BRAF: v-raf murine sarcoma viral oncogene homolog B1
BRCA1: breast cancer 1, early onset
BSA: bovine serum albumin

C₁₂FDG: Fluorescein-Di-Beta-D-Galactopyranoside
Cdc42: cell division and control 42
CD36: Cluster Determinant 36
CDK: cyclin-dependent kinases
CDKN2A: Cyclin dependent kinase 2A
C/EBP β : CCAAT/enhancer binding protein β
CHK: checkpoint kinase
CoSmad4: Co- Suppressor of Mothers Against Decapentaplegic 4
CXCL: (C-X-C) motif ligand 1

ds DNA: double stranded DNA
DAPK: death associated protein kinase
DDR: DNA damage response
DMEM: Dulbecco's modified Eagle's medium
DROSHA
DSB: double stranded breaks

E2F1: E2 promoter binding factor
E2F4/5: transcription factor activating adenovirus E2 gene 4/5
ECM: extracellular matrix
EdU: 5-ethynyl-2'-deoxyuridine
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ELISA: enzyme linked immunosorbent assay
EMT: epithelial to mesenchymal transition
ERK: extracellular signal-related kinase

FACS: fluorescence-activated cell sorting
FAS: First apoptosis signal
FBS: fetal bovine serum
bFGF: Basic Fibroblast growth factor
FITC: Fluorescein Isothiocyanate
FOXO: forkhead homeobox type O

Gy: Gray
GADD45B: growth arrest and DNA damage-inducible 45
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GBM: Glioblastoma
G-CIMP: GC island methylator phenotype
GDF: growth and differentiation factors
GFAP: Glial fibrillary acidic protein

H2AX: Histone 2A
HCC: Human hepatocellular carcinoma
HDF: Human diploid fibroblasts
HIF: hypoxia inducible factor
HP-1: Heterochromatin protein 1
HRAS: Harvey rat sarcoma

ID: Inhibitor of differentiation
IDH1: Isocitrate dehydrogenase 1
IFN- γ : Interferon
IGFBP: Insulin growth factor binding protein
IKK α : I kappa B kinase- α
IL1R: interleukin-1 receptor

IMR90: Human lung fibroblasts

JAK: a Janus kinase
JNK: c-Jun-N-terminal kinase

KEGG: Kyoto encyclopedia of genes and genomes
KRAS: Kirsten Rat Sarcoma

Lgl: lethal giant larvae
Lgl3SA: lethal giant larvae 3 serine to alanine
LAP: latency associated peptide
LGG: lower grade gliomas
LIF: Leukemia inducible factor
LLC: TGF β large latent complex
LTBP: latent TGF β binding protein

miRNA: microRNA
 MAPK: mitogen activated protein kinase
 MDM2: Mouse double minute 2 homolog
 MEF: mouse embryonic fibroblasts
 MEK: MAPK/ERK kinase
 MH1: Mad homology 1
 MGMT: Methyl Guanine Methyl Transferase
 MKK7: Mitogen activated protein kinase kinase 7
 MRI: Magnetic Resonance Imaging
 MRN complex, MRE11-RAD50-NBS1 complex
 MTK1/MKK4: Mitogen activated protein kinase kinase kinase 4
 MYC: myelocytomatosis

NF1: Neurofibromin 1
 NK cells: Natural killer cells
 NKG2D: Natural Killer cell lectin-like receptor subfamily K
 NOD/SCID: Non-obese diabetic/severe combined immunodeficiency
 NOD/SCID IL2R γ^{null} : Non-obese diabetic/severe combined immunodeficiency Interleukin 2 receptor gamma null

OIS: oncogene induced senescence
 OLIG2: Oligodendrocyte transcription factor

p16INK4A: p16 Inhibitor of CDK4
 p21CIP1: p21 CDK-Interacting Protein 1
 Par6: partitioning defective 6
 PARP1: Poly [ADP-ribose] polymerase 1
 PAK2: p21/Cdc42/Rac1-activated kinase 2
 PBS: phosphate buffer saline
 PCNA: Proliferating cell nuclear antigen
 PDGFB: platelet derived growth factor B
 PDGFRA: Platelet derived growth factor receptor A
 PriGO cells: primary glioblastoma cells
 PI3K: Phosphatidylinositol-3-Kinase
 PKC ϵ : protein kinase c iota
 PML: Promyelocitic leukemia
 PTEN: Phosphatase and tensin homolog

Rac1: ras-related C3 botulinum substrate 1
 Rho: rhodopsin
 Rock1: Rho-associated, coiled-coil containing protein kinase 1
 RAS: Rat sarcoma
 Rb: Retinoblastoma
 RFK: The amino acid sequence RFK
 ROS: Reactive Oxygen Species

RPA: replication protein A
RR: Ribonucleotide reductase
RRM2: Ribonucleotide Reductase, M2 Subunit
R Smad: receptor Smad
RTK: Receptor tyrosine kinase
RT-PCR: reverse transcriptase polymerase chain reaction

siRNA: small interfering RNA
ss DNA, single strand DNA
SA β Gal: senescence associated beta galactosidase
SAHF: Senescence associated heterochromatin foci
SASP: senescence-associated secretory phenotype
SMAD: Suppressor of Mothers Against Decapentaplegic
SOX2: (sex determining region Y)-box 2
STAT: Signal Transducer and Activator of Transcription

TAK1: Transforming growth factor-beta-activated kinase 1
TCGA: The Cancer Genome Atlas
TERT: Telomerase reverse transcriptase
TGF β : Transforming growth factor β
TGFBRII: TGF β receptor-II
THBS: thrombospondin
TIF1 γ : Transcription intermediate factor 1 γ (TIF1 γ also known as TRIM33)
TMB: Tetramethylbenzidine
TMZ: Temozolomide
TNF- α : Tumor necrosis factor alpha
TP53: Tumorprotein p53
TRAF6: Tumor necrosis factor receptor-associated factor 6
TRIM3: Tripartite motif-containing protein 3
TUJ1: beta-III tubulin

UVB: Ultraviolet B

VEGF: vascular endothelial growth factor

XRCC1: X-ray repair cross-complementing protein 1

YAP: Yes associated protein 1

INTRODUCTION

Cancer: The human body is a multicellular organism that relies on the coordinated behavior of the individual cells. Cells send, receive, and interpret a complex set of signals instructing them on the proper development of the organism. This depends on appropriate cell division, differentiation, and death among a host of other activities. Uncontrolled cellular proliferation (neoplasia) can lead to a mass that is contained (benign) or one that has cellular invasion into the surrounding tissue (malignant). Invasion from the site of origin into the bloodstream or the lymphatics can lead to neoplastic cells colonizing distant organs (metastasis). Furthermore, it has been suggested that cancer has ten main hallmarks. They include cancer cells sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality and genomic instability, having abnormal metabolic pathways, evading the immune system, inducing tumor promoting inflammation and angiogenesis, as well as activating invasion and metastasis (1) .

Cancer is a leading cause of morbidity and mortality in the developed world. According to the World Health Organization (WHO), there were 14 million new diagnoses of cancer in the world in 2012 with the number of new cases expected to rise by 70% in the next twenty years (<http://www.who.int/mediacentre/factsheets/fs297/en/>). Cancer is the leading cause of mortality in Canada with almost 30% of all deaths caused by cancer in 2007 (Stats Canada). In addition, the economic toll of cancer is such that the total economic cost of cancer in 2010 was 1.16 trillion dollars. Thus, development of better modalities of cancer prevention and treatment are imperative (<http://www.statcan.gc.ca/pub/84-215-x/2010001/table-tableau/tbl001-eng.htm>).

Glioblastoma: Glioblastoma (GBM) is the most common primary malignant tumor of the adult brain (2). It is also one of the most lethal human cancers (3). Although not as large a burden on the global population as lung cancer, GBM still has an incidence of 3.5 per 100,000 new cases per year in the developed world (4). In the United States there is an incidence of 10,000 new cases annually with almost 50,000 patients currently battling GBM (5-7).

Historically, GBM has been classified as a grade IV astrocytoma by the WHO based purely on histopathological examination. Grade I tumors are non-invasive and considered mostly benign whereas the rest of the grades are increasingly invasive and malignant (8). Simple clinical observations have led to further division of GBM into primary and secondary GBM. Primary GBM arises in patients de novo whereas secondary GBM is progression of previous lower grade astrocytoma to GBM (9).

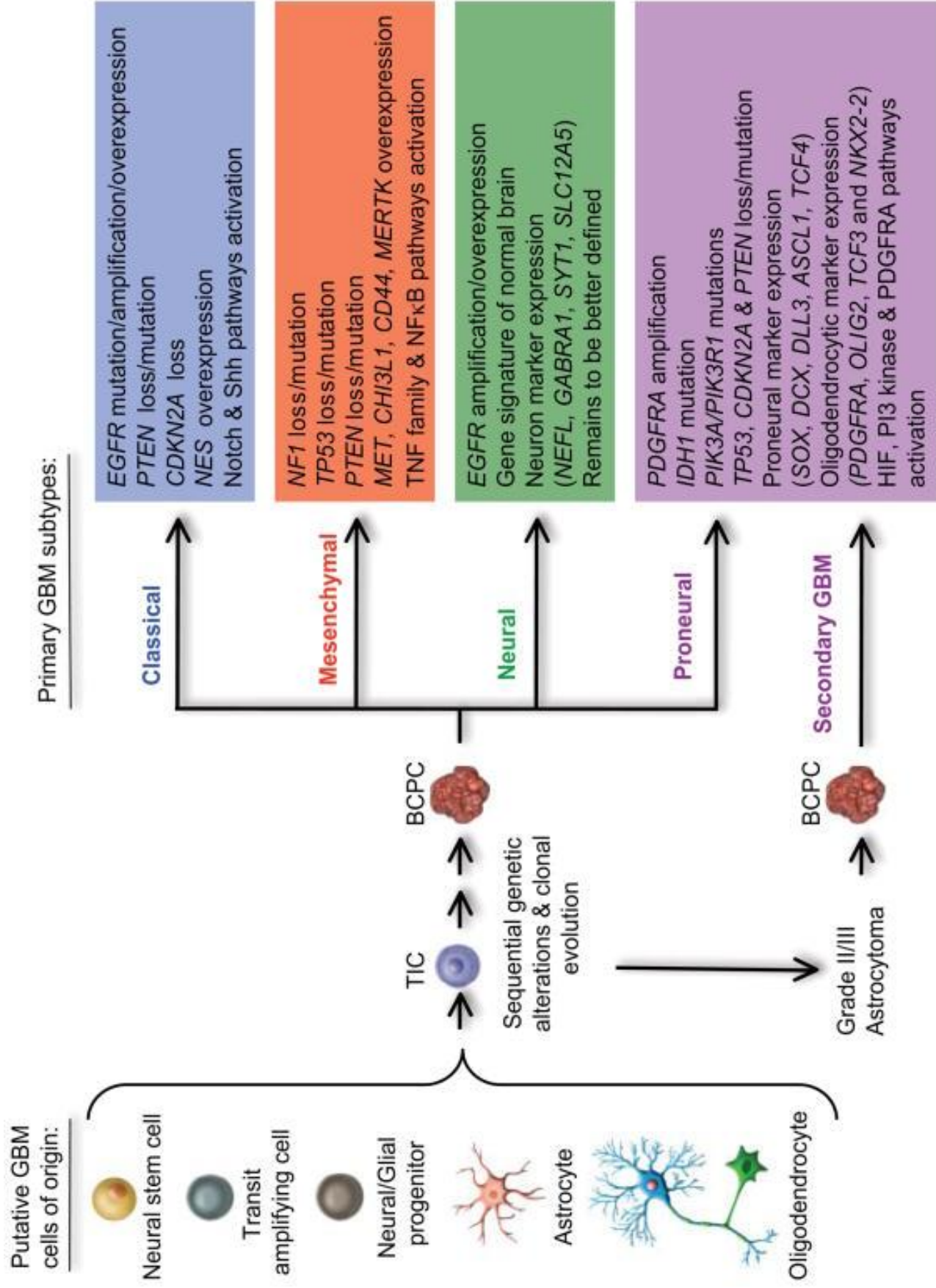
Treatment generally consists of surgical debulking followed by adjuvant chemotherapy and radiation therapy. Despite maximal therapy, the 10 year survival rate is 10% and the median survival time is 14 months (10). Given the highly invasive nature of GBM, total resection is not feasible and the disease inevitably recurs.

Heterogeneity of GBM: There has been an explosion of molecular data collected on many cancers recently, with GBM being one of the most extensively studied. Undoubtedly this has led to a better understanding of the biology of GBM. Molecular analysis of GBM has shed light on the extensive heterogeneity present in GBM (11).

Primary glioblastoma is now commonly placed in molecular subclasses (12) (**Fig 1.1**). Using unsupervised hierarchical clustering of transcriptional data from the Cancer Genome Atlas (TCGA) and combining it with DNA copy number alterations as well as sequencing data,

Figure 1.1: Sequential Genetic Changes Observed in the Pathogenesis of Different Subtypes of Glioblastoma. Some cells in the normal brain undergo genetic alterations, which leads to a population of tumor-initiating cells (TICs), which can then further accumulate genetic and epigenetic changes and become brain cancer-propagating cells (BCPC). The latter cells are responsible for the formation of glioblastoma. GBM indicates glioblastoma multiforme; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin homolog; TNF, tumor necrosis factor; PDGFRA, platelet-derived growth factor receptor-A; IDH, isocitrate dehydrogenase; PI3K, phosphoinositol 3-kinase; HIF, hypoxia-inducible factor.

Fig 1.1



Veerhak *et al.* divided GBM into 4 molecular subtypes: proneural, neural, classical, and mesenchymal (13). Subtypes are associated with different mutations, for example the classical subtype frequently has amplifications and mutations in epidermal growth factor receptor (*EGFR*) as well as loss of Cyclin dependent kinase inhibitor 2A (*CDKN2A*) and phosphatase and tensin homolog (*PTEN*). The proneural subtype commonly has alterations in platelet-derived growth factor subunit A (*PDGFA*) with frequent mutations in isocitrate dehydrogenase-1 (*IDH1*). The mesenchymal subtype was found to be enriched in mutations or loss of *NF1*, *TP53*, and *CDKN2A* (13). However, the neural subtype was not associated with any clear unique genetic signature. Noushmehr *et al.* expanded the framework of the integrated analysis by performing unsupervised hierarchical clustering of DNA methylation profiles to discover a subset of tumors being enriched for CpG island methylator phenotype (G-CIMP) in the proneural subclass. The G-CIMP subclass was also tightly associated with *IDH1* mutations (14).

Data also suggests that the proneural subtype is present in all patients and the other subtypes evolve from it through acquisition of additional mutations (15). Using mathematical modeling, Ozawa *et al.* suggested a temporal sequence of driver events that lead a proneural like precursor cell into most non G-CIMP mesenchymal subtype GBMs (15). They listed the evolutionary sequence as beginning with chromosome 7 gain and chromosome 10 loss. *CDKN2A* loss and/or *TP53* mutation with alterations canonical for specific subtypes lead to the mesenchymal subtype. Further computational modeling led to identification of *PDGFA* gain and *PTEN* loss as initial driving events in gliomagenesis. *PDGFA* was validated as a driving event in a mouse model of gliomagenesis with further *NF1* loss converting the proneural subtype into the mesenchymal subtype (15). Later studies have found other molecular mechanisms that also play a role in converting the proneural subtype into the mesenchymal subtype (16).

Further heterogeneity in GBM was demonstrated at the intratumoral level by Patel *et al.* 2014. Using single cell RNA sequencing to profile 430 cells from five primary glioblastomas, it was found that all five tumors, while consisting of a dominant subtype, contained a mixture of individual cells with different GBM subtypes. The proneural subtype was found in some cells of all tumors, independent of the dominant subtype of the tumor, further giving evidence to the idea that all GBMs arise from proneural like precursor cells (17).

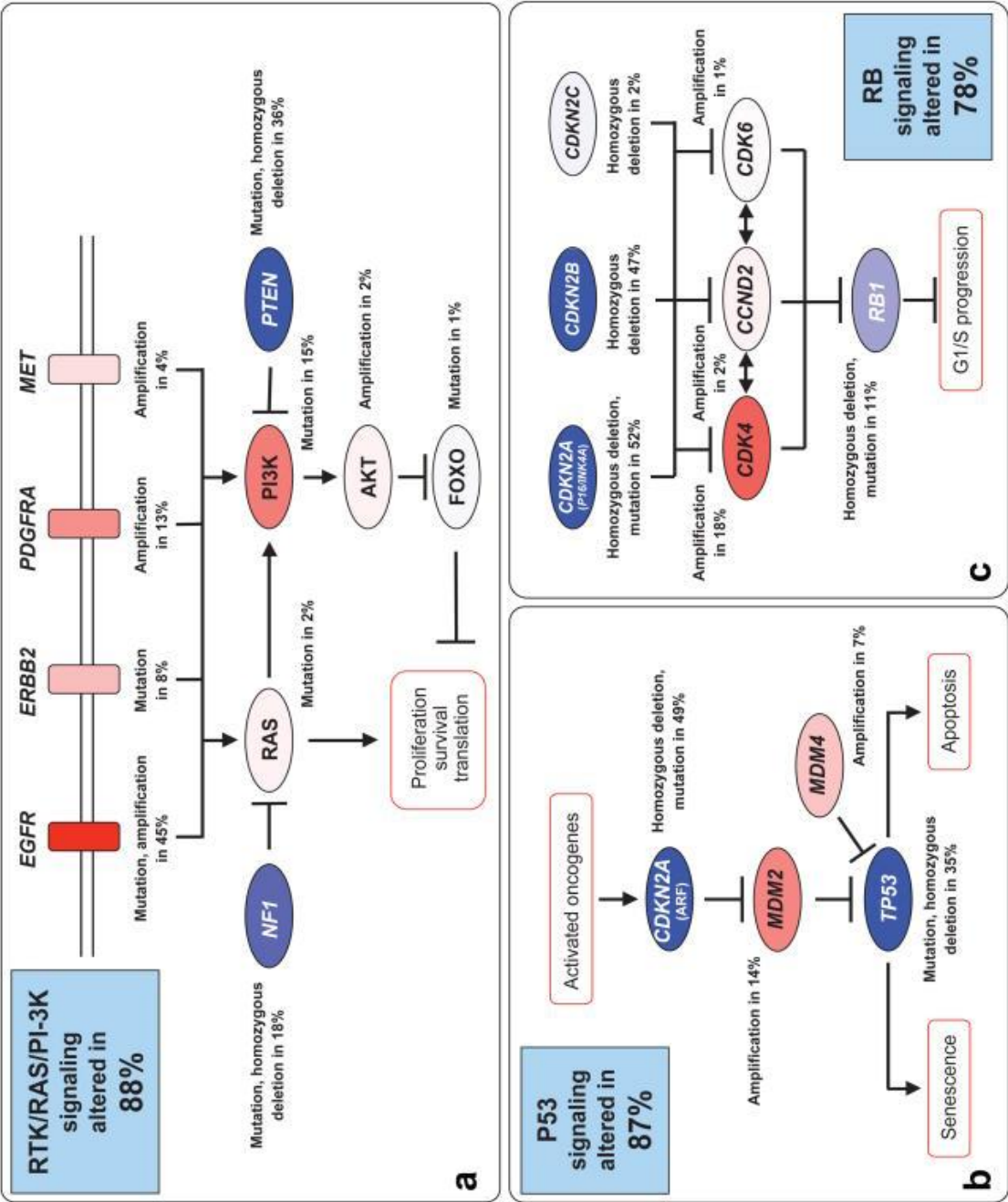
Increased complexity of GBM biology has been demonstrated further. It has become apparent that primary and secondary GBM possess different molecular correlates (9). Recent integrated analysis of gliomas, including genome wide analysis of lower grade gliomas (LGG) (grade II and III gliomas), demonstrated a different mutational profile between the various gliomas (18). In addition, LGGs with *IDH* mutation with concomitant 1p/19q codeletion and Telomerase reverse transcriptase (*TERT*) mutation were most strongly associated with the oligodendroglial histological type whereas LGGs with *IDH* mutation without 1p/19q codeletion often had mutations in tumorprotein p53 (*TP53*) and alpha-thalassemia/mental retardation (*ATRX*) and were strongly associated with grade II and III astrocytoma. Gliomas with wild type *IDH* and *TERT* as the only mutation were primarily grade IV astrocytoma (GBM) and were associated with the worst prognosis (19). Hence, lower grade astrocytomas that convert to GBM and primary GBM arise from different temporal sequence of driver mutations.

Despite such extensive heterogeneity in glioblastoma, integrated analysis of DNA copy number, gene expression, DNA methylation, and nucleotide sequence aberrations has shown that three core pathways are commonly disrupted and are key drivers of tumorigenesis (19) (**Fig 1.2**). They include the receptor tyrosine kinase (RTK)/ Rat sarcoma (RAS)/ phosphatidylinositol-3-kinase (PI3K) pathway (altered in 88% of GBMs), p53 signalling (altered in 87% of GBMs), and

Figure 1.2: Primary sequence alterations and significant copy number changes for components of the (a) RTK/RAS/PI-3K, (b) p53, and (c) RB signaling pathways are shown.

Red indicates activating genetic alterations, with frequently altered genes showing deeper shades of red. Conversely, blue indicates inactivating alterations, with darker shades corresponding to a higher percentage of alteration. For each altered component of a particular pathway, the nature of the alteration and the percentage of affected tumors affected are indicated. Blue boxes contain the final percentages of glioblastomas with alterations in at least one known component gene of the designated pathway.

Fig 1.2



Retinoblastoma (Rb) pathway (altered in 78% of GBMs). Although targeting of individual components of these pathways has not led to any drugs with meaningful improvements in survival, likely due to cross talk between pathways, targeting these pathways in combination may be of therapeutic value (20). For example, clinical trials of inhibitors of EGFR (gefitinib) and PDGFR (imatinib) have been disappointing (21,22). A phase II study investigating imatinib in recurrent gliomas demonstrated a partial response in only 5 out of 112 patients (23). Redundant pathways in RTK activation facilitate downstream signalling that causes resistance of tumors to therapy (24).

Genomic analysis of GBM has provided a wealth of data that is starting to show clinical implications. For example, it has been observed that GBM subclasses with *IDH* mutations (albeit rare) have better overall survival and do not gain significant benefit from intensive radiation and chemotherapy (13,25). It is also known that patients having promoter methylation of methyl guanine methyl transferase (*MGMT*), a DNA repair enzyme, leads to better response to Temozolomide (TMZ) (26). However, the clinical implications, such as overall survival or response to therapy, for the majority of GBM subclasses have not been identified. While GBM has been extensively studied at the molecular level, a deeper translation of genomic data to the bedside is required to enhance patient care.

Cancer Stem Cells:

Recently, GBM has been suggested to follow the cancer stem cell hypothesis (27), an idea that originated in embryonic tumors and has now broadened to encompass a spectrum of cancers (28-30). Teratocarcinomas were considered to originate from stem cells, as along with differentiated

cells, there were many types of undifferentiated cells that were noted in an aberrancy of embryonal development (28). Pierce *et al.* further noted that undifferentiated cells in these tumors possessed higher mitotic activity and the yield of teratocarcinoma formation correlated with the frequency of transplantation of the undifferentiated cells (31). Pierce's group would further go on to show a clonal origin of mouse teratocarcinoma by generating tumors from implantation of a single cell. The malignant cell not only propagated cancer but also gave rise to a relatively "benign" population of cells (32,33).

Similar observations were made in neuroblastomas, within a tumor existed a population of cells with variable amounts of differentiation (34). Prognosis depended on the degree of differentiation; surgery could cure differentiated tumors as they were focal (35), unlike undifferentiated tumors which were widely disseminated (36). In 1937, Furth and Khan demonstrated initiation of leukemia from donor mice into recipient mice, albeit only 5% of the time, hinting that leukemia possessed a heterogeneous population of cells of which a small percentage had tumorigenic potential (37). In summary, these data suggested that tumor growth and progression was caused by a relatively rare population of undifferentiated cells that formed a hierarchical organization of cancer, forming the backbone of the cancer stem cell hypothesis (38).

Bonnet and Dick applied these principles to show human acute myeloid leukemias (AML) followed the cancer stem cell hypothesis (39), followed by Al-Hajj *et al.* for breast cancer (29). Subsequently, Singh *et al.* and Galli *et al.* discovered a rare population of cells isolated from patient GBM samples that also appeared to be consistent with the cancer stem cell hypothesis (40,41). CD133⁺ GBM cells formed a rare fraction of GBM cells that were positive for markers of neural stem cells, such as Nestin, and retained the capacity to form neurospheres,

another property assigned to neural stem cells (41). CD133⁺ cells also were capable of differentiating into multiple lineages upon treatment with fetal bovine serum (FBS) for seven days. Importantly, CD133⁺ cells maintained the ability to self-renew compared to CD133⁻ cells (27). Singh *et al.* would later report a xenograft assay suggesting the tumor initiating capability of CD133⁺ cells in vivo. Injection of 100 CD133⁺ cells into mice brains could recapitulate the original tumor whereas injection of up to 10⁵ CD133⁻ cells failed to do so, establishing further support for the cancer stem cell hypothesis in GBM (40).

An argument put forth recently to explain the failure of radiation and chemotherapy in treating GBM is that current therapy targets the less malignant undifferentiated cells rather than the malignant stem cells. Chen *et al.* found TMZ treatment in a mouse model of glioma did not eradicate cancer cells with properties of stem cells which gave rise to highly proliferative cells forming the bulk of the tumor (42). Similarly, Bao *et al.* suggested GBM may be resistant to radiation therapy due to GBM cancer stem cells having enhanced capacity for DNA damage repair (43). Irradiation of CD133⁺ cells led to a greater proportion of surviving cells in both cell culture and mice xenografts compared to CD133⁻ cells. Inhibiting the DNA damage response led to a reversal of radioresistance in CD133⁺ cells, suggesting a novel mechanism to improve the radiation response of GBM in patients by specifically targeting unique mechanisms to GBM cancer stem cells (43).

Another therapeutic proposal that utilizes the cancer stem cell model of GBM is to induce differentiation of malignant stem cells into differentiated cells with reduced malignant potential (44). Vescovi *et al.* determined the proliferative potential of GBM could be reduced by inducing differentiation of CD133⁺ GBM cancer stem cells into the astrocytic lineage by treatment with bone morphogenic proteins (BMP4). Cheng *et al.* found through pulse chase experiments that

GBM cancer stem cells implanted into mice brains gave rise to vascular pericytes that supported the neovasculature of the tumor. Specifically inhibiting the differentiation of GBM cancer stem cells into vascular pericytes dramatically reduced the tumor burden in mice (45).

Despite the promising aspects of applying the cancer stem cell hypothesis to GBM, there are numerous issues surrounding it. Firstly, there are no definitive markers of cancer stem cells in GBM. Bier *et al.* proved CD133 is not a reliable marker as they were able to confirm tumorigenic activity of CD133- cells derived from patients with GBM (46). Secondly, the relative infrequency of cancer stem cells in GBM has also been questioned. Using the neurosphere assay, Singh *et al.* could only obtain cultures of cancer stem cells from patient samples 20% of the time (40), however, Pollard *et al.* and Al-Mayani *et al.* were almost always able to produce cultures (47,48). Laminin coated flasks were used to grow primary cells as a monolayer. Cells cultured in this manner not only retained the undifferentiated characteristics described earlier but also formed tumors when xenografted into mice brains that resembled the original tumor (47). The ability of an assay to underestimate the frequency of tumorigenic cells has been questioned in the study of melanoma. Human melanoma cells initially transplanted into Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice resulted in only 1 in a million cells capable of forming tumors (49). However, Quintana *et al.* were able to detect tumorigenic cells 1 in 4 times by switching to a more immunocompromised mouse model Non-obese diabetic/severe combined immunodeficiency Interleukin 2 receptor gamma null (NOD/SCID IL2R γ^{null}) (50).

A central controversy towards the cancer stem cell hypothesis in GBM is whether or not evidence actually exists to support the hierarchical model as the cause of the extensive

heterogeneity present in GBM (38). Considering that differentiated neurons could be induced to become stem cells severely complicates the hierarchical model presented by the cancer stem cell hypothesis (51). Furthermore, Suva *et al.*, using induction of transcription factors [sex determining region Y-box 2 (SOX2), oligodendrocyte transcription factor (OLIG2) among others]] were able to reprogram differentiated GBM cells into undifferentiated GBM “stem cells” (52). These data suggest determining the cell of origin of GBM may well be impossible at this time and that merely targeting GBM cells with stem cell components may not be adequate (38).

Cells harvested from human patients with GBM (primary glioblastoma cells) have been given various identities; some groups call them “Brain Tumor Initiating cells” (BTICs), others have referred to them as “Glioblastoma Stem Cells” (GSCs) (40,45). Although primary glioblastoma cells may not adhere to all the postulates of the cancer stem cell hypothesis, such as being the cell of origin of GBM or being rare in GBM tumors, they have been shown to possess certain stem cell properties. For example, they often are undifferentiated cells positive for Nestin, form neurospheres, and have the ability to differentiate into multiple lineages (47). Hence, we simply refer to them as primary glioblastoma cells. Such cells isolated at our facility were termed PriGO cells in response to a requirement of the ethics review process of their isolation from patients at the Ottawa Hospital. These cells also were positive for Nestin, able to differentiate into multiple lineages, and most importantly, grew into invasive tumors when xenografted intracranially into immunocompromised mice.

Primary glioblastoma cells provide a more accurate model to study GBM behavior. Most GBM research in the past was conducted with established cell lines that have been in culture for decades such as U87MG (53). Having been cultured in the presence of serum, an agent known to induce aberrancy in primary glioblastoma cells, and passaged countless times, they no longer

represent GBM properly (54). For this reason, primary glioblastoma cells are cultured in serum free media and not maintained beyond 20 passages (40).

Consequently, primary glioblastoma cells transplanted into mice brains form tumors that closely represent the original disease (40). Necrosis and neovascularization, hallmarks of GBM, are often seen in these tumors (40). In contrast, tumors formed from cell lines such as U87MG often form well circumscribed masses (55). Hence, investigation of mechanisms of invasion and tumorigenicity in primary glioblastoma cells may lead to more efficacious therapeutic development.

Senescence

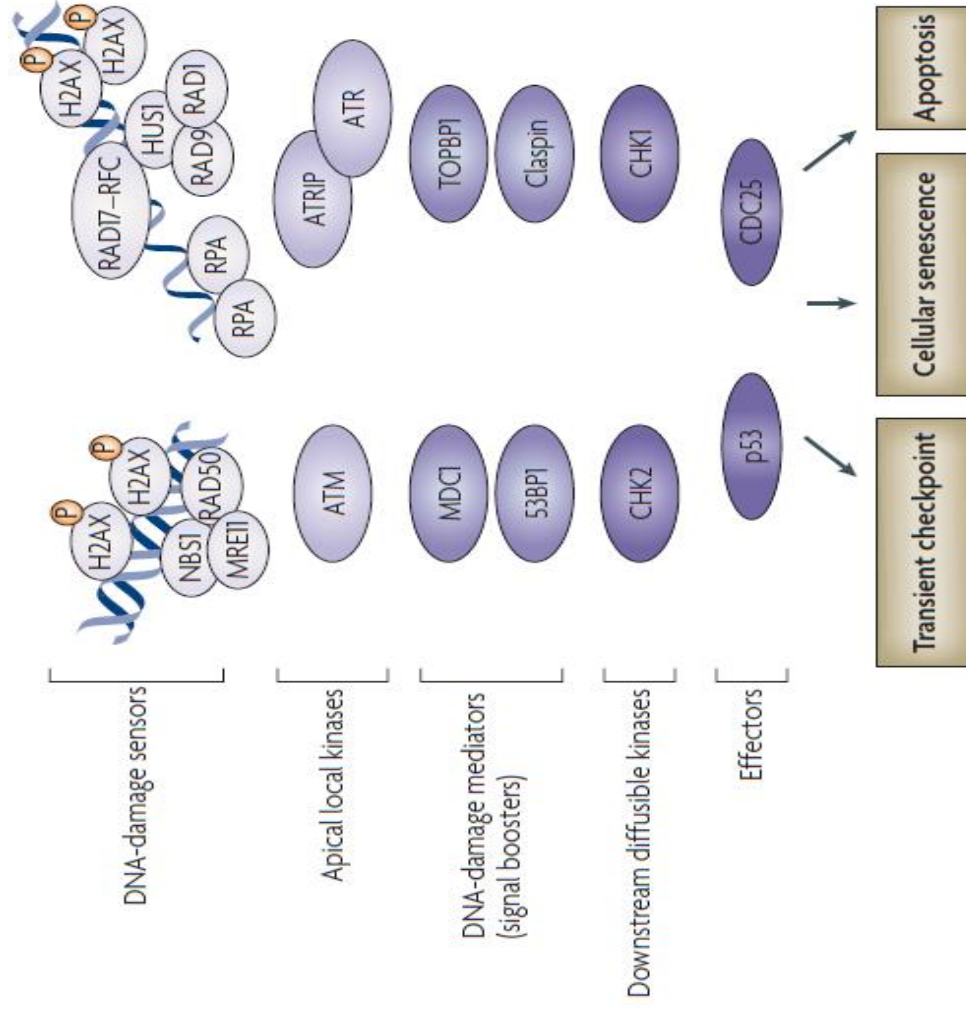
Senescence is a cellular stress response that results in irreversible arrest of growth despite continued metabolic activity (56). It was first described by Hayflick *et al.* as a mechanism for cell cycle arrest of fibroblasts at the end of their replicative lifespan (57). This was attributed to the eventual loss of protective nucleotide sequences at the end of DNA strands (telomeres) with repeated cycles of replication (58). Subsequently, it was found that cells could undergo senescence prematurely, before the end of their replicative lifespan, independent of the loss of telomeres (59) (60,61).

Molecular mechanism of senescence: All of the above mentioned causes of senescence depend on the central mechanism of DNA damage to exert their effect. DNA damage results in the activation of pathways that ultimately converge on p53 and the important regulators of cell cycle arrest such as retinoblastoma (Rb) (62) (**Fig 1.3**).

The tumor suppressor protein p53 is widely known as the guardian of the genome and its loss is known to occur in at least half of all human cancers. Normally, p53 gets degraded in the proteasome after being ubiquitinated by several E3 ligases (63), the most important being Mouse double minute 2 homolog (MDM2). Stress signals causing DNA damage trigger p53 activation by preventing its degradation and accumulation (64). As a transcription factor, p53 can cause expression of numerous genes leading to eventual outcomes such as apoptosis, quiescence, or senescence. One of the mechanisms by which p53 induces cell senescence is expression of p21Waf1 (65), which indirectly regulates Rb activity.

Figure 1.3: Schematic diagram showing the DNA damage response. Double stranded DNA damage results in activation of ATM mediated signalling converging on p53 activation compared to single stranded DNA damage that involves the ATR signalling cascade.

Fig 1.3

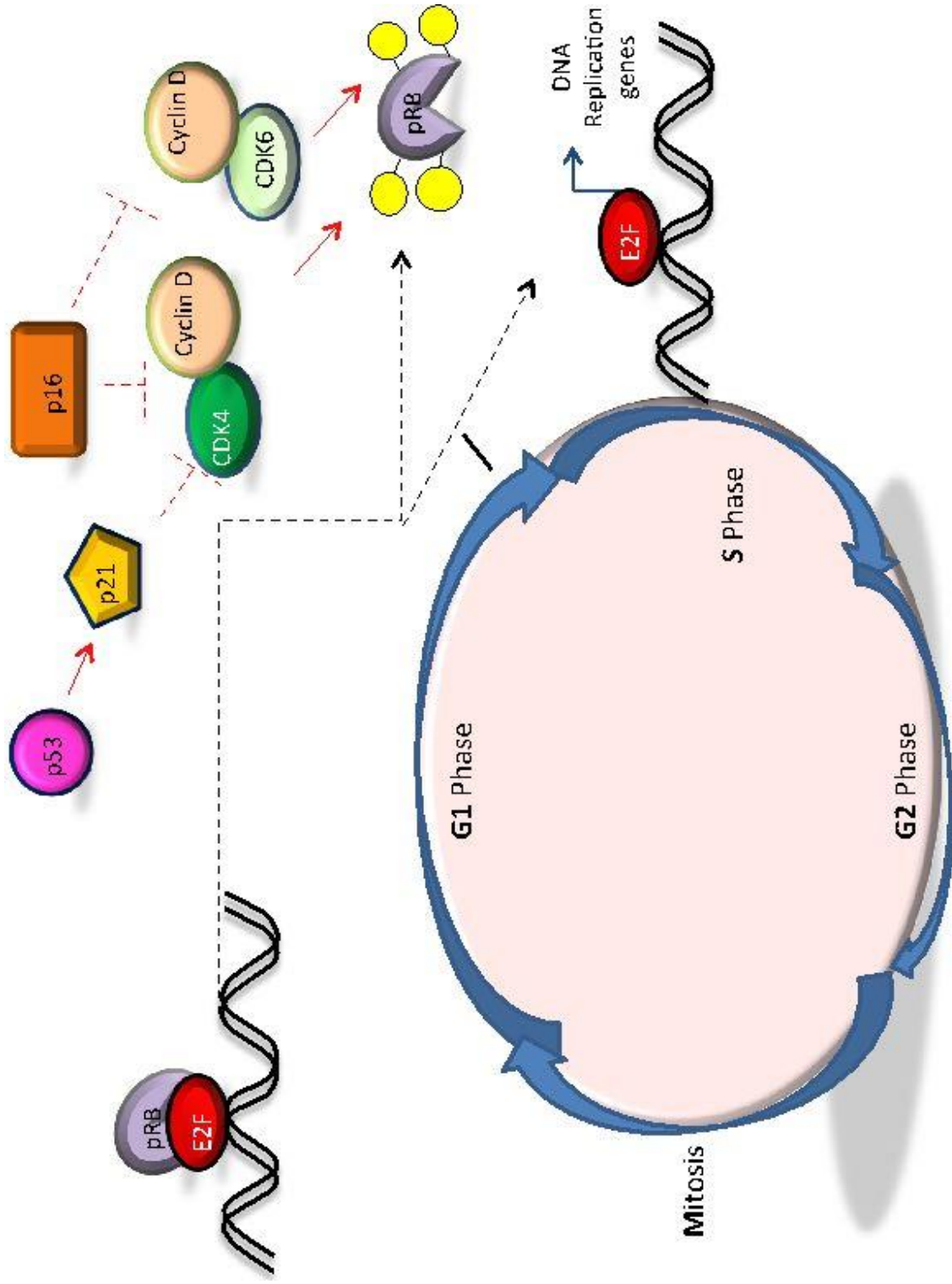


Retinoblastoma is considered the “master brake” of the cell cycle and plays a key role in senescence (66). By repressing E2F transcription factors in its active hypophosphorylated state, it inhibits transcription of genes essential for DNA replication and subsequent proliferation (67). Activity of Rb is regulated by the cyclin-dependent kinases (CDKs) which phosphorylate Rb, inactivating it and allowing the transcription of E2 promoter binding factor (E2F) responsive genes (**Fig 1.4**). The key proteins regulating the activity of CDKs-dependant Rb activity are p16 inhibitor of CDK4 (p16^{INK4A}) and p21^{Waf1}, both potent inhibitors of the cell cycle (68). p16 displaces cyclin D from CDK4 and CDK6 and prevents Rb phosphorylation, and arrest cells in the G1 phase (69). p21 can bind to all CDKs at any stage of the cell cycle but it does not necessarily displace cyclin molecules from their CDK targets (68).

The p53 mediated p21 activation is one of the end products of the DNA damage response (DDR) (70). The DDR is a signal amplification cascade that is triggered by the cell in response to discontinuity in the DNA double helix. It serves to prevent propagation of altered DNA into the daughter cells as well as to coordinate DNA repair (65). DNA damage that leads to double stranded breaks (DSBs) such as ionizing radiation are sensed by the MRE11-RAD50-NBS1 (MRN) complex that recruits the large kinase Ataxia-telengectasia mutated (ATM) (71). ATM undergoes autophosphorylation and also phosphorylates histone H2AX which recruits more ATM molecules causing a positive feedback loop and spreading the phosphorylation of H2AX along the chromatin (72). Once a threshold of ATM activity is reached, checkpoint kinase (CHK) 2 is activated by ATM phosphorylation (73). CHK2 then diffuses through the nucleus and phosphorylates numerous substrates which eventually lead to the activation of proteins such as p53 and stable cell cycle arrest through the induction of p21 (74).

Figure 1.4: This simplified model, focusing on early cell cycle entry, illustrates that hypophosphorylated, active pRb represses E2F-mediated transcription. The action of CDKs, exemplified by the cyclin D dependent CDK4 and 6, phosphorylate pRb and thus release E2F to activate transcription of early DNA replication genes. p16^{INK4a} and p21^{Waf1}, the latter usually activated by p53, inhibit CDKs and retain pRb in its active cell cycle inhibitory state.

Fig 1.4



Molecular Mechanisms of Cellular Senescence

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DNA damage in the form of single strand formation also leads to activation of the DDR (65). While the end effectors in this response are the same, p53 and p21, the ssDNA binding protein replication protein A (RPA) binds to the exposed strand generating a signal for the recruitment of ATM and Rad3-related (ATR) (75). ATR phosphorylation of CHK1 leads to its diffusion throughout the nucleus and subsequent downstream activation of p53 and p21 (76). Another mechanism by which ssDNA damage can cause senescence in epithelial cells is the downregulation of Poly [ADP-ribose] polymerase 1 (PARP1) with activation of the stress response leading to p16 induced cell cycle arrest without activation of the ATM or ATR dependent DNA damage response (77).

The mechanism by which the cell is driven into a permanent state of arrest is not clear. However, in some cell types senescence is associated with changes in chromosome structure that results in more stable repression of E2F responsive genes (78). Narita *et al.* demonstrated that senescent IMR90 human fibroblasts form heterochromatin that has methylation on lysine 9 in histone H3 (K9M-H3) with accompanying HP-1 (adaptor proteins involved in heterochromatin formation). Senescence associated heterochromatin foci (SAHF) formation of E2F responsive genes required Rb activity and was detected in senescent cells but not quiescent cells (67). However, this is not universal as some cell types, such as mouse embryonic fibroblasts (MEFs) can undergo senescence without robust SAHF formation (79).

Another study by Passos *et al.* suggests a feedback loop is triggered by the DNA damage response (DDR). This loop is mediated by p21 activation producing mitochondrial dysfunction leading to reactive oxygen species (ROS) generation that further establishes DNA damage foci, propagating the DDR feedback loop (80). After a delay of several days this feedback loop places the cell in a state of senescence (80).

Causative Agents of Senescence:

Telomere Attrition: The failure to maintain telomeres results in what is now called replicative senescence as it depends on repeated loss of telomeres with each round of DNA replication, essentially conferring cells a finite number of divisions (57,81). Telomeres normally contain protective proteins that once removed trigger the DDR akin to dsDNA damage leading to p53 activation (82). Furthermore, telomeres are sensitive to DNA damage themselves as they are not as readily accessible by the DNA damage repair apparatus (83).

Replicative stress: Replicative stress, not to be confused with replicative senescence, is the slowing or stalling of replication fork progression and/or DNA synthesis, usually results in the generation of stretches of single stranded (ss)-DNA (84). This results from the helicase continuing to unwind the DNA despite the stalling of the replication fork (85). The exposed SS-DNA can initiate the DDR, as explained later through RPA binding and recruitment of ATR (84,86). Specific detection of replicative stress is challenging as many of the markers for DNA damage are non-specific such as γ H2AX (84). Although the clearest readout of replication stress is the measurement of DNA synthesis by DNA combing assays, other methods of detecting replication stress include ATR dependant phosphorylation of RPA as well as RPA foci indicating generation of ss-DNA (87-89).

Oncogene induced senescence: Primary cells could be induced prematurely to undergo senescence by oncogenes, termed oncogene induced senescence (OIS) (90). For example, expression of the oncogene, Harvey rat sarcoma (*HRAS*), leads to a biphasic response

characterized by an initial phase of hyperproliferation followed by the slowing of proliferation and eventual entrance into senescence (91). Expression of other oncogenes such as v-raf murine sarcoma viral oncogene homolog B1(*BRAF*), *c-myc*, and E2 promoter binding factor (*E2F1*) has demonstrated the same biphasic response as *HRAS* (92-94). OIS is thought to be a result of the DDR, due to failure of the replication fork to progress, with RPA binding and ATR activation (95).

DNA damaging agents: Ionizing radiation exerts its effects on cells by creating double stranded (ds)-DNA breaks eliciting the DNA damage response, activating the ATM-p53-p21 response pathway (96,97). In WI-38 human fibroblasts, ionizing radiation induces both apoptosis and senescence through p53 activation (98). Senescent cells demonstrate DNA damage foci alongside increased levels of cell cycle arrest markers (65).

Chemotherapeutics also function by causing DNA damage. Hydroxyurea, an inhibitor of the enzyme ribonucleotide reductase (RR), has long been used for the treatment of certain leukemia (99). Inhibition of RR leads to a lack of conversion of ribonucleotides to deoxyribonucleotides, stalling the replication fork and leading to replicative stress induced senescence (100). Hydroxyurea is known to induce senescence in human diploid fibroblasts with increased levels of p53 and p21 (101). Recently developed potent inhibitors of RR include Triapine, an agent currently in early clinical trials in cervical cancer (102).

Oxidative stress: Many types of stress stimuli including oncogene activation and telomere attrition increase the level of ROS (103,104). High intracellular ROS levels induce senescence by activating the p38 MAPK pathway leading to increased expression of p53 and p21 (105).

Furthermore, hyperoxia can induce senescence in cells and treatment with anti-oxidants can delay or prevent senescence (106,107).

Culture stress: Premature senescence can be induced by culture stress and is independent of telomere attrition (108). This can be due to inadequate culture conditions such as an abnormal concentration of growth factors, ambient oxygen levels as well as the lack of normally surrounding extracellular matrix components and cell types (59,109-111). For example, most cells experience plasma *in vivo*, not serum and Loo *et al.* found serum treatment led to the induction of premature senescence in MEFs (112). Senescence due to culture stress can also occur without detectable DDR signaling (113).

Detection of senescence: Although no single specific marker to detect cellular senescence currently exists, senescence is usually identified by a combination of signature features including cessation of proliferation (59). The best biochemical test to identify senescence is senescence associated beta galactosidase activity (SA β Gal) (114). SA β Gal detects the activity of the lysosomal β -galactosidase enzyme at a pH of 6 where the increased lysosomal activity of senescent cells is displayed rather than that of non-senescent cells which usually occurs at a pH of 4 (115). Senescent cells also have increased expression of genes involved in the p53/Rb network of tumor suppressors such as p21/p16 (116), although they indicate cell cycle arrest and not necessarily senescence. Another general feature of senescent cells, although non-specific, is morphological changes including flattening and enlargement.

Senescent cells also demonstrate signs of DNA damage and markers of DNA damage usually offer supporting evidence of cells having undergone senescence (82). Markers commonly

used include γ H2AX, although recent literature debates its usefulness (65). Firstly, γ H2AX as mentioned before indicates dsDNA damage and hence may not be seen in cells having undergone senescence in response to ssDNA damage, in which case other markers such as RPA can be present instead (84). Secondly, recent evidence points to a different apparatus of DNA damage response being used depending on the cell type. While fibroblasts utilize the ATM-ATR dependent DNA damage response, epithelial cells undergo senescence in response to ssDNA damage by forming persistent and large X-ray repair cross-complementing protein 1 (XRCC1) foci that activate the p38 mitogen activated protein kinase (MAPK) signaling cascade ultimately leading to p16-mediated cell cycle arrest (77). Finally, cells can senesce without activation of the DDR such as with ectopic expression of p21 and p16 (117,118). Thus DNA damage is very often present in senescent cells but it is not absolutely required.

In an effort to detect senescence more specifically, factors involved in SAHF formation have also been looked at. Initially, tri-methylated lysine 9 histone H3 (H3K9Me_{2/3}) seemed promising but it also had limitations in confirming senescence in cells (67). However, other components of SAHF formation have been more promising as markers of senescence. Ferbeyre *et al.* not only found promyelocytic leukemia (PML) to be upregulated and forming nuclear bodies in senescent cells but regulated senescence directly (119).

Function of Senescence: Senescence has been shown to have numerous physiological roles. Firstly, it is a naturally occurring phenomenon during development (120). Numerous senescence positive structures in the embryo have been identified including the neural tube (121). Although the majority of studies involve mice, it has been shown to occur in human embryos as well (122).

Senescence also occurs in adult cells such as megakaryocytes as well as in the human placenta (123,124).

Senescence has been found to be both beneficial and detrimental in disease processes. Induction of senescence in hepatic stellate cells limits fibrosis following chronic liver injury (125). Similarly, myofibroblasts generated during wound repair undergo senescence to limit fibrosis (126). Other instances where cells undergo senescence to limit fibrosis include renal and cardiac cells following ischemia (127,128). Senescence has also been shown to protect against atherosclerosis (129).

However, cellular senescence has also been found to be associated with premature ageing. Firstly, in a mouse model, senescence was recently shown to promote ageing and age related deterioration of several organs such as the kidneys, heart, and fat. Clearance of p16 positive cells prolonged lifespan and preserved functioning of glomeruli and adipocytes (130). Another example is mice with overly active p53 activity which demonstrated premature degenerative changes such as loss of subcutaneous fat and osteoporosis and cells from these mice were prone to undergo senescence (131,132). Other negative impacts of senescence include aggravation of pulmonary fibrosis, association with obesity and promotion of type 2 diabetes (122).

Senescence in cancer: Senescence has complementary roles in cancer as well, exerting pro and anti-tumorigenic effects depending on the circumstances. Senescence is known to inhibit tumorigenesis but is highly dependent on two critical tumor suppressor networks of p53 and Rb (113). Cells with these pathways intact undergo senescence in response to oncogenic stimuli to prevent neoplastic transformation. Premalignant human nevi contain senescent cells whereas the

malignant counterpart melanoma is devoid of such cells (92,133). Similar results were obtained in mice models of oncogenic Ras expression (90). These data indicate that malignant cells escape the inherent mechanism of senescence present in the premalignant lesions.

Aside from prevention of tumorigenic cells to emerge, senescence has also been shown to inhibit tumor progression. Ventura *et al.* demonstrated reactivation of p53 in mice with established sarcomas resulted in dramatic tumor regression with several characteristics of senescence (134). Similarly, Xue *et al.* showed reactivation of p53 in invasive hepatocarcinomas caused induction of senescence in these tumors. Interestingly, senescence induction elicited an innate immune response that resulted in clearance of the tumors (135). This provided evidence that not only can tumor cells still retain the capacity to undergo senescence but that senescence induction in tumor cells may be a viable therapeutic option.

Senescent cells also partake in extensive signalling to the environment through the increased expression of growth factors such as insulin growth factor binding protein (IGFBP), chemokines (CXCL), inflammatory cytokines (IL-6) and proteases termed the senescence-associated secretory phenotype (SASP) (121). SASP can play a role in tumor suppression but paradoxically has also been known to exert a protumorigenic influence (135,136). IGFBP7 is secreted by senescent human melanocytes and can induce senescence in neighboring non-senescent melanocytes (137). Senescent fibroblasts can secrete IL-6, IL-8, and plasminogen activator inhibitor-1 (PAI-1) to promote tumor suppression (138-140). However, malignant tumorigenesis can be promoted by IL-6 and IL-8 in other circumstances. IL-6 and IL-8 can also stimulate invasion of the basement membrane by premalignant epithelial cells (141,142). Finally, vascular endothelial growth factor (VEGF) can be secreted by senescent fibroblasts to stimulate angiogenesis (143).

Senescence in GBM: GBM, like most other cancers, bypasses replicative senescence in order to proliferate continuously. There are high levels of mutations in the TERT promoter as well as mutations in the alpha-thalassemia/mental retardation (ATRX) gene to facilitate limitless replication (144,145). GBM also frequently has mutations in the p53 and Rb pathways such as mutations in the CDKN2A locus (146).

However, despite these losses, GBM cells have still been shown to undergo senescence in response to various stimuli. Irradiation in U87MG cells primarily stimulates senescence as does treatment with the DNA topoisomerase 1 inhibitor SN-38 in cells with intact p53 (53,147). Furthermore, U-1242 MG cells that have lost p16 undergo senescence with ectopic expression of p16 (148). Finally, treatment of multiple GBM cell lines with the anti-cancer agent berberine resulted in senescence through inhibition of EGFR signaling (149). It should be noted though that these data are mostly compiled from established cell lines that likely do not well represent the original disease. It remains to be seen if primary glioblastoma cells also harbor the capability to undergo senescence.

TGF β

Transforming growth factor (TGF) β is a cytokine that belongs to the TGF β superfamily (150). It plays a key role in complex functions of epithelial and neural tissues, the immune system, as well as wound repair (151). TGF β also regulates cellular proliferation, differentiation, survival, and adhesion to prevent malignancy from occurring (151). However, along the evolutionary path to cancer, cells develop the ability to negate its effects or even manipulate the signalling pathway to promote tumor growth and invasion, metastasis, and immune suppression (151).

The TGF β superfamily of proteins has been identified in numerous organisms (152,153). While seven members have been identified in *Drosophila*, there are over 30 members in humans (154). The superfamily can be further divided into two branches. One branch comprising of activin, nodal, lefty, myostatin, and TGF β and the other consisting of bone morphogenic proteins (BMPs), anti-mullerian hormone (AMH) and growth and differentiation factors (GDFs) (155-157). The two branches often have diverse yet complementary effects and vary in their temporal and spatial expression patterns. Embryonic stem cell differentiation, body axis formation, left-right symmetry, and organogenesis are heavily influenced by activins, nodals, BMPs, and AMH, and GDFs (151). BMPs are also involved in bone growth and repair while myostatin causes inhibition of muscle development (158,159). Virtually all cell types are responsive to TGF β and it is expressed in many cell types (151). On the other hand myostatin is expressed in very few types of cells (151). BMP2, as an example, is expressed throughout the life of an organism whereas AMH is only expressed during embryonic development (151,160).

TGF β has three isoforms (TGF β 1, β 2, and β 3) with 70-80% homology between the three (161). Although the effects are similar *in vitro*, they are quite diverse *in vivo* (162,163). In the context of my studies I will elaborate on TGF β 1. TGF β 1 is the most commonly studied of the three isoforms and contains 390 amino acids. The N-terminal consists of a signal peptide that facilitates secretion from the cell while the C-terminal region contains the eventual mature TGF β molecule. The two ends are linked by a pro-region latency associated peptide (LAP) (164). Upon cleavage of the LAP in the golgi by furin type enzymes, TGF β forms eight disulfide links within the protein and a ninth link to another TGF β molecule to form a 25 kDa dimer (165). However, there is still non-covalent interaction between the LAP and the mature TGF β . Furthermore, this

complexes with the latent TGF β binding protein (LTBP) to form the TGF β large latent complex (LLC) which is ultimately secreted from the cell (166).

Unregulated signaling of TGF β can lead to severe complications such as fibrosis or autoimmune disease (167). Since TGF β binds to its receptors with high affinity, release of TGF β from the LLC represents tight control over TGF β activation (165). Activation of TGF β can be mediated by numerous factors including proteases (plasmin, metalloproteases), integrins, pH, reactive oxygen species, and thrombospondin (THBS) (165,168). The mechanistic details involved in activation are not fully understood but it is known that some factors may be cell or tissue specific whereas others are seen more ubiquitously (169).

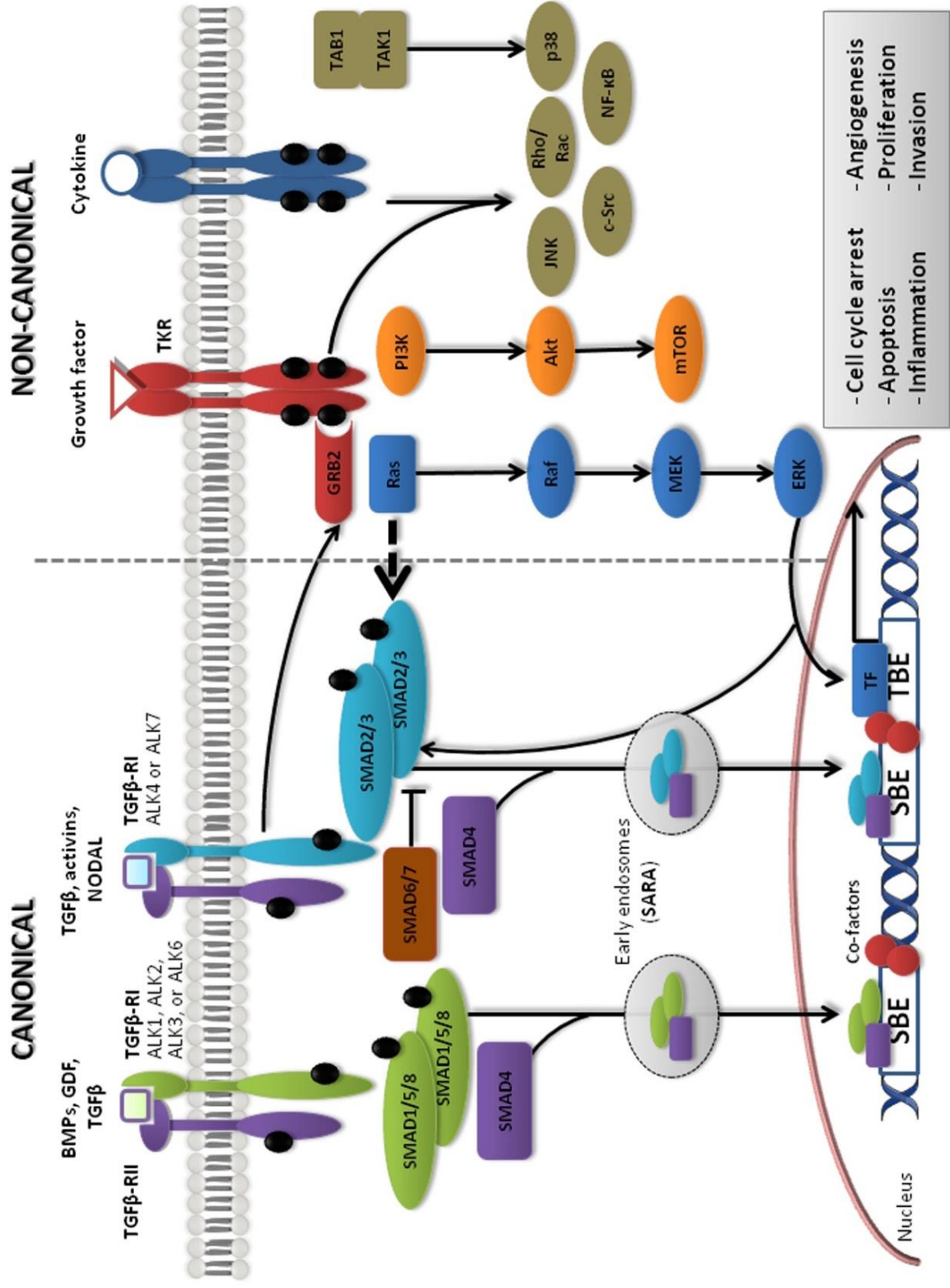
TGF β signaling: TGF β canonical signaling begins with the homodimeric molecule binding to the TGF β receptor type 1 (a serine kinase) causing phosphorylation of the TGF β type 1 receptor (a threonine kinase) and leading to the formation of a heteromeric complex (151). The type 1 receptor subsequently phosphorylates receptor Smad (R Smads) proteins (normally present in the cytoplasm) leading to signal transduction. Smad (suppressor of mothers against decapentaplegic) proteins contain 2 regions, a MH1 region that binds to DNA and a MH2 (mad homology) region that binds to various coactivators and corepressors (160). The R Smads then complex with CoSmad4, the only known CoSmad in humans (157). This complex shuttles to the nucleus for gene transcription (**Fig 1.5**). RSmads 2 and 3 are activated by the TGF β branch of the superfamily and Rsmads 1, 3, and 8 are activated by the BMP branch (151).

Each RSmad-CoSmad cofactor combination allows transcription of specific genes based on interactions with the regulatory region of the target genes. Many genes can be simultaneously

activated or repressed by a single TGF β stimulus through the numerous combinations with the various cofactors available (170). Hence, the cellular context of the specific signal based on the different Smads present lends the TGF β pathway tremendous pleiotropy with severe consequences if gone awry (151).

Figure 1.5: Overview of the TGF β family pathways, canonical and non-canonical.

Fig 1.5



Non-canonical TGF β signaling can be through branching Smad pathways or Smad-independent pathways. Removal of *SMAD4* in numerous organs in mice does not lead to improper development of such organs, provided the TGF β receptors are still functional (171,172). Transcription intermediate factor 1 γ [(TIF1 γ also known as tripartite motif-containing **protein 3** (TRIM33)] facilitates TGF β -induced erythrocyte differentiation through receptor activated Smad 2/3 in a Smad 4-independent manner (173). Smad 2/3 activated by TGF β also controls mouse keratinocyte differentiation by binding to I κ B kinase α (IKK α) (174). In vascular smooth muscle cells, receptor activated Smad 2/3 also bind to p68 (a component of microRNA (miRNA) processing complex DROSHA) to control production of miRNA miR-21, important in inducing a contractile cell phenotype (175).

Numerous TGF β signaling cascades that are Smad-independent are known despite the details of their activity yet to be elucidated. The activation of the TGF β receptor complex can also stimulate the JNK and p38 mitogen activated protein kinase (MAPK) pathway through interaction with the interleukin-1 receptor-effector module IL1R-TRAF-6 (tumor necrosis factor receptor-associated factor 6) - transforming growth factor-beta-activated kinase 1 (TAK1) (176). The Rho-Rock1 (rhodopsin-ras-related C3 botulinum substrate 1) and Cdc42 (cell division and control 42)/Rac1(ras-related C3 botulinum substrate 1)-PAK2 (p21/Cdc42/Rac1-activated kinase 2) complexes can also be stimulated by TGF β receptor activation (177,178). TGF β signaling without involvement of the type 1 receptor is also known. The type 2 receptor can phosphorylate Par6 allowing it to dissolve tight junctions in epithelial cells in the process of epithelial to mesenchymal transition (EMT) (179). Furthermore, other signaling pathways can also interact with the TGF β signaling cascade such as the MAPK pathway. Growth factor mediated MAPK-

ERK (extracellular signal-related kinase) activation causes phosphorylation of Smad 2/3 at several serine/threonine sites, attenuating TGF β signaling such as in colorectal cancer (180).

TGF β in tumor suppression: Cellular utilization of TGF β signaling as a tumor suppressor mechanism is through cell autonomous tumor suppressor mechanisms including cytostasis, senescence, differentiation, and apoptosis (151). TGF β characteristically suppresses cells in the G1 phase of the cell cycle (160). Smad3/4 complexes with FoxO transcription factors, with requirement of Sp1, stimulates the expression of p15 and p21 CDKIs, key mediators of cytostasis (181-183). The cell type determines the specific CDKI activated in response to TGF β signaling as p57 is expressed in hematopoietic stem/progenitor cells whereas p15 and p21 are expressed in astrocytes and neural progenitor cells respectively (182,184,185). TGF β also mediates repression of c-myc, a potent transcriptional inducer of cell growth and division, through a complex of proteins including Smad3/4, p107, E2F4/5 (transcription factor activating adenovirus E2 gene 4/5), and C/EBP β (CCAAT/enhancer binding protein β) (186,187).

TGF β also suppresses growth by promoting differentiation. Mesenchymal precursors differentiate into fibroblasts and myofibroblasts, whereas other members of the family such as BMP4 promote mesenchymal precursors into astroglia (151,155). ID (Inhibitor of Differentiation/DNA binding) proteins repress differentiation and TGF β downregulates ID1 in epithelial and endothelial cells in culture. The ATF3 (activating transcription factor 3), a repressor of ID1, is stimulated by TGF β induced Smad3 signalling (188).

TGF β in senescence: Senescence is another modality of TGF β mediated tumor suppression. TGF β signaling has been shown to induce replicative senescence as well as premature senescence. The human adenocarcinoma cell line A549 underwent replicative senescence in response to TGF β treatment. TGF β led to positive SA β Gal as well as morphological changes consistent with the senescent phenotype by downregulating telomerase activity (189). Human diploid fibroblasts (HDFs) underwent premature senescence upon treatment with subcytotoxic doses of UVB (ultraviolet B) through TGF β signalling (190). The signaling mechanisms leading to TGF β mediated senescence can be complex. Human hepatocellular carcinoma (HCC) cells underwent senescence in response to TGF β by upregulating p21 and p15 in a p53 independent manner (191). However, there was an increase in accumulated reactive oxygen species, a phenomenon also observed by Passos *et al.* They determined a positive feedback loop of senescence induction. The loop relied on TGF β signaling mediated ROS generation from the mitochondria to induce a state of perpetual DNA damage to lock the cell into a senescent state (80). Human corneal epithelial cells also demonstrated a senescence response to TGF β treatment with upregulation of p16 and p21. TGF β also induced a SASP response in these cells with increased levels of Il-6, Il-8, and TNF- α (192). These observations indicate the possibility of TGF β as a therapeutic strategy for the treatment of certain cancers by promoting premature senescence.

TGF β in apoptosis: Although TGF β has been observed to induce apoptosis *in vivo*, the molecular mechanisms involved have not yet been delineated (151). *In vitro*, TGF β has been shown to induce apoptosis in several cell lines. Although the mechanisms are not clear, involvement of Smad dependent and independent pathways have been noted. For example,

apoptosis in the hepatoma cell line with induction of death associated protein kinase (DAPK) has been shown (193). Apoptosis induction in hepatocytes through GADD45B has also been shown (193). Gastric carcinoma cell lines can also undergo apoptosis through the induction of the death receptor FAS in response to TGF β (193).

TGF β in the environment: TGF β also exerts a tumor suppressive effect by interacting with cells in the stroma, especially fibroblasts. Evidence suggests inhibition of paracrine factors expressed by stromal fibroblasts limits the surrounding epithelial proliferation (151). Mice generated with targeted deletion of the *TGFBRII* (TGF β receptor-II) in fibroblasts developed prostatic and forestomach hyperplasia that eventually progressed to neoplasia (194). In addition to fibroblasts, TGF β exerts influence on immune cells to curb inflammatory reactions. Its important immunosuppressive effects were demonstrated upon conditional deletion of *TGFBRII* in the hematopoietic system of mice leading to lethal inflammatory disease (195,196). Furthermore, TGF β exerts its immunosuppressive effects by targeting CD4⁺ cells (Th1 and Th2), CD8⁺ cells, dendritic cells, natural killer (NK) cells, and macrophages as well as promoting generation of regulatory T cells that in turn also inhibit NK cells and macrophages (151). These combined effects are important in developing immune tolerance which is critical in the intestinal mucosa (197). Loss of immune tolerance in the intestinal mucosa is important in the development of colon cancer, as it is often a progression from inflammatory bowel diseases (ulcerative colitis and crohn's disease) with malfunctioning TGF β signalling central to the pathogenesis (197).

Given the importance of the tumor suppressive actions of TGF β , it is not surprising that many types of cancers evolve mutations in the TGF β signaling pathway to escape its effects

(151). Biallelic inactivating mutations of *TGFBR2* are often a consequence of mutations in replication mismatch repair genes, seen frequently in cancers such as colon, gastric, and pulmonary among others (198). Frameshift and missense mutations in the *TGFBR1* gene have also been observed in numerous cancers (151). Although receptor Smads (RSmads) are critical to TGF β signalling, they are often not mutated in cancers (151). However, rare types of colon cancer have been found to contain mutations in *SMAD2* (199). On the other hand, mutations in CoSmads are often seen in cancer. *SMAD4* is often deleted or inactivated in pancreatic cancer to almost the same degree of prevalence as mutations in p53, *KRAS* (Kirsten Rat Sarcoma), or p16 (200). In addition, overexpression of Smad antagonists such as Smad6 and Smad7 negatively affects TGF β signaling and is seen in endometrial cancers (201,202).

Cancer cells often maintain TGF β signaling but lose the tumor suppressive arm of the pathway in order to escape the tumor suppressive effect of TGF β , usually by having defective or loss of cytostatic genes (151). Breast cancer cells isolated from the pleural fluid of human patients with metastatic disease to the lungs demonstrated intact TGF β receptor, functioning Smads, and responsiveness of certain TGF β genes upon stimulation of the pathway, but not p15 induction or c-myc repression (186).

Pro-tumorigenic effects of TGF β : Losing the tumor suppressive arm of the TGF β signaling pathway but maintaining other aspects of the pathway confers a selective advantage to cancer cells as TGF β can also exert pro-tumorigenic effects. Firstly, TGF β is a key player in epithelial to mesenchymal transition (EMT), a normal process in development that is driven by numerous transcription factors such as Snail, Slug, and Twist among others (151,203). As mentioned

earlier, phosphorylation of Par6 by TGFBR2 also plays a role in modulating the cytoskeleton of epithelial cells (179). However, TGF β mediated EMT is a pathological process in neoplasia that facilitates metastasis. EMT grants cancer cells motility and invasive properties by losing E-cadherin and other epithelial cell junctions and gaining a mesenchymal cell cytoskeleton (204). Mouse models have shown EMT in response to TGF β (155,205). In addition, gene expression analysis of human breast cancer cells identified an active TGF β pathway. Inhibition of this pathway led to a change from a mesenchymal phenotype to more of an epithelial one (206).

TGF β also supports an invasive phenotype by mobilization of myofibroblasts. These highly motile cells share features of fibroblasts and smooth muscle cells and are generated from mesenchymal precursors in response to TGF β (207). Their protumorigenic effects include production of factors that enhance neoangiogenesis (such as vascular endothelial growth factor (VEGF)), cancer cell proliferation and tumor invasion (208). TGF β itself can also produce autocrine mitogenic factors that enhance tumor proliferation (151). Finally, while the development of immune tolerance is key to inhibiting tumorigenesis in the mucosa, immunosuppression can be protumorigenic for cells that have lost the tumor suppressive arm of the TGF β pathway allowing cells to evade the immune system. For example, TGF β can repress production of perforin, granzymes, Fas ligand, and IFN- γ in CD8⁺ cytotoxic cells to prevent destruction of tumor cells (209).

TGF β in GBM: TGF β signaling is known to play an important role in GBM. Many of the tumor suppressive effects of TGF β are negated and the tumor promoting aspects enhanced (151). TGF β has been shown to enhance glioma cell proliferation by inducing the mitogen platelet derived

growth factor B (PDGFB) (210). In addition, frequent deletion of the p16 Inhibitor of CDK4 [(p15INK4B) locus on chromosome 9p21 (with p16INK4A and alternate reading frame (ARF) also deleted as a consequence)] disables the cytostatic mechanism of TGF β (211). Angiogenic factors such as VEGF are regulated by TGF β and hypoxia inducible factor (HIF) in GBM. The VEGF promotor has DNA binding sites for both Smad and HIF-1 (212). TGF β treatment of the cultured cell line U87 led to increased angiogenesis in a zebrafish glioma model study. This study also demonstrated a crucial involvement of the c-Jun-N-terminal kinase (JNK) pathway in angiogenesis development as JNK inhibitors abrogated TGF β mediated angiogenesis (213).

Expectedly, the immunosuppressive effects of TGF β favor GBM proliferation. TGF β inhibits CD8⁺ T cells and NK cells in human glioma patients by repressing the activating receptor natural killer cell lectin-like receptor subfamily K (NKG2D) and in vitro knockdown studies of TGF β in cell lines demonstrated enhanced cytotoxic activity of these cells (214). Finally, Penuelas *et al.* demonstrated the role of TGF β in the self-renewal of primary glioblastoma cells. In their model, TGF β increased the self-renewal capacity of primary glioblastoma cells by Smad dependant transcription of Leukemia inducible factor (LIF) through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Furthermore, TGF β inhibited differentiation of these cells and promoted oncogenesis *in vivo* (215).

Thrombospondin (THBS), an activator of latent TGF β

THBS is an extracellular matrix (ECM) glycoprotein that is abundant in adult tissues. There are five known members (THBS1, THBS2, THBS3, THBS4, and THBS5) (216). THBS1 was

originally discovered to be associated with activated platelets but was later found to be stored in granules inside platelets and released upon platelet activation (217). Hence, its original function was thought to be involved in the fibrin clot during hemostasis (218). However, it has since become known to also play an important role in the tumor microenvironment and tumor progression which varies depending on the cellular and molecular component (219).

The gene for human *THBS1* is on chromosome 15q15 and is 20 kb with 22 exons. Several transcription factor binding sites are present in the promoter region, notably serum response factor (218). Serum as well as TGF β , PDGF, basic fibroblast growth factor (bFGF), and p53 can upregulate THBS1 as well as stress conditions such as heat shock or hypoxia. Increasing cell density decreases the expression of THBS1 (218,220).

THBS1 is a 450 kDa homotrimer with each polypeptide chain containing 1152 amino acid residue (180kDa) that is heavily modified post-translationally with N-linked glycosylation and β -hydroxylation of asparagine residues (218,221). Each subunit contains globular domains at both the N and C terminal ends connected with a thin strand and two disulfide bonds at cysteine 252 and 256 (221). There is a region homologous to procollagen near the N-terminal and may play a role in the assembly of the homotrimer (216). There are three repeats (Type 1, 2, and 3) with type 3 being rich in aspartic residues that are involved in calcium binding as well as interacting with integrins in cells (218).

Function of THBS1: One of the key functions of THBS1 is the activation and hence, regulation of TGF β activity (222). In fact, TGF β null mice display a similar phenotype to THBS1 null mice (223). THBS1 binds directly with latent TGF β *in vitro* and the two are associated together in

biological fluids such as platelet releasate (224). The amino acid sequence RFK located between the first and second type 1 repeats interacts with latent TGF β and is responsible for activation (225). The binding of THBS1 to latent TGF β is thought to confer a conformational change that disrupts the non-covalent interactions of the latency associated peptide (LAP) with TGF β (165). Furthermore, THBS1 does not interact with the latent TGF β binding protein (LTBP) associated with latent TGF β (226). This does not require cells or other activating factors such as proteases (222). This property is unique to THBS1 as the other THBSs lack the RFK sequence and thus only THBS1 is capable of activating latent TGF β (227).

THBS1 in tumor suppression: THBS1 is thought to play the role of tumor suppressor in cancer development that is independent of its TGF β modulating activity. One of the first identified roles in cancer for THBS1 was its anti-angiogenic activity (228). The procollagen homology domain and the type 1 repeats are responsible for this activity both *in vitro* and *in vivo* (229). In addition, THBS1 modulates anti-angiogenic activity by binding to cell surface receptors such as CD36 (230). The THBS1-CD36 interaction acts as a negative regulator of angiogenesis by inducing apoptosis of endothelial cells as well as inhibiting their migration and proliferation (231,232). THBS1 is now also being recognized to regulate other aspects of tumor biology such as adhesion, invasion, migration, proliferation, apoptosis, immunity response and treatment response of cancer cells.

An example of the growing role and novel functions being identified of THBS1 in cancer biology is the discovery of THBS1 having tumor suppressor function that was independent of its anti-angiogenic activity (233). Baek *et al.* found THBS1 suppressed Kras driven lung

tumorigenesis by inducing senescence in a mouse model of lung cancer. THBS1 was upregulated by oncogenic Kras in a p53 dependent manner leading to a positive feedback loop of p53 stabilization by sequestering ERK in the cytoplasm. This was demonstrated by *THBS1* null mice with oncogenic Kras induction having a significantly shorter overall survival compared to THBS1 wild type mice (233). THBS1 was also shown to interact with CD47 on mice endothelial cells to promote replicative senescence and inhibit angiogenesis (234).

GADD45B and its involvement in senescence:

The growth arrest and DNA damage-inducible 45 (GADD45) family of genes include *GADD45A*, *GADD45B*, and *GADD45G* (235). The genes code for proteins that have a high degree of homology and similar but not exact functions (236). They were discovered in response to stress stimuli and are involved in regulating numerous biological processes such as cell cycle control, survival, and apoptosis (237-239). The regulation of these genes is variable depending on the nature of the stress stimuli, its magnitude and the cell type (107).

GADD45 proteins are 18kDa in size and have similar structures (236). For example, human GADD45A and mouse GADD45G were found to both have 5 α -helices and 5 β -sheets, demonstrating the degree of conservation in the family of proteins (240,241). This is reflected by 55% homology between the three proteins. Additionally, the proteins can dimerize to form homo-dimers or hetero-dimers (236).

The GADD45 proteins carry out their function by interacting with similar sets of proteins such as CDK1, proliferating cell nuclear antigen (PCNA), p21, and mitogen activated protein kinase kinase 4 (MTK1) (242-245). Binding of GADD45 proteins to MTK1 leads to

autophosphorylation of MTTK1, activating its catalytic kinase domain (246). Through these interactions, GADD45 proteins have been shown to stimulate the p38-c-JNK MAPK pathway to regulate apoptosis, survival, or growth arrest in response to stress stimuli (245). The expression of GADD45 proteins increases in response to genotoxic stress by agents such as p53 or BRCA1 (breast cancer 1, early onset) (236). NF- κ B induces upregulation of GADD45B but downregulation of GADD45A and GADD45G (236,247).

GADD45 proteins have also been shown to be involved in the senescence response of cells with emphasis on the nature of the stress. GADD45A deficiency led to increased senescence with myc overexpression in a mouse model of mammary tumorigenesis (248). On the other hand, oncogenic Hras activity did not lead to increased senescence in GADD45A null MEFs (249). In contrast, GADD45B has been shown to have pro-survival effects by inhibiting the MAP kinase, MKK7 (mitogen activated protein kinase kinase 7) with subsequent loss of JNK kinase activation, the downstream target of MKK7 (250). In another study, GADD45B null MEFs underwent premature senescence more readily in the presence of oxidative stress compared to wild type MEFs. Interestingly, GADD45B null MEFs underwent arrest in the G2 phase rather than G1, unlike other senescent MEFs (107).

Further evidence of GADD45B having a pro-survival role was demonstrated by bone marrow cells from *GADD45B* null mice undergoing apoptosis more readily in response to radiation and chemotherapy (251). Fibroblasts with overexpressed GADD45B were more resistant to apoptosis (252). However, the importance of the nature of the stimulus was again demonstrated as *GADD45B* null or wildtype MEFs were not more prone to apoptosis in response to TNF- α (253). Hence, the role of these proteins, while important, cannot be generalized and varies on multiple factors including the stress stimuli and the cell type.

Rationale:

GBM is one of the most lethal human cancers. Despite aggressive therapy consisting of maximal surgical resection, radiation and chemotherapy, the median survival is still only 14 months (254). There has been a minimal improvement in meaningful therapy in decades. Hence, urgent development of novel therapeutics is required.

Senescence has recently been identified as a potent tumor suppressor mechanism (255) (256). Senescence not only prevents tumor formation from oncogene activation but studies have also shown induction of senescence in tumors can cause regression and be a viable therapeutic strategy in the treatment of cancer. For example, reactivation of p53 in mice with established sarcomas results in dramatic tumor regression with several characteristics of senescence (134). Similarly, reactivation of p53 in invasive hepatocarcinomas caused induction of senescence and clearance of the tumors (135). Another potential advantage of exploiting senescence as a therapeutic mechanism includes senescent cells partaking in extensive signalling with the environment, effecting neighboring cancer cells and utilizing the immune system to clear tumor cells that have undergone senescence (135).

Premature senescence in glioblastoma is not well understood. Many glioblastoma cells have telomerase promoter mutations that allow them to bypass replicative senescence to continue proliferation (204). In addition, glioblastoma cells are expected to be resistant to oncogene induced premature senescence due to a high frequency of mutations in the *CDKN2A* locus (257). Much of this data has been collected from the use of established cell lines which are now thought to no longer properly represent GBM behavior. Primary glioblastoma cells are now looked as a better model of study as they more closely mimic GBM characteristics in mice xenografts such

as invasiveness (258). Thus an important question is whether primary glioblastoma cells are capable of undergoing premature senescence and if so can it be used to reduce their malignant potential. Hence, the aim of my study is to evaluate the capacity for primary glioblastoma cells to undergo premature senescence in response to various stress inducing agents and to determine the underlying mechanisms.

Resistance to therapy is thought to be in large part due to the tremendous heterogeneity present in GBM. Alongside histological heterogeneity, it is now known that GBM is also one of the most heterogeneous cancers at the molecular level. GBM is now further subdivided into multiple molecular subtypes based on a genetic expression pattern. Furthermore, heterogeneity is present in GBM not only in cells isolated from different patients but also at the single cell level with individual cells having expression patterns of multiple subtypes. While this has aided significantly in the biological understanding of the disease, the clinical relevance of molecular heterogeneity in GBM remains largely unknown. Veerhak *et al.* suggested, based on correlational studies, the various subtypes render different responses to intense therapy (13). Experimental data is now starting to emerge that demonstrates some of the subtypes respond differently to therapeutics such as radiation. Bhat *et al.* determined the proneural subtype was sensitive to radiation therapy whereas the mesenchymal subtype was largely resistant with respect to cell death (16). Still, it remains to be discovered if molecular heterogeneity in GBM influences the ability of primary glioblastoma cells to undergo premature senescence. Another aim of my project will be to evaluate the capability of the various subtypes to undergo senescence in response to various stress inducing agents.

Hypothesis: Induction of premature senescence in primary glioblastoma cells can inhibit their proliferative potential. Primary glioblastoma cells respond variably to stress inducing agents with respect to undergoing premature senescence due to inter-patient heterogeneity.

Objectives:

- 1) **Determine the capability of the various primary glioblastoma subtypes to undergo premature senescence in response to various stress inducing agents.**
- 2) **Determine the molecular mechanisms governing premature senescence in primary glioblastoma cells.**

MATERIALS AND METHODS

Cell lines and Cell culture:

Patient samples for isolation of primary glioblastoma cells were obtained after informed consent following a protocol that was approved by the Ottawa Health Science Network Research Ethics Board. Primary glioblastoma cells were isolated as described previously (258) following the method described by Pollard *et al.*(47). Briefly, Surgical samples were harvested from consented patients undergoing surgery for suspected glioblastoma (and without a history of previous lower grade brain tumor) using a Nico Myriad surgical device (NICO Corporation, Indianapolis, IN, USA). Cultures were digested with Accutase, filtered through 100 μ M and 40 μ M nylon mesh filters, and plated on laminin-coated plates as described by Pollard *et al.* (47). Accutase and laminin were from Sigma-Aldrich, Oakville, ON, Canada. Cultures were grown in Neurobasal A medium supplemented with B27, N2 (all from Life Technologies, Burlington, ON, Canada), EGF and FGF (Peprotech, Rocky Hill, NJ, USA). In this method, cells are grown as adherent cells on laminin-coated plates in neural stem cell media. Routine cell culture was performed in 5% O₂, the physiologic concentration of O₂ in the brain (259). Cells were used at low passage number and were routinely checked and shown to be free of mycoplasma.

WI-38 is a diploid human cell culture line composed of fibroblasts derived from lung tissue (260). The cell line was originally procured from the American Type Culture Collection (ATCC[®] CCL-75[™]) and kindly gifted to us by Dr. Doug Gray from the Ottawa Hospital Research Institute, Ottawa. These cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 10% (v/v) of a 2:1 mixture of donor bovine serum and fetal bovine serum.

Antibodies and reagents

TUJ1 rabbit monoclonal antibody was from Covance (Princeton, NJ, USA product number MRB-435P). GFAP mouse monoclonal antibody (product number G3893), Propidium Iodide solution and Triapine (3-AP) were from Sigma-Aldrich (Oakville ON Canada,). Nestin mouse monoclonal antibody (product number MAB1259), recombinant BMP4 and recombinant TGF β were from R&D Systems (Minneapolis MN, USA). p21 mouse monoclonal antibody, pSMAD2 rabbit monoclonal antibody and SMAD2 rabbit monoclonal antibody were from Cell Signaling Technology (Danvers MA, USA, product numbers DCS60, 138D4 and D43B4). PML mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz CA, USA, product number PG-M3). LSKL peptide was from AnaSpec (Fremont CA, USA). LDN 212854, SB 431542 and U0126 was from Tocris Bioscience (Bristol UK). Click-iT EdU Alexa Fluor 488 labeling kit was from ThermoFisher Scientific (Waltham MA, USA). FITC-labeled Annexin-V was from Molecular Probes (Eugene, OR, USA).

Immunofluorescence

Immunofluorescence microscopy was performed as described earlier (258). Briefly, cells were plated on laminin-coated coverslips and following the experimental procedure, cells were fixed with ice-cold 4% paraformaldehyde for 30 minutes. Cells were blocked in 5% serum in PBS (goat or chicken) then incubated with the primary antibody for 1 hr in 5% serum at the following concentrations: Nestin 1:100, GFAP 1:600, TUJ1 1:500, PML 1:50, and γ H2AX 1:50. The relevant secondary antibody was incubated in 5% serum at 2 μ g/ml for 1 hr. Slides were analyzed using a Zeiss Axioskop 2 microscope from Carl Zeiss Inc.

For differentiation experiments using TUJ1 and GFAP antibodies, images were taken of five random fields of view (chosen under DAPI filter) per condition per experiment. Analysis was carried out in ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The proportion of positive cells to total nuclei was assessed per condition $\pm$ SE for each experiment.

Western blot analysis

Western blot analysis was performed as described earlier (261). Briefly, total cell proteins obtained after lysis of cell pellets were subjected to SDS-PAGE and run on a 4–12% gradient bis-tris polyacrylamide gel (Life Technologies – Invitrogen). The proteins were then transferred onto a polyvinylidene difluoride nylon membrane (Bio-Rad Laboratories, Hercules, CA, USA) (GE Healthcare Life Sciences). Membranes were probed with primary antibodies at 4°C overnight, followed by goat secondary antibodies conjugated to horseradish peroxidase (HRP; Bio-Rad Laboratories) for 1 hr. To control for total protein loading, membranes were stripped of the primary antibodies and reprobed with anti-GAPDH (Sigma-Aldrich). Secondary HRP conjugated antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate reagents (Thermo Scientific Pierce). Western blots were visualized by chemiluminescence from HRP conjugated secondary antibodies and detected with the Alpha Innotech Fluorchem FC2 system (Santa Clara, CA, USA) and quantitated using Alphaview software from ProteinSimple.

SA β Gal staining

SA- β -gal staining was performed essentially by the method described previously (262). Briefly, PriGO cells were seeded at 10% confluence (~ 25,000 cells per coverslip) on laminin-coated coverslips in each well of a six-well dish and subjected to treatment. Cells were maintained at 37 °C, 5% CO₂ and 5% O₂. Cells were washed twice with PBS and fixed for 10 min with 4% (wt/vol) paraformaldehyde at room temperature. Cells were washed again twice with PBS. Cells were stained overnight in dark at 37 °C in freshly made SA β Gal mixture consisting of a final concentration of 40 mM citric acid/sodium phosphate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM sodium chloride, 2 mM magnesium chloride, 1 mg/ml X-Gal, and distilled water at a pH of 6.0. Cells were then washed thrice with PBS and coverslips were mounted on slides in Dako mounting media. Cells were observed using a Zeiss Axioskop 2 microscope. Total and SA- β -gal-positive cells were counted in a minimum of five separate fields for each condition.

Cell proliferation assays

Live and total cell counts were analyzed by viable cell counting at the indicated time points after radiation/triapine/serum treatments using a trypan blue exclusion Vi-Cell XR cell counter (Beckman Coulter, Mississauga, ON, Canada). Cell proliferation was also determined by EdU labeling using a Click-iT EdU Alexa-Fluor 488 Imaging kit from ThermoFisher Scientific. Briefly, PriGO cells were cultured on the coverslips overnight (1×10^4 /ml). Cells were incubated with 10 μ M EdU for 24 hrs following which the cells were fixed with 1 ml of 3.7% formaldehyde in PBS for 15 minutes at room temperature. Cells were washed twice with 1 ml of 3% BSA in PBS. Cells were permeabilized with 1 ml of 0.5% Triton® X-100 in PBS at room

temperature for 20 minutes. The permeabilization buffer was removed by washing the cells twice with 1 ml of 3% BSA in PBS. 0.5 ml of Click-iT® reaction cocktail was added to each well, followed by incubation for 30 minutes at room temperature in dark. Cells were washed again with 1 ml of 3% BSA in PBS. Total and EdU-positive nuclei were counted under a Zeiss Axioplan 2 microscope at excitation and emission wavelengths of 495 and 519 nm, respectively. A minimum of five random fields were counted for each condition.

Thrombospondin-1 ELISA

Human thrombospondin-1 protein in the culture supernatants/serum was quantified using human thrombospondin 1 ELISA kits from RayBiotech (Norcross GA, USA) essentially as per the manufacturer's recommendations. Briefly, 100 µl of the standard or the sample was added to the pre-coated 96-well Strip Microplate for 2.5 hrs at room temperature with gentle shaking. Then, 100 µl of biotinylated detection antibody was added to each well for 1 hr at room temperature. Subsequently, 100 µl of streptavidin-conjugated HRP was added to each well for 45 min at room temperature. The color reaction was developed by adding 100 µl of TMB One-Step Substrate reagent to each well for 30 min at room temperature. The reaction was stopped by adding 50 µl of stop solution to each well. The plates were read at 450 nm immediately.

Morphology

Slides were analyzed using a Nikon Eclipse TE 2000-U microscope using × 20 magnification. Representative pictures were taken using a Coolpix MDC lens (Nikon Canada inc., Mississauga, ON, Canada) and a Nikon 5400 digital camera.

Apoptosis analysis by intracellular PI or Annexin-V staining

Following serum and triapine treatment, PriGO cells were evaluated for cell death by using intracellular PI staining. Cells were harvested following accutase treatment, washed with PBS, and fixed with methanol for 15 min at 4°C. The methanol was washed away with PBS, and cells were treated with 25 µl of 10 µg/ml RNase A, followed by staining with 25 µl of 1 mg/ml PI solution (Sigma-Aldrich) at 4°C for 1 hr. The DNA content was analyzed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) and the FACSDiva software. The subdiploid DNA peak (<2 N DNA), immediately adjacent to the G0/G1 peak (2 N DNA), represents apoptotic cells and was quantified by histogram analyses. PI histogram figures were obtained with WinMDI version 2.8 software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA).

Apoptosis was also measured by staining cells (1.0×10^5 /ml) with FITC-labeled Annexin-V (Molecular Probes, Eugene, OR) for 15 min at room temperature in the dark. Annexin-V-positive cells were quantified by flow cytometry. The data was analyzed using WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

Radiation treatments

Cells were irradiated in a Pantak cabinet X-Ray unit, operating at 250 kVp, with a dose rate of approximately 80 cGy per minute.

Microarray expression analyses and bioinformatics

Total RNA was isolated from cells using Qiagen RNeasy Plus mini kits. Expression analysis was done using Affymetrix Human Gene 2.0 ST arrays at StemCore Laboratories (Ottawa, Canada)

with one sample per condition. Full microarray data was deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nih.gov/geo/>). To assess the likely glioblastoma subtypes of our samples, we downloaded the file TCGA_unified_CORE_ClaNC840.txt from https://tcga-data.nci.nih.gov/docs/publications/gbm_exp/. This file contains the normalized expression levels of 840 genes, 210 each for proneural, neural, classical and mesenchymal subtypes, across 173 tumor samples. Of those tumor samples, 53 are proneural, 26 are neural, 38 are classical and 56 are mesenchymal. To relate this to our own data, we first downloaded the annotation file for our microarray chip, HuGene-2_0-st-v1.na35.hg19.probeset.csv.zip, from Affymetrix's online technical support page (http://www.affymetrix.com/support/technical/byproduct.affx?product=hugene-1_0-st-v1). We used that file to associate the 840 Gene IDs in the TCGA file to transcript IDs in our microarray report. This allowed us to find one or more transcript IDs for all but 73 of the 840 Gene IDs. Transcript IDs for an additional 63 Gene IDs were found by using online databases, primarily GeneCards (<http://www.genecards.org/>), to identify alternative or retired Gene IDs matching the TCGA Gene IDs. Those alternative IDs were then found in the Affymetrix file. The remaining 10 Gene IDs from the TCGA signature, for which no alternative symbol could be found that matched a transcript ID in the Affymetrix documentation, were ignored in the remainder of the analysis. For 51 Gene IDs, we found multiple transcript IDs purportedly reporting expression for that gene. In such cases, we arbitrarily chose the numerically lowest transcript ID to represent the gene. We correlated the expression values of every TCGA sample to every other TCGA sample, and to each of our samples. We then plotted heatmaps of these correlation coefficients. From those heatmaps, the within-subtype similarities of the TCGA samples are visually apparent, as are the similarities of our samples to the TCGA samples representing the four subtypes.

qPCR

For mRNA analysis, mRNA was isolated from cells using Qiagen's RNeasy Plus Mini Kit and reverse transcribed using the Qiagen Quantitect RT Kit and Bio-Rad T100 thermal cycler. The resulting cDNA was mixed with primers (Integrated DNA Technologies) and iTaq Universal Probes Supermix (Bio-rad), then run and analyzed using a 7500Fast system (Applied Biosystems). PCRs were performed using the following primers:

For PUM1 (reference gene), 5'-TGAGGTGTGCACCATGAAC -3' and 5'-CAGAATGTGCTTGCCATAGGG 3'.

For GADD45B: 5'-ACGAGGACGACGACAGAGAT-3' and 5'-GCAGGATCCTTCCATTGAGA-3'.

RNA interference

RNA interference to deplete cells of thrombospondin 1 and GADD45B were done as described previously (263). siGenome Human THBS1 and GADD45B siRNAs were purchased from Dharmacon (Lafayette, CO, USA). The first THBS1 siRNA had the sense RNA sequence GGACUGCGUUGGUGAUGUA and the second had the sense RNA sequence GUACAGAAACGUAGUCGUC. The first GADD45B siRNA had the sense RNA sequence GUACGAGUCGGCCAAGUUG and the second had the sense RNA sequence CGGCCAAGUUGAUGAAUGU. Non-Targeting siRNA # 2 from Dharmacon was used as a non-targeting duplex control. RNA duplex concentrations were determined by measuring absorbance at 260 nm and calculating concentrations using extinction coefficients provided by the manufacturer.

PriGO cells were plated in six-well dishes at a density of 10^5 cells/plate the day before transfection, in NA media supplemented with N2, B27, EGF and FGF. On the day of transfection, 20 μ l of RNA duplex solution was added to 165 μ l OptiMEM I (Invitrogen Canada Inc., Burlington, ON, Canada). In a separate tube, 4 μ l of Oligofectamine (Invitrogen Canada Inc., Burlington, ON, Canada) was added to 11 μ l of OptiMEM I. This was mixed gently and incubated 10 min at room temperature. The diluted Oligofectamine was then added to the RNA duplex in OptiMEM I and incubated for another 20 min at room temperature after gentle mixing. Media was removed from the cells, and the cells were washed once with 3 ml of warm OptiMEM I. 800 μ l of OptiMEM I was added per well, and the Oligofectamine/RNA duplex mix was then added (200 μ l per well) and mixed by rocking the plate from side-side for 30 seconds. Final concentrations of RNA in the transfections were 20 nM for all siRNA. Control RNA concentrations were matched to the specific siRNA duplex used. Cells were incubated 4 hrs at 37°C, after which 2 ml of NA media was added. Fresh media was replaced in each well 48 hrs later.

For senescence analysis, cells were assayed 7 days after transfection with siRNA being washed off after 48 hr. Initial plating of cells was 15,000 to 20,000 in a 6-well dish on laminin-coated cover slips.

Transduction with lentiviral vectors

Replication-incompetent lentiviral particles were made by the four-plasmid transfection method described by Wiederschain *et al.* (264). PriGO8A cells were transduced with 100 μ l lentiviral vector containing supernatant and 900 μ l of supplemented NA media. Cells were incubated for 24 hr. For inducible expression, cells were first transduced with lentivirus made with Tet-

activator plasmid pLVX-tet-on-advanced (Clontech, Mountain View, CA, USA) and selected with G418 (500µg/ml); these cells were then transduced again with lentivirus made with the inducible cDNA vectors and selected with puromycin (0.5µg/ml).

Plasmid Constructs:

The p21 lentiviral vector was created using p21 cDNA from the A172 cell line with the 2 primers: 5'-GGATCCAGGCACCGAGGCACTCAGAG-3' and 5'-GTCGACGGACTGCAGGCTTCCTGTGG-3'. The cDNA was then cloned into the pLenti-CMV GFP Puro (Addgene Plasmid 17448) vector and sequenced.

The Lgl3SA lentiviral vector used was generated previously as described by Gont *et al.* (258).

The GADD45B lentiviral vector was created using GADD45B cDNA from PriGO8A cells with the 2 primers: 5'-GGATCCATGACGCTGGAAGAGCTCGTGG-3' and 5'-GAATTCTCAGCGTTCCTGAAGAGAGATG-3'. The cDNA was then cloned into pLVX-Tight-Puro (Clontech, Mountain View, CA, USA).

Statistical analysis:

SigmaPlot12 and Microsoft Excel software were used for statistical analyses. Comparisons between two groups were done using t-tests for normally distributed data with equal variance. For groups that were not normally distributed or that did not show equal variance, the Mann-Whitney Rank Sum test was used. For comparisons between multiple groups the Kruskal-Wallis One Way Analysis of Variance on Ranks and the All Pairwise Multiple Comparison Procedures (Dunn's Method) were used. A p value <0.05 was considered significant.

RESULTS

Objective # 1. Determine the capability of PriGO cells to undergo premature senescence.

1.1: Characterization of PriGO cell lines.

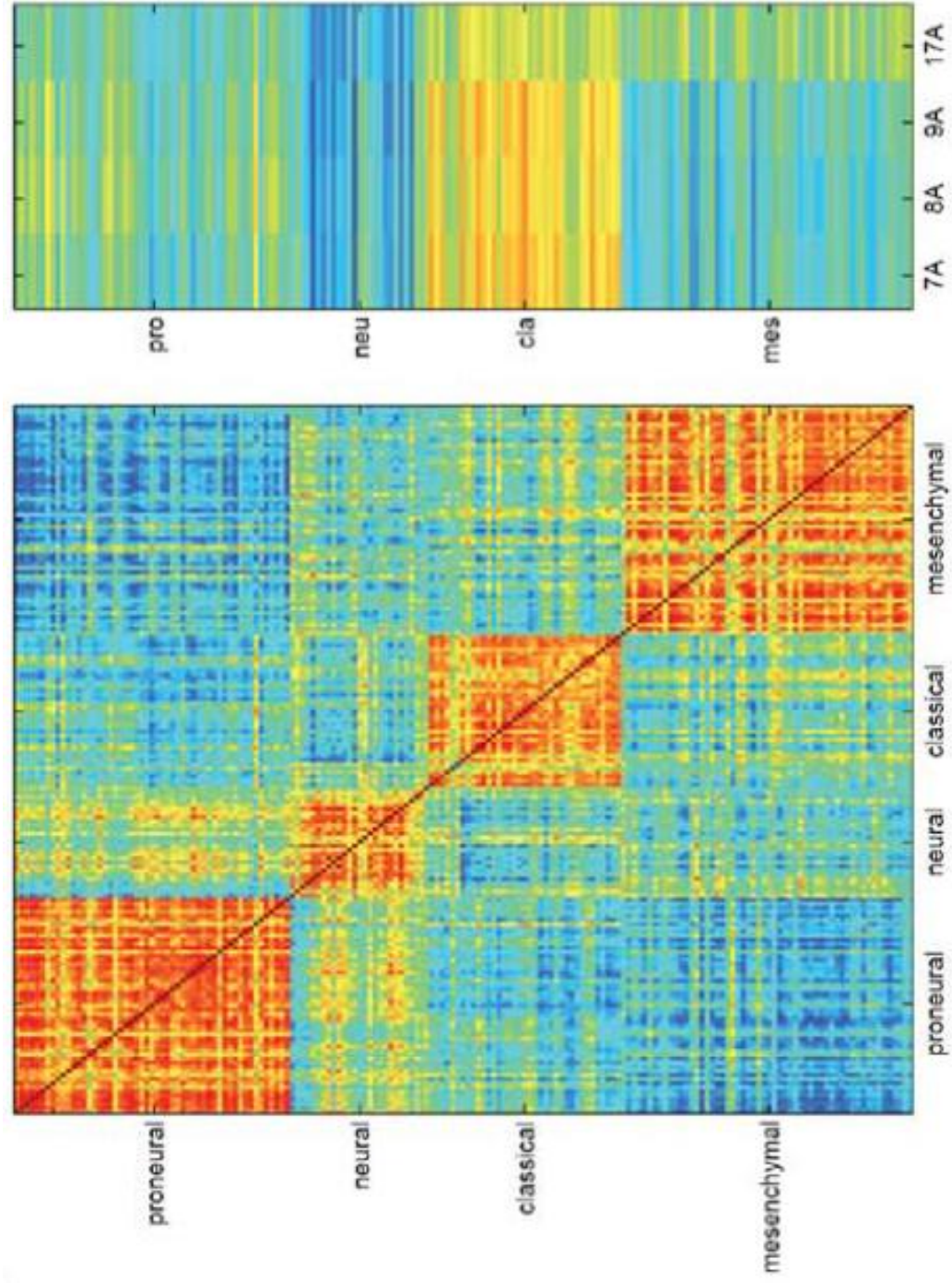
In an effort to study a better model of glioblastoma behavior, PriGO cells harvested from human patients with glioblastoma have been cultured in our laboratory. The cell line best characterized previously is PriGO8A but other cell lines such as PriGO7A, PriGO9A and PriGO17A have also been studied (258). These PriGO cells were grown as a monolayer on laminin coated plates but retained the ability to form neurospheres in the absence of laminin, a property observed in neural stem cells. Other neural stem cell features such as Nestin positivity and the ability to differentiate along multiple lineages was also observed in these PriGO cells. Importantly, all of the mentioned PriGO cell lines formed invasive tumors in mice xenografts (265).

Molecular studies have proven invaluable in classification and prognosis of glioblastoma but little data exists correlating it with treatment, a consequence of clinical significance (25,266). One of the aims of my project was to determine a correlation of the effect of stress inducing agents to the molecular subtype of PriGO cells, specifically with respect to the capability to undergo premature senescence. To determine the molecular subtype of the four PriGO cell lines, microarray analysis was performed and the gene expression profile was analyzed. It was determined that PriGO7A, PriGO8A and PriGO9A are predominantly of a classical subtype whereas PriGO17A had a substantial mesenchymal component (**Fig 1**).

Figure 1. Molecular subtypes of PriGO cells.

Glioblastoma cell lines were classified into molecular subtypes by isolating total RNA from cells using Qiagen RNeasy Plus mini kits. Expression analysis was done using Affymetrix Human Gene 2.0 ST arrays at StemCore Laboratories with one sample per condition. Bioinformatics analysis was performed and plotted as heatmaps.

Fig 1:



Objective 1.2: Determine the effect of serum on classical and mesenchymal PriGO subtypes with respect to senescence and cell death.

1.2.1: Serum induces senescence in classical PriGO cells in contrast to the mesenchymal PriGO subtype.

Previously, Lee et al described primary glioblastoma cells treated with serum as initially undergoing a growth delay followed by the appearance of cells with altered chromosomal numbers (54). Another study described mouse embryonic stem cells having undergone senescence in response to serum, an initial growth delay followed by cells with aneuploidy (112). Furthermore, human fibrosarcoma cells underwent senescence in response to serum (267). Hence, it was hypothesized that serum may induce senescence in PriGO cells.

To determine serum induced senescence in PriGO cells, firstly PriGO8A cells were treated with 10% serum for 7 days followed by Sa β Gal staining. Serum treatment resulted in a significant 50% increase in Sa β Gal positivity and morphological changes of an enlarged and flattened shape, characteristic of the senescence phenotype (**Fig 2 A, B, and C**). Serum significantly reduced proliferation as determined by EDU incorporation (**Fig 2 D, E**). Furthermore, serum treatment for 7 days led to a significant increase in PML bodies compared to untreated cells as detected by immunofluorescence (**Fig 3 A, B**). Serum also caused an increase in p21 as detected by Western blot analysis (**Fig 3 C**). However, PriGO8A cells did not express p16 basally and this did not increase with serum treatment (**Fig 4 B**).

Interestingly, there was also no increase in the DNA damage response marker γ H2AX in PriGO8A cells treated with serum for 7 days compared to untreated cells, as detected by immunofluorescence (**Fig 3 D**). Finally, serum did not cause cell death as demonstrated by PI

Figure 2. Serum induces senescence in PriGO8A cells:

(A). PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 10% foetal bovine serum for seven days. Cells were then fixed and stained for SA β gal activity. Images shown are representative of cells stained for SA β gal using differential interference contrast microscopy.

(B): PriGO8A cells were treated as in A. Cells were then fixed and stained for SA β gal activity. The left graph shows total cells (light grey bars) and SA β gal-positive cells per field. The right graph shows the same data plotted to show the change in the percentage of SA β gal-positive cells. Data are from analysis of five random fields per condition. Error bars show the standard deviation. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$.

(C): PriGO8A cells were treated as in A then fixed and stained for SA β gal activity as before. Images shown are representative phase contrast images of single cells (photographed in black and white).

(D): PriGO8A cells were treated as in A. On day 6 they were labelled for 24 hrs with EdU. Cells with EDU incorporation were detected using the Click-IT EdU detection kit from Thermo Fisher. A representative picture of EdU incorporation in the presence and in the absence of serum is shown

(E): Quantitation of EDU incorporation, data are from five randomly chosen fields. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$.

Fig 2:

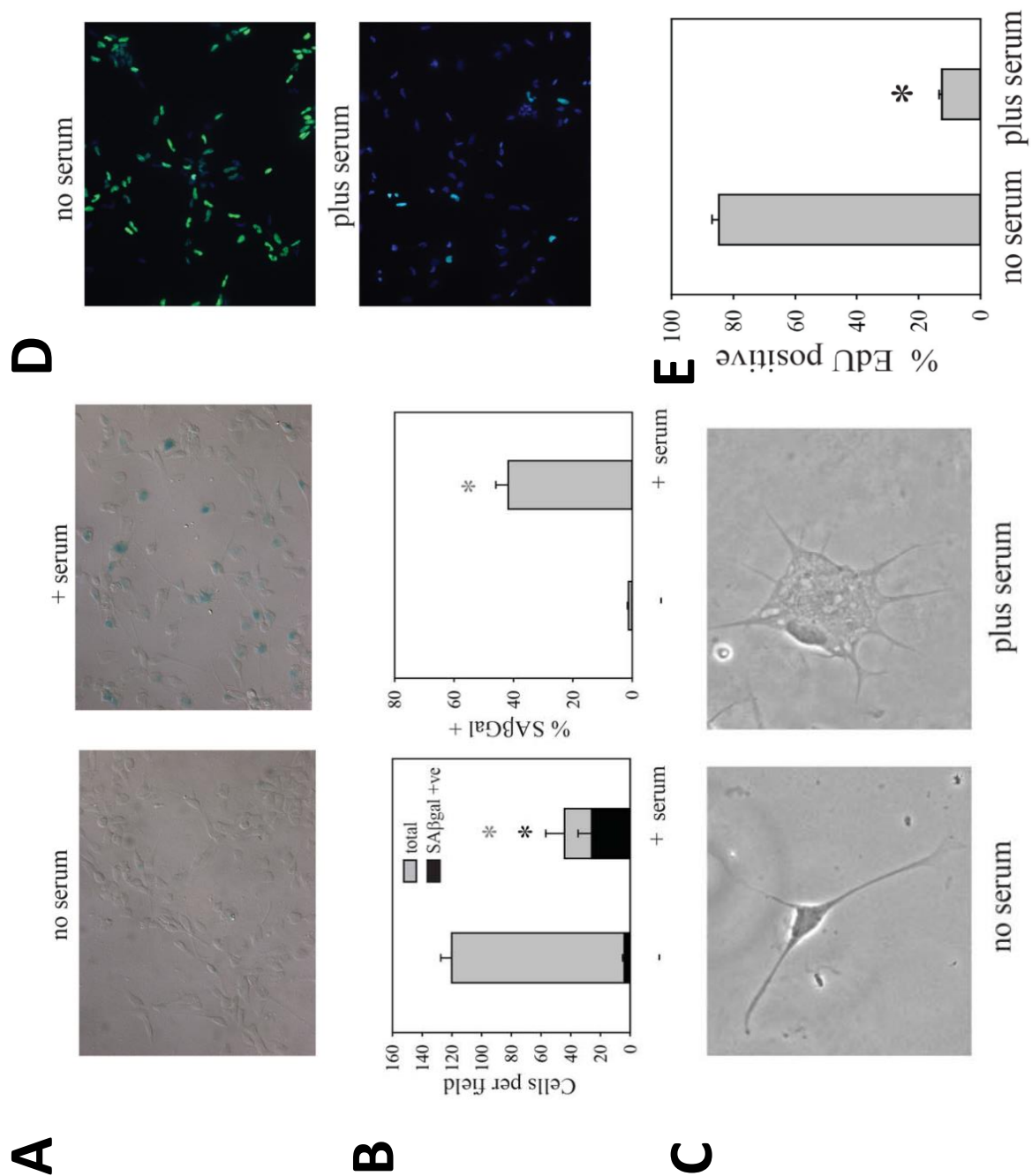


Figure 3. Further Characterization of serum-induced senescence in PriGO8A cells:

(A): PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 10% serum for 7 days and then fixed and analyzed for PML bodies by immunofluorescence. Representative images are shown with PML in green and DAPI staining in blue.

(B): Quantitation of PML immunofluorescence. Data are from 100 cells per condition. Whiskers show 10th and 90th percentiles. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$.

(C): PriGO8A cells were treated with the indicated doses of radiation or 10% serum. Two days later, total cell extracts were analyzed by Western blotting for levels of p21. GAPDH was used as a loading control. Results shown is a representative of two experiments

(D): PriGO8A cells were treated as in A. Cells were then fixed and analyzed for γ H2AX foci by immunofluorescence. Data are from approximately 100 cells per condition. The percent of cells with different numbers of foci from 0 to 14 foci per nucleus are plotted. Results shown is a representative of two experiments

Fig 3:

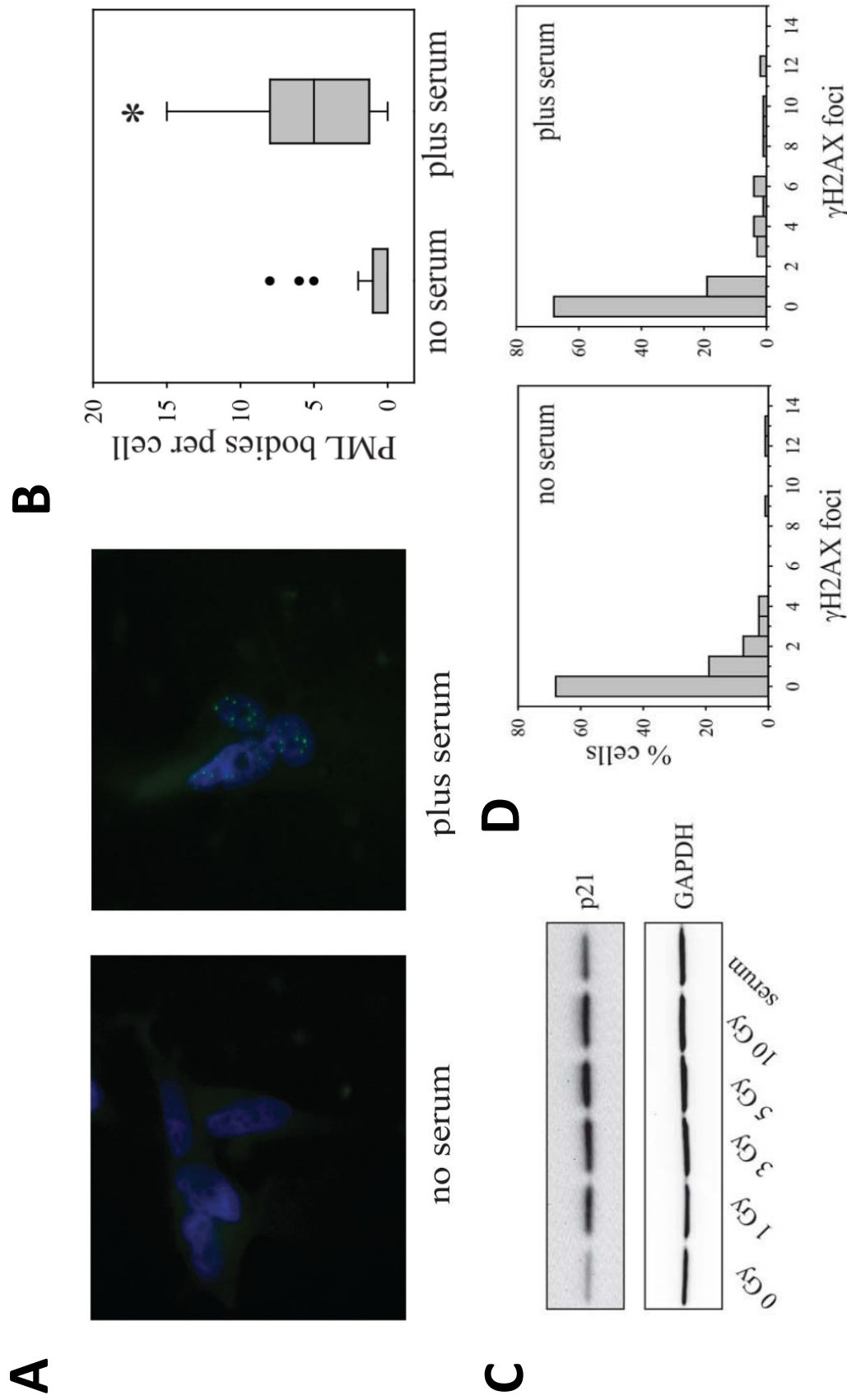


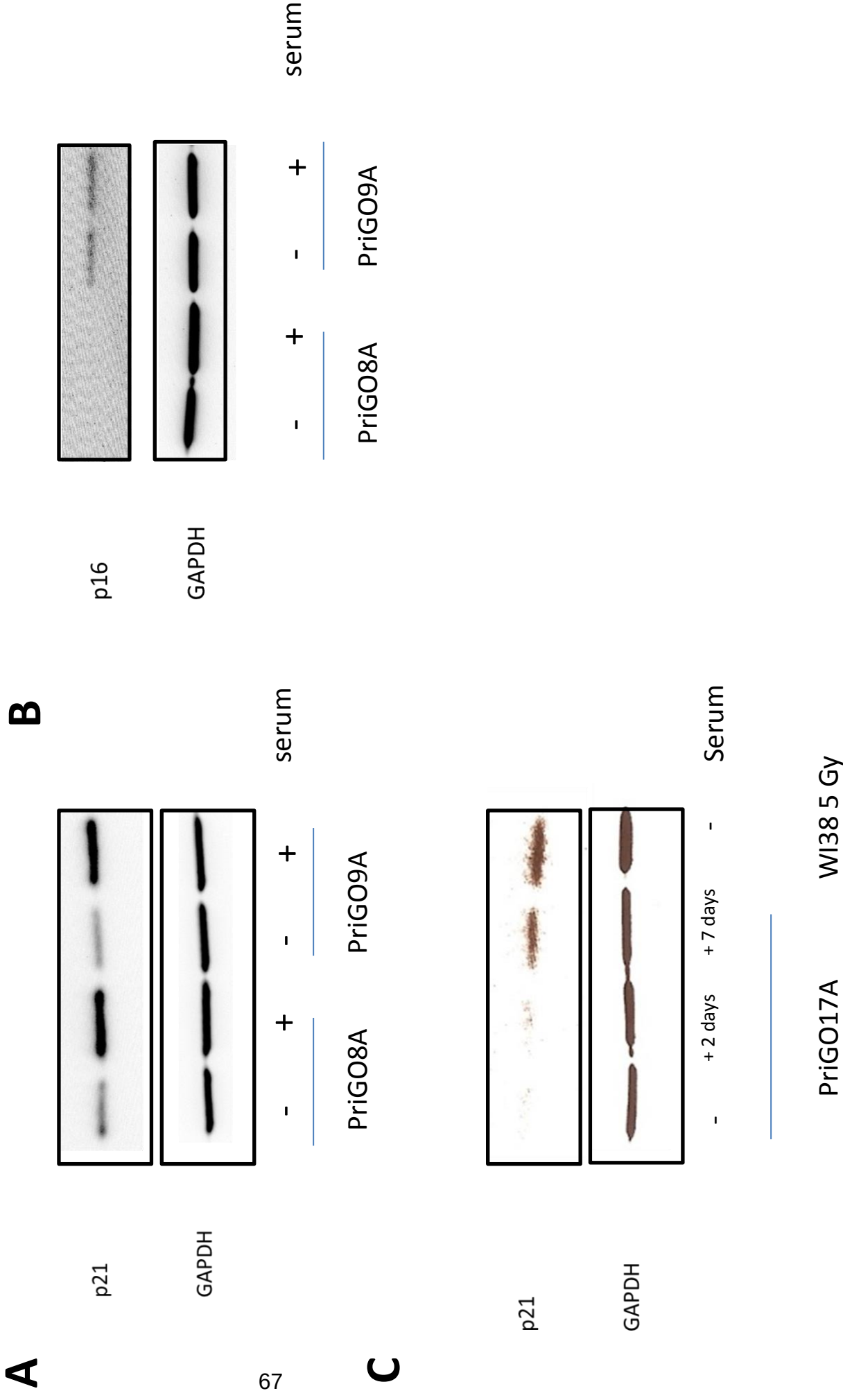
Figure 4. Serum induces p21 expression in PriGO cells.

(A) PriGO8A, and PriGO9A cells ($1 \times 10^5/\text{ml}$) were cultured for 24 hrs following which they were treated with 10% serum as in Figure 23. Total cell extracts were harvested 7 days later and subsequently analyzed for p21 by Western Blot analysis.

(B) PriGO8A and PriGO9A cells were treated with serum as in A. Total cell extracts were obtained 7 days later and p16 was analyzed by Western Blot analysis.

(C) PriGO17A cells were treated with serum as above. Total cell extracts were harvested 2 days and 7 days later and subsequently analyzed for p21 by Western Blot analysis. WI38 cells irradiated with 5 Gray were used as a control. A representative picture of three experiments is shown.

Fig 4:



staining but did significantly reduce the cell number by almost 50% compared to untreated cells demonstrating that the cytoreductive effect of serum was due to senescence and not apoptosis (**Fig 5 A and B**).

The ability of serum to induce senescence in PriGO cells was assessed in other cell lines as well. PriGO9A cells treated with serum also showed an increase in Sa β Gal positivity, but lower than PriGO8A cells (**Fig 6 A**). Serum treatment led to a significant 30% decrease in cell number, again less than that observed in PriGO8A cells (**Fig 6 B**). Serum also induced morphological changes in PriGO9A cells that were consistent with the senescent phenotype (**Fig 6 C**). Once again, similar to PriGO8A cells, there was no appreciable change in γ H2AX detection in PriGO9A cells treated with serum (**Fig 6 D**). To confirm serum induced senescence in PriGO9A cells, other markers were analyzed as with PriGO8A cells. There was an increase in PML body detection (**Fig 7 A and B**) and a decrease in EDU incorporation in serum treated cells (**Fig 8 A and B**). PriGO9A cells also demonstrated an increase in p21 levels with serum treatment (**Fig 4 A**). Although PriGO9A cells do show basal expression of p16, there was no increase in response to serum (**Fig 4 B**). These results suggest that the classical subtype of PriGO cells (PriGO8A and PriGO9A) underwent senescence in response to serum.

Next, it was investigated whether the same effect would be observed in the mesenchymal subtype of PriGO cells (PriGO17A). Serum treatment did not show any evidence of senescence induction in PriGO17A cells. There was no change in Sa β Gal positivity nor were there any appreciable differences in morphology with serum treatment (**Fig 9 A and B**). Serum did not cause a significant change in cell number (**Fig 9 C**) nor was there any decrease in EDU incorporation (**Fig 10 A and B**). Serum treatment also did not lead to an increase in the level of

Figure 5. Serum-induced senescence does not cause cell death in PriGO8A cells:

(A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were treated with 10% serum for 48 hours. Cells were then stained with Propidium Iodide and analyzed for apoptosis by flow cytometry. Results shown are a representative of three experiments.

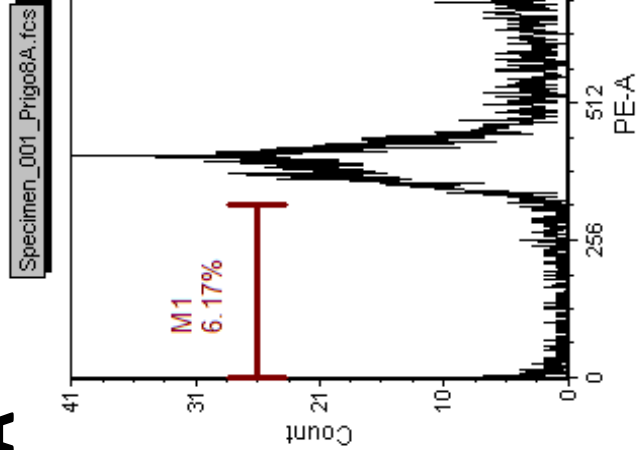
(B) Quantitation of PI staining. Results shown are a mean $\pm$ SD. N=3. * indicates $p < 0.05$.

(C) Cells were treated with 10% Serum as in A. Cells were cultured for 48 hrs following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are a mean $\pm$ SD. N=3. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$.

Fig 5:

PriGO8A

A

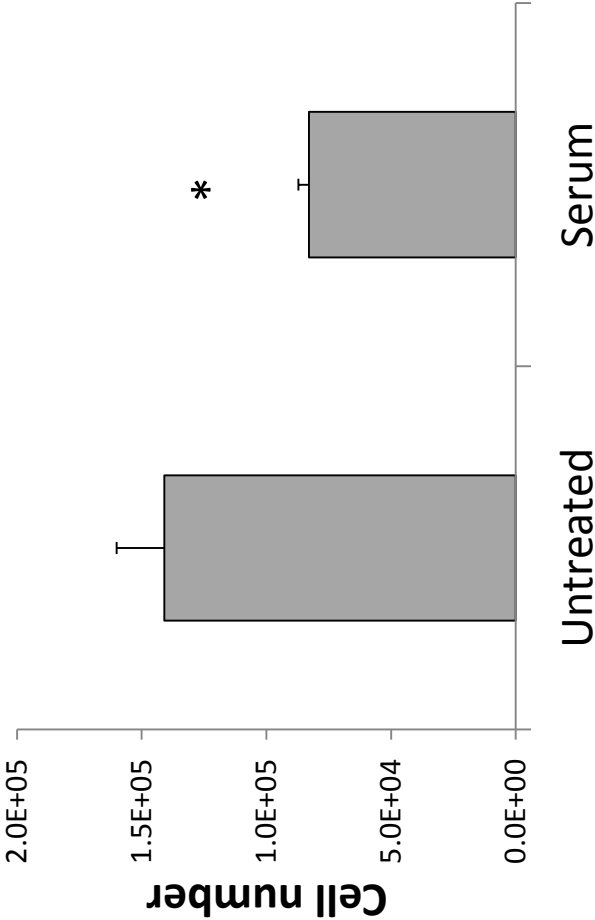


Untreated

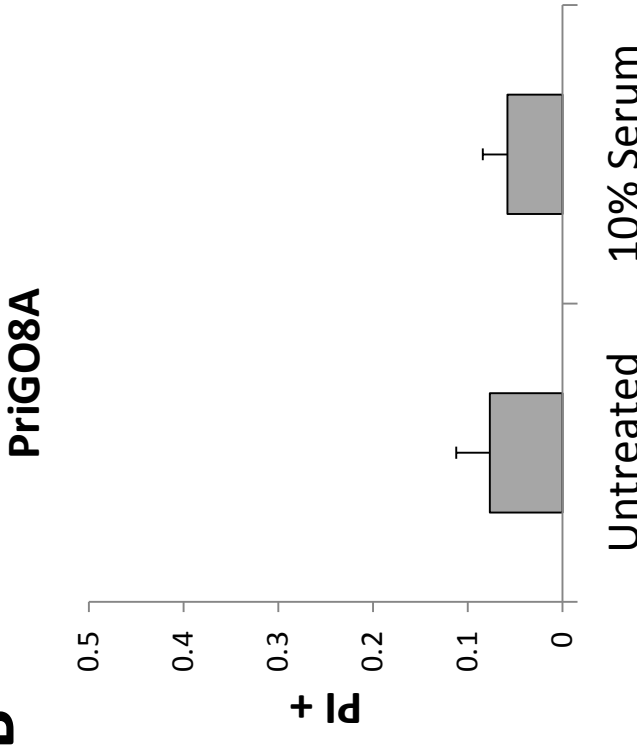
10 % FCS

C

PriGO8A



B



Untreated

10% Serum

PriGO8A

PI +

Figure 6. Serum induces senescence in PriGO9A cells:

(A) PriGO9A cells (1×10^5 /ml) were cultured for 24 hrs following which they were treated with 10% serum. The cells were cultured for 7 days following which they were assayed for SA β gal activity as described before. Statistical significance was determined using two-tailed t tests. * indicates $p < 0.05$.

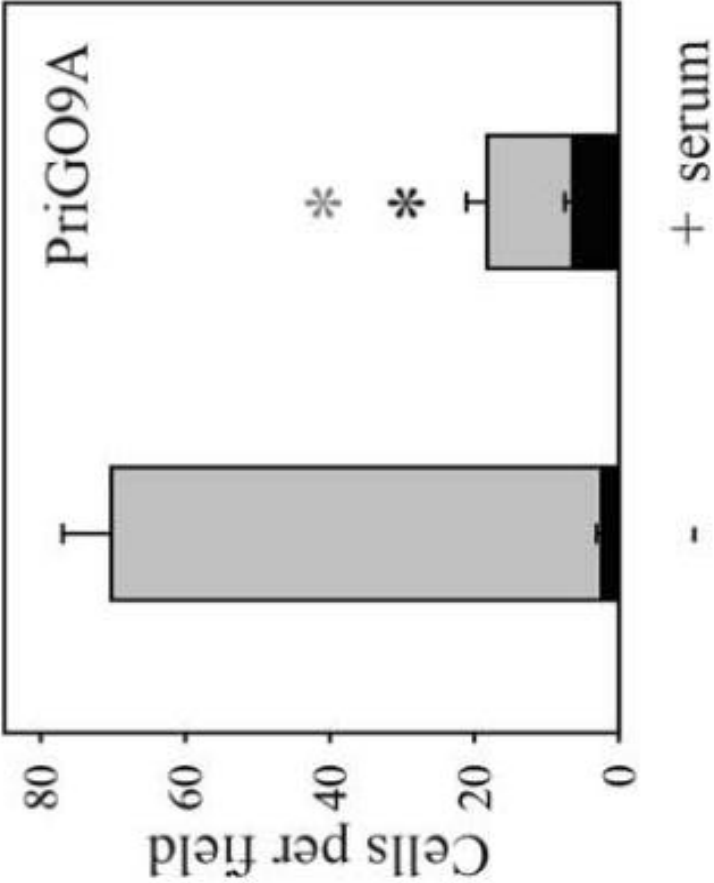
(B) Cells were treated with 10% serum as above. Live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown in A and B are a mean $\pm$ SD. N=3. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$.

(C) Morphology of PriGO9A cells treated with 10% serum compared to untreated cells. (40x magnification). Results shown are a representative of three experiments.

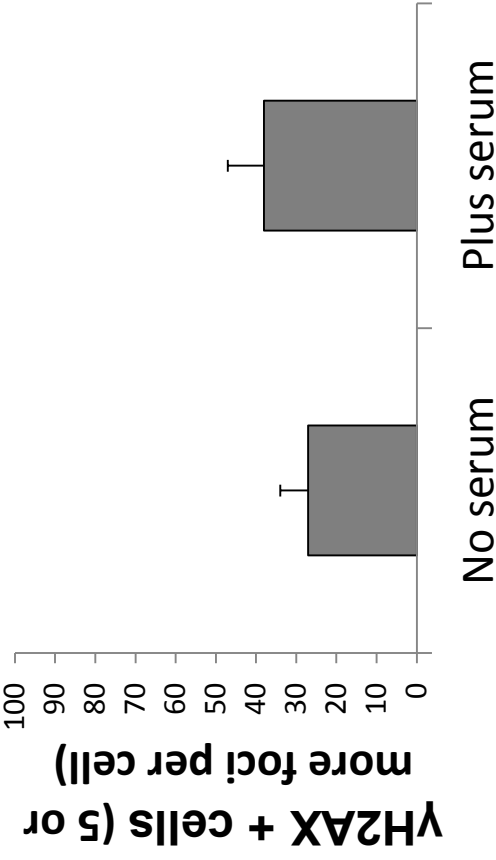
(D) Cells were treated with 10% serum as above. Cells were cultured for 7 days, then fixed and analyzed for γ H2AX foci by immunofluorescence. Data are from 100 cells per condition. The percent of cells with different numbers of foci from 0 to 14 foci per nucleus are plotted.

Fig 6:

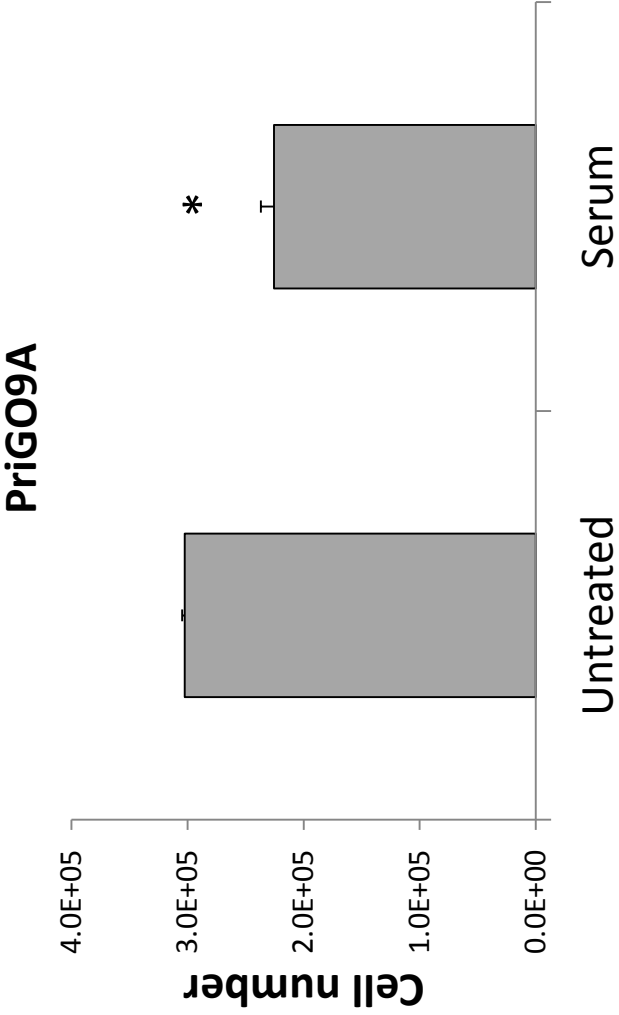
A



D



B



C

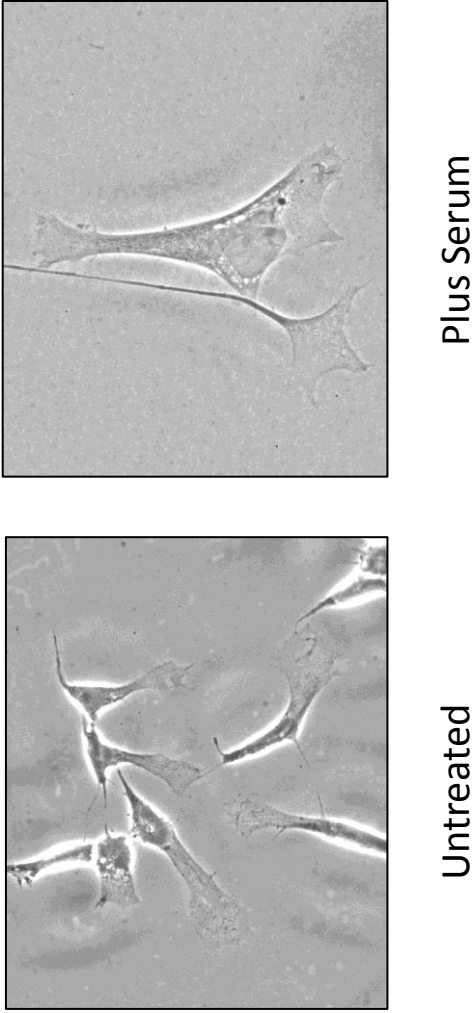


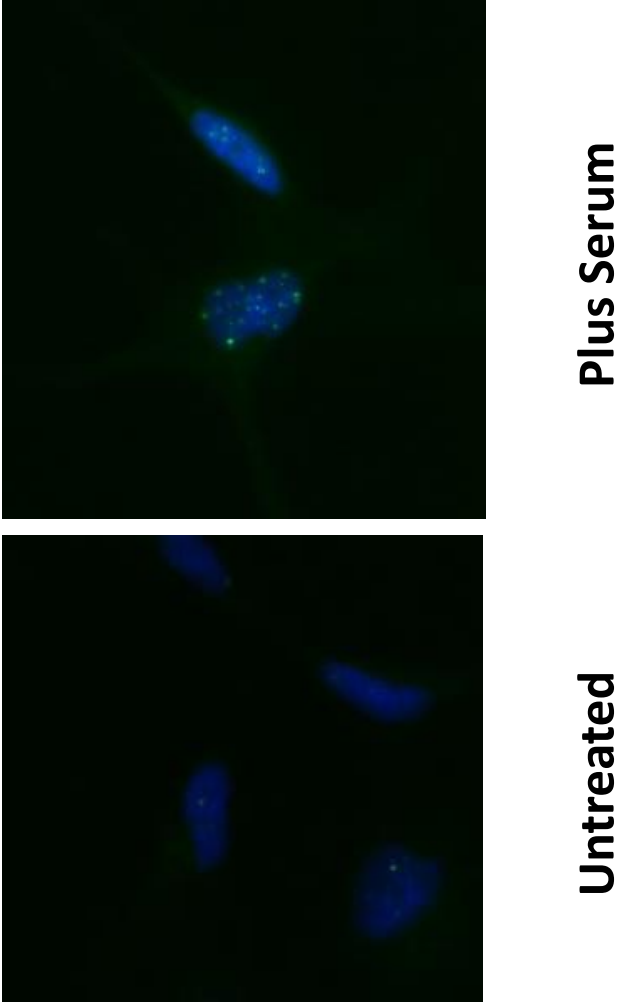
Figure 7. Induction of PML bodies in PriGO9A cells in response to serum.

A): PriGO9A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 10% serum for 7 days and then fixed and analyzed for PML bodies by immunofluorescence. Representative images are shown with PML in green and DAPI staining in blue.

(B): Quantitation of PML immunofluorescence. Data are from 100 cells per condition. Data shown is a mean of two experiments.

Fig 7:

A



B

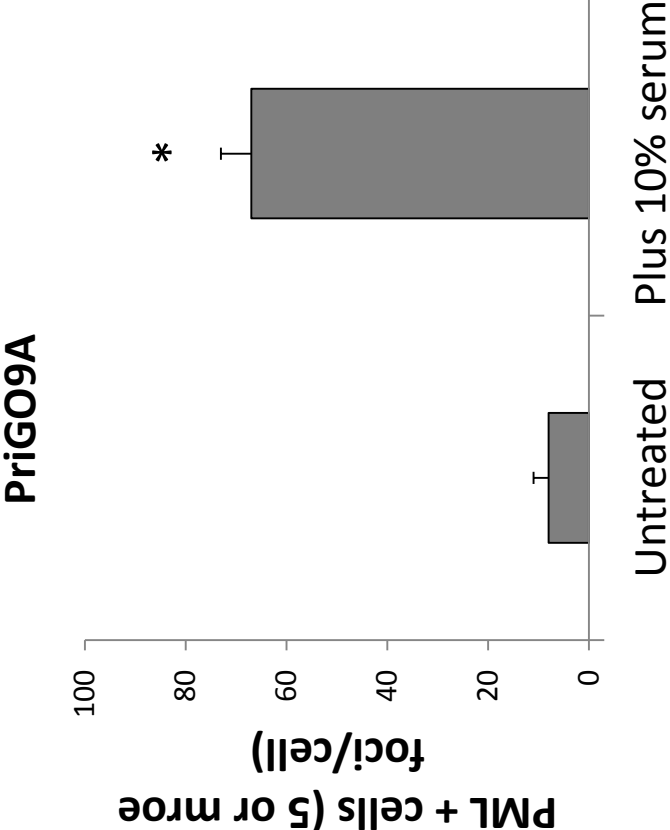


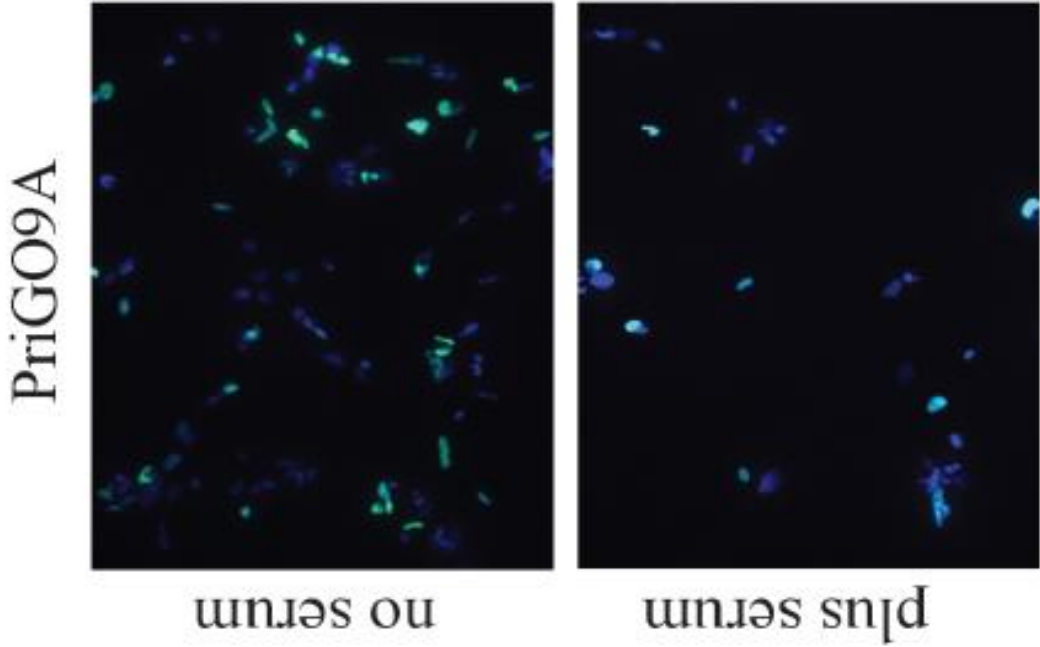
Figure 8. Serum inhibits proliferation of PriGO9A cells as determined by EdU incorporation.

(A) PriGO9A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 10% serum. On day 6, cells were labelled for 24 hrs with EDU. Cells with EDU incorporation were detected using the Click-IT EDU detection kit from Thermo Fisher. A representative picture of EdU incorporation in the presence and in the absence of serum is shown.

(B) Quantitation of EdU incorporation, data are from five randomly chosen fields. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$.

Fig 8:

A



B

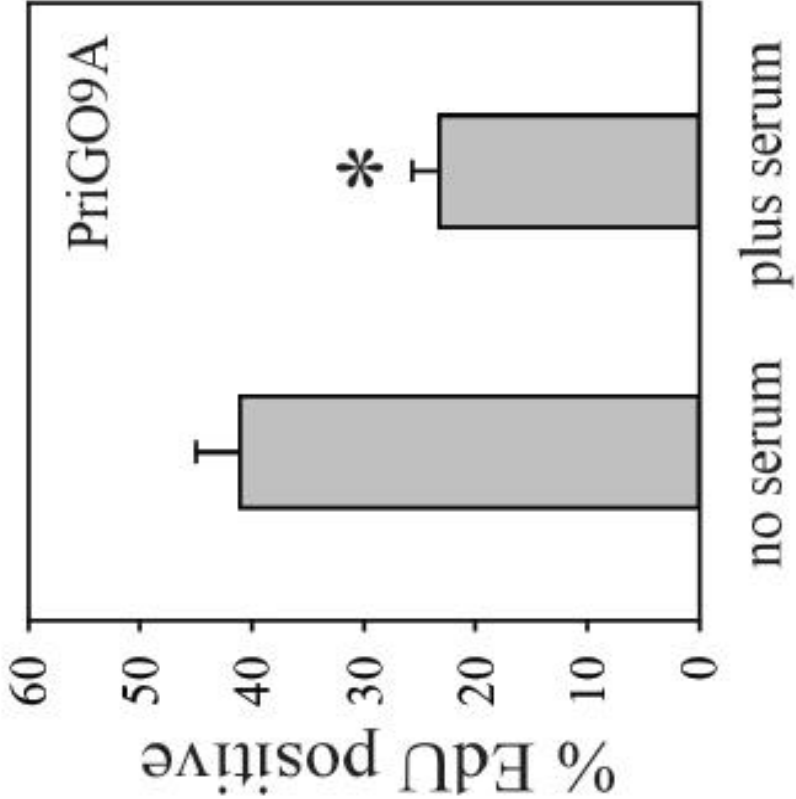


Figure 9. PriGO17A cells are resistant to serum-induced senescence.

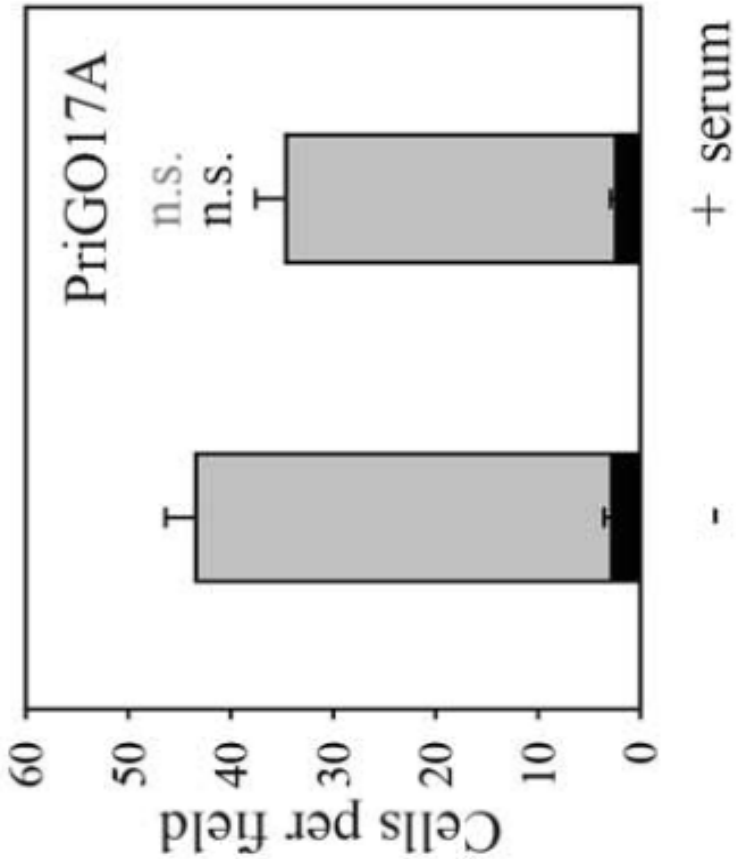
(A) PriGO17A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 10% serum. On day 7, cells were assayed for SA β Gal activity as described before. Statistical significance was determined using two-tailed t tests. * indicates $p < 0.05$.

(B) Morphology of PriGO17A cells treated with 10% serum compared to untreated cells (40x magnification). Results shown are a representative of three experiments.

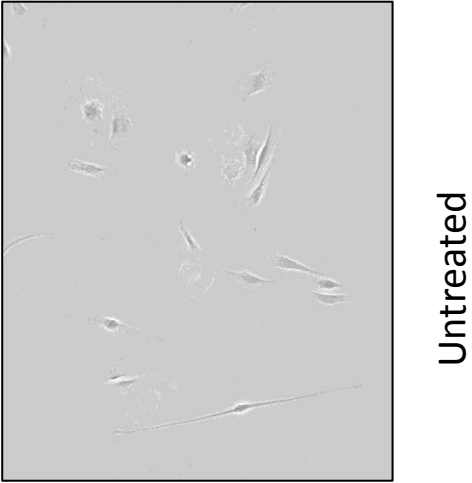
(C) PriGO17A cells (1×10^5 /ml) were treated with 10% serum as described above. After 48 hrs, live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown in A and B are a mean $\pm$ SD. N=3. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$.

Fig 9:

A



B



C

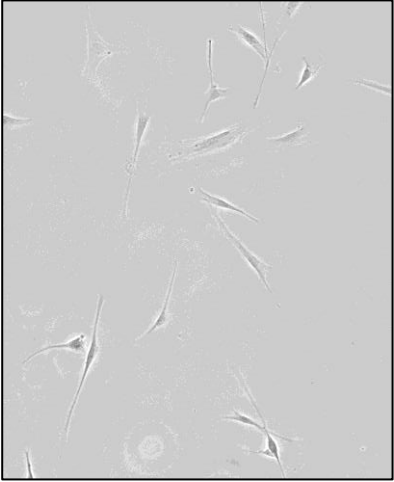
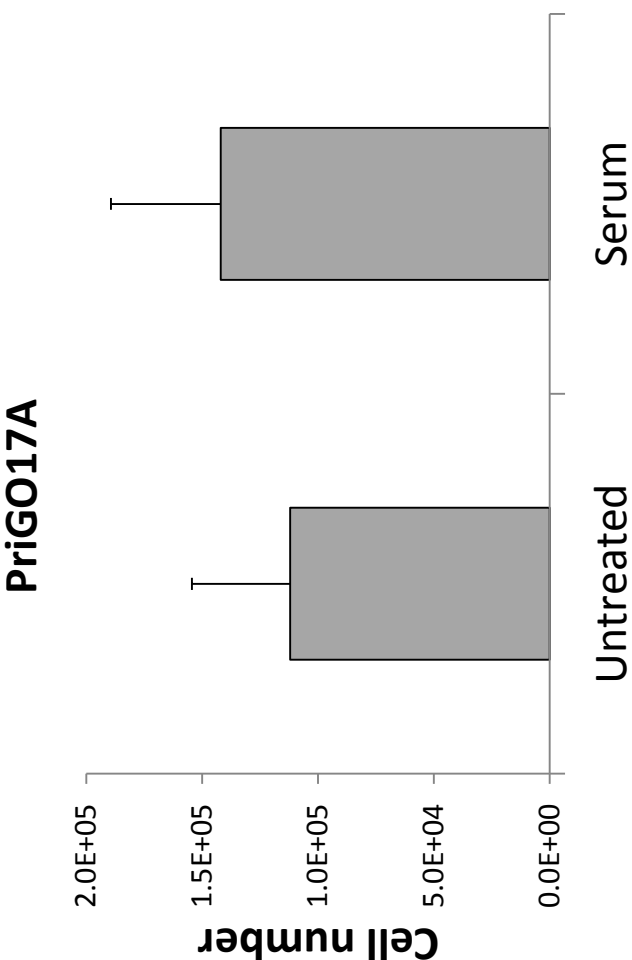


Figure 10. Serum fails to inhibit proliferation of PriGO17A cells as determined by EdU incorporation.

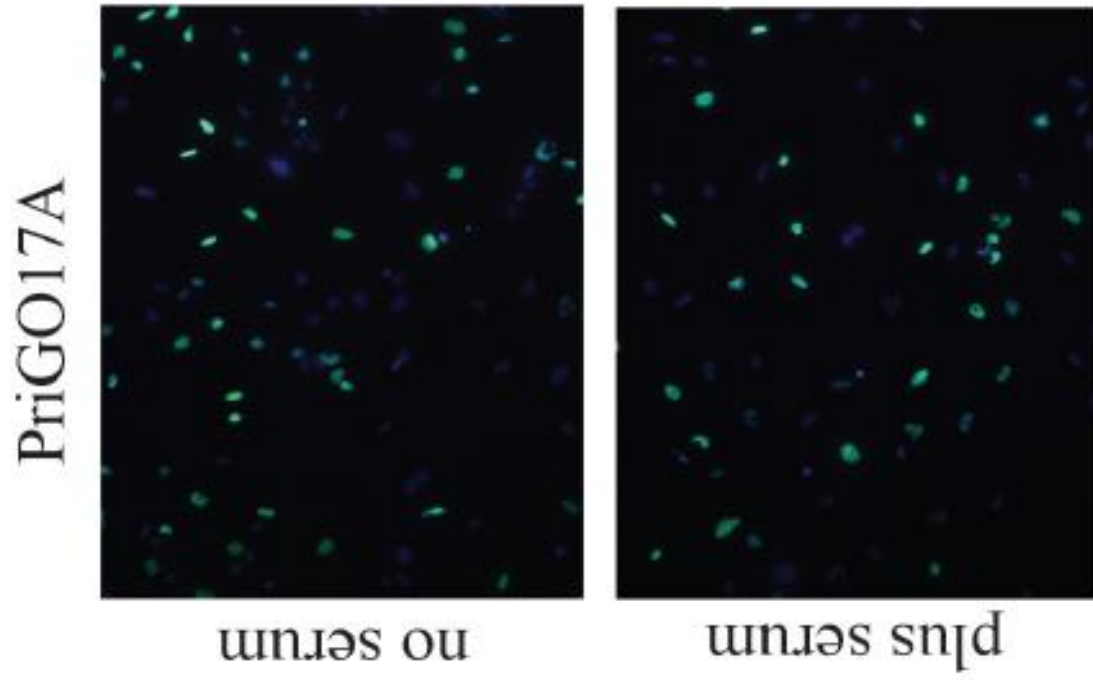
(A) PriGO17A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 10% serum as in Figure 23. On day 6 they were labelled for 24 hrs with EdU. Cells with EdU incorporation were detected using the Click-IT EdU detection kit from Thermo Fisher. A representative picture with EdU incorporation in the absence and in the presence of serum is shown

(B) Quantitation of EdU incorporation, data are from five randomly chosen fields. Results shown in A and B are a mean $\pm$ SD. N=3. Statistical significance was determined using two tailed t-test.

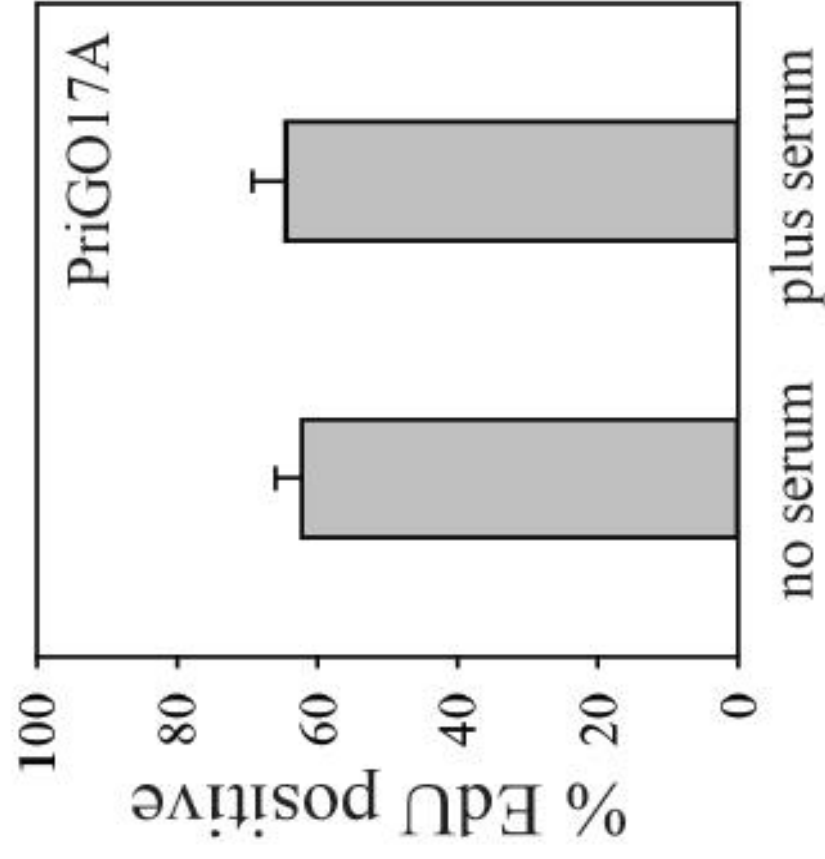
* indicates $p < 0.05$.

Fig 10:

A



B



p21 after 48 hrs, although it did so at 7 days post serum treatment (**Fig 4 C**). PriGO17A cells may undergo a cytostatic response to serum at that time such as differentiation. These results suggest that serum induced senescence in classical PriGO cells in contrast to the mesenchymal PriGO17A subtype that was resistant to serum-induced senescence.

1.2.2: Serum in contrast to BMP4 induces aberrant differentiation in classical PriGO cells:

Serum is known to induce aberrant differentiation in PriGO cells (54). This effect was also observed in PriGO8A cells. Cells treated with serum resulted in an increase in GFAP expression, as well as the TUJ1 expression, a marker for the neuronal lineage, often in the same cell (**Fig 11 A and B**). This indicates that serum induced cellular differentiation does not proceed towards only 1 lineage. However, this raises the question whether PriGO cells always undergo aberrant differentiation or is this phenomenon limited to serum treatment.

PriGO cells retain the ability to differentiate along a single lineage, not always differentiating in an aberrant manner. In contrast, treatment of PriGO8A cells with BMP4, an agent known to induce differentiation of primary glioblastoma cells into the astrocytic lineage (44), led to over 90% of cells expressing GFAP compared to untreated cells (**Fig 12 A and B**). Furthermore, there was minimal detection of TUJ1 in either BMP4 treated or untreated cells.

Finally, it was questioned whether senescence and differentiation are separate processes in PriGO cells. Although serum induces both differentiation and senescence in PriGO8A cells, I determined whether these two processes are linked or is serum just having a pleiotropic effect. As mentioned above BMP4 can induce differentiation of PriGO8A cells. However, it is not known whether the cytostatic effect of BMP4 is limited to differentiation or can some of it also be attributed to senescence in PriGO cells. PriGO8A cells treated with BMP4 did not result in an

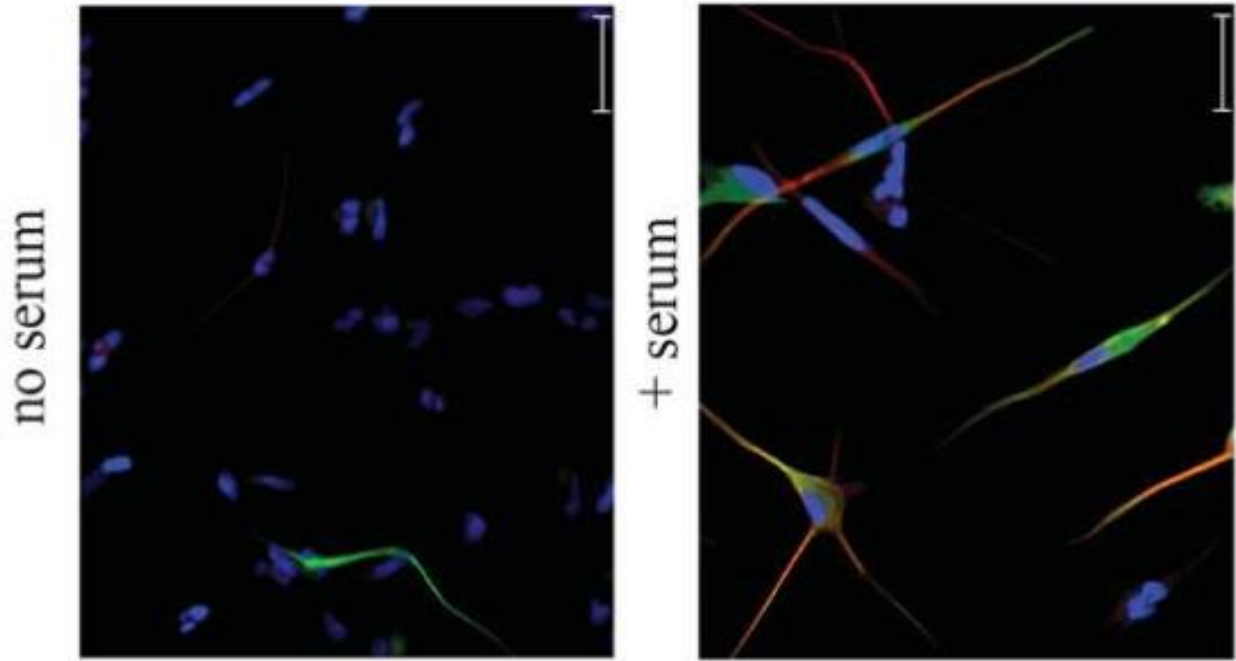
Figure 11. Serum induces aberrant differentiation in PriGO8A cells.

(A) PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 5% serum as in Figure 8. On day 7, cells were fixed and stained for immunofluorescence for GFAP (green) and TUJ1 (red) with appropriate antibodies. Nuclei were stained with DAPI (blue). A representative picture of immunofluorescence in the presence and in the absence of serum is shown.

(B) The panel shows quantitation of the numbers of TUJ1+, GFAP+ and double positive cells in the presence and the absence of serum. Results shown in are a mean $\pm$ SD. N=3. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$.

Fig 11:

A



B

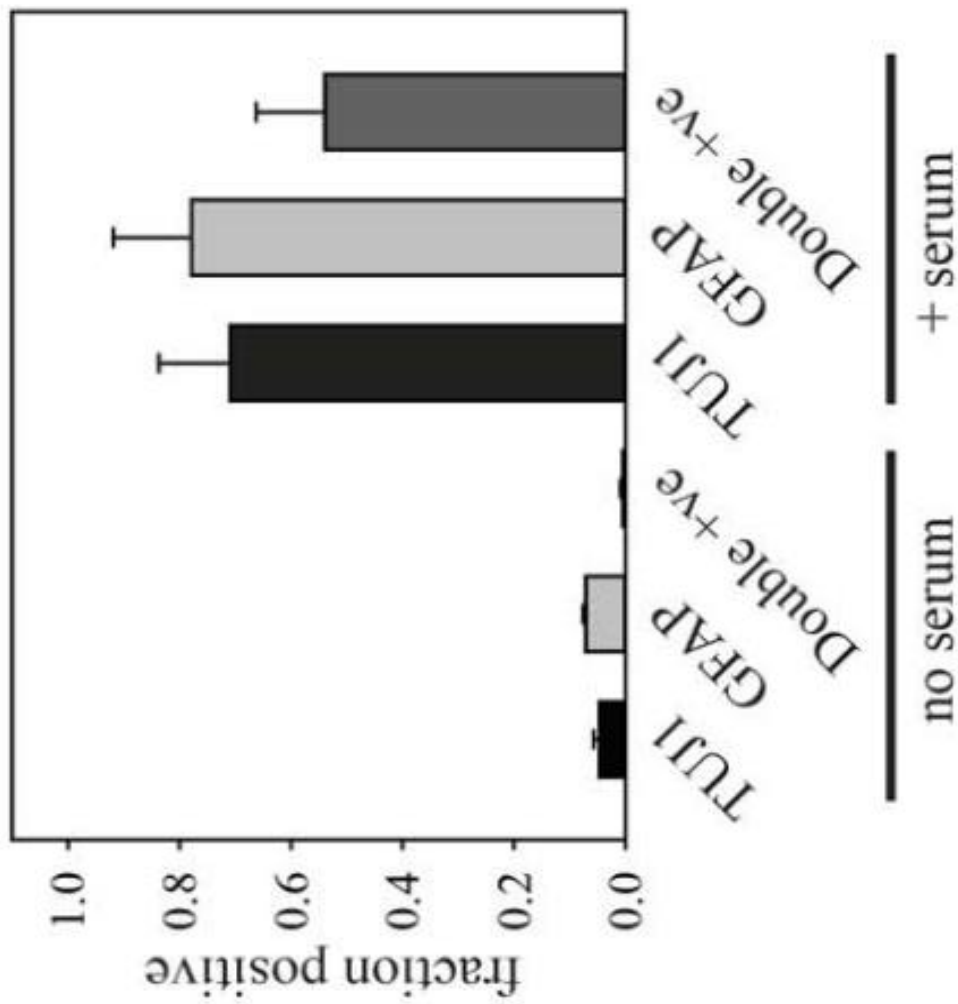
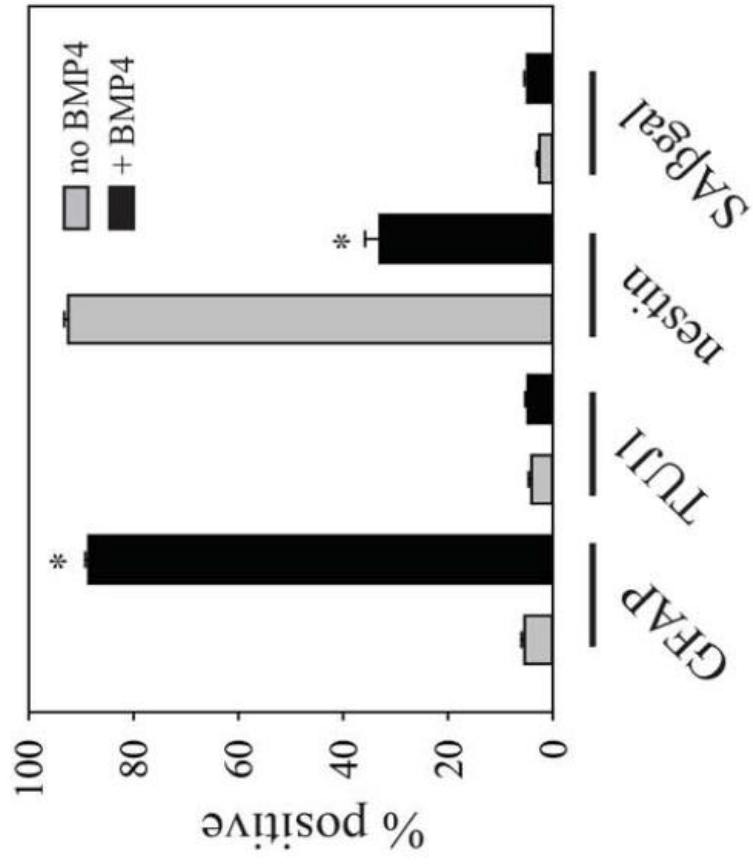
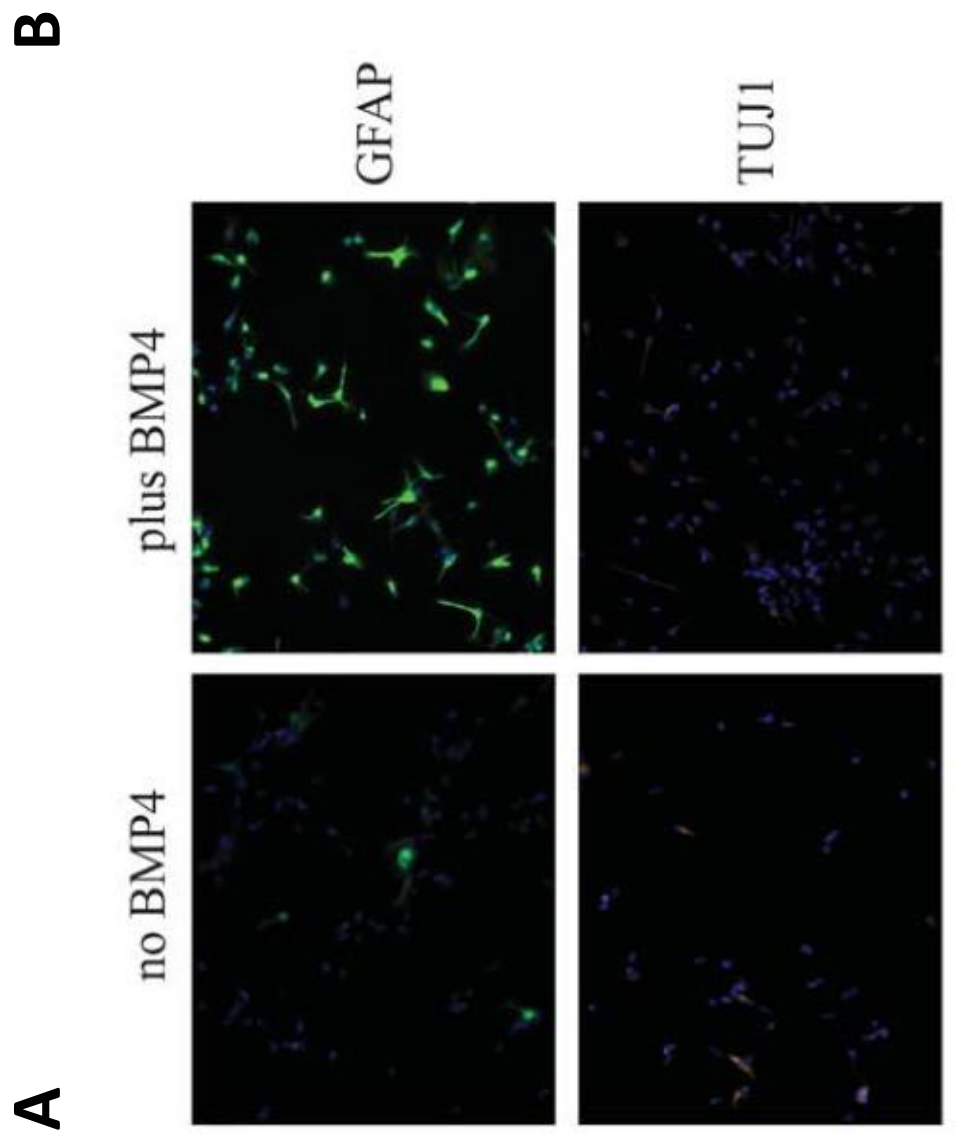


Figure 12. BMP4 induces differentiation but does not induce senescence in PriGO8A cells.

(A) PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 100 ng/ml of BMP4. Seven days later, cells were fixed and stained for immunofluorescence for GFAP, TUJ1 and Nestin with appropriate antibodies. A representative picture of immunofluorescence in the presence and in the absence of BMP4 is shown.

(B) PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 100 ng/ml of BMP4. Seven days later, cells were assayed for SA β Gal activity as described before. The bar graph shows quantitation of GFAP+, TUJ1+, Nestin+ and SA β Gal positive cells. Data are from five randomly selected fields per condition. Results shown in are a mean $\pm$ SD. N=3. Statistical significance was determined using two-tailed t tests. * indicates $p < 0.05$.

Fig 12:



increase in Sa β Gal positivity (**Fig 12 B**) suggesting BMP4 does not cause senescence in PriGO8A cells. Hence, serum induced senescence is not a feature of differentiation.

BMP's, including BMP4, are known to be present in serum. BMP4 is thus likely to be one of the agents in serum inducing differentiation in PriGO8A cells. To further confirm that BMP4 does not induce senescence in PriGO8A cells, it was determined whether inhibition of BMP4 signalling would affect the capability of serum to cause senescence. PriGO8A cells were pre-treated with LDN 212854, an inhibitor of the BMP type 1 receptor, followed by treatment with serum. LDN did not cause a significant decrease in serum induced Sa β Gal positivity in PriGO8A cells, confirming BMP4 does not cause senescence in PriGO8A cells (**Fig 13**). Hence, my results suggest that serum induced differentiation and senescence in PriGO8A cells are not linked.

Objective 1.3: Determine the susceptibility of classical and mesenchymal PriGO subtypes to ionizing radiation with respect to senescence

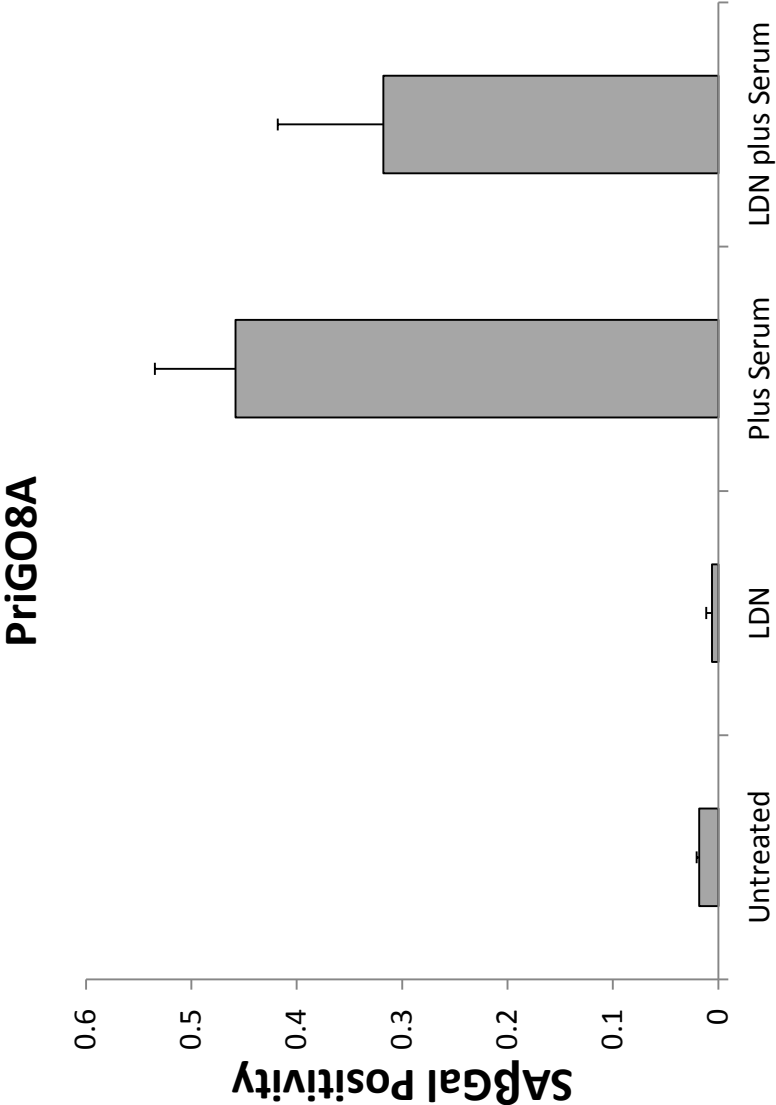
1.3.1: Classical and Mesenchymal PriGO cells do not undergo senescence in response to ionizing radiation.

Ionizing radiation is a well-known stimulus causing cells such as fibroblasts to undergo premature senescence (268). Radiation leads to double stranded DNA (dsDNA) breaks, activating the DDR, resulting in either apoptosis or senescence (65). Although established glioblastoma cell lines such as U87MG have been shown to undergo senescence in response to radiation (269), it is unknown if PriGO cells are capable of undergoing senescence in response to

Figure 13. Serum-induced senescence is not mediated by BMP4 in PriGO8A cells

PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were pretreated with BMP4R inhibitor LDN 212854 (5 η M) for 24 hours. Subsequently, cells were treated with 10% serum for 7 days following which cells were analyzed for SA β Gal activity as described before. Results shown in are a mean $\pm$ SD. N=3. Statistical significance was determined using two-tailed t tests. * indicates $p < 0.05$.

Fig 13:



radiation. Furthermore, serum induced senescence may depend on activation of the DDR in response to dsDNA damage.

To determine if dsDNA damage induces senescence in PriGO cells, PriGO8A cells were treated with x-ray radiation at 0, 1, 3, 5, and 10 Gray (Gy) and Sa β Gal was performed 7 days later. Although the percentage of Sa β Gal positive cells increased in a dose dependant manner, the absolute number of Sa β Gal positive cells did not significantly change following radiation (**Fig 14 A**), suggesting radiation did not induce senescence but rather may have caused selection of senescent cells.

The same phenomenon was observed for PriGO9A cells (**Fig 14 B**). While radiation treatment caused an increase in the percentage of senescent cells it did not lead to an increase in the actual number of senescent cells, once again suggesting selection rather than senescence may explain the increase in the percentage of senescent cells. Radiation of PriGO17A cells did not lead to an increase in the total number of Sa β Gal positive senescent cells. However, it also did not lead to an increase in the percentage of Sa β Gal positive cells either (**Fig 14 C**). This suggests PriGO17A cells do not respond to radiation unlike the PriGO8A and PriGO9A cells.

Objective 1.3.2: Determine the susceptibility of classical and mesenchymal PriGO subtypes to ionizing radiation with respect to cell death.

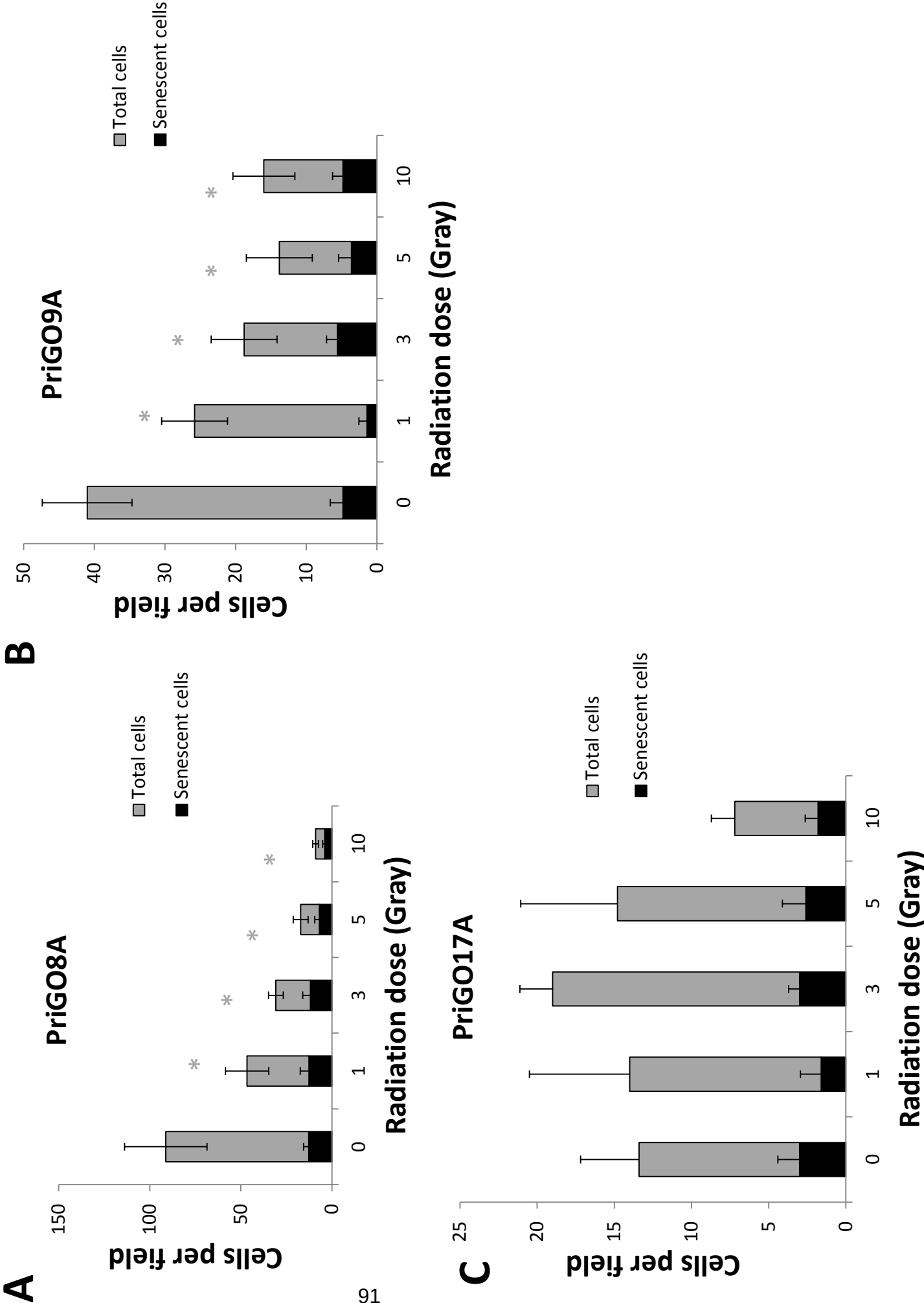
Classical PriGO cells are susceptible to ionizing radiation with respect to cell death whereas the mesenchymal PriGO cells are resistant.

Although none of the PriGO cells underwent senescence in response to ionizing radiation, PriGO8A and PriGO9A cells appeared to be selected for senescence. This raised the question

Figure 14. Radiation does not induce senescence in PriGO cells:

(A) PriGO8A, (B) PriGO9A, and (C) PriGO17A cells (1×10^4 /ml) were cultured for 24 hrs following which they were irradiated with a dose of 1, 3, 5 or 10 Gray (Gy). Cells were cultured for another 7 days following which cells were fixed and stained for SA β gal activity. The graphs show total cells (light grey bars) and SA β gal-positive cells (dark black) per field. Data are from analysis of five random fields per condition. Results shown are a mean $\pm$ SD. N=3. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, n=3.

Fig 14:



whether these cells were undergoing cell death in response to radiation. Furthermore, the PriGO17A cells did not respond to radiation at all. There is one report which suggests that the mesenchymal subtype of primary glioblastoma cells were radioresistant with respect to cell death, especially compared to the proneural subtype (16). Given that the effect of radiation on the classical subtype of PriGO cells is not known, I next determined the differences between the classical and mesenchymal subtype cells to respond to radiation with respect to cell death.

First, I determined the effect of radiation on PriGO8A cells with respect to cell death. PriGO8A cells were irradiated with 1, 5, or 10 Gray (Gy) and cell counts were performed after 6, 24, and 48 hrs later. There was a dose dependent decrease in cell number with an increase in radiation dose (**Fig 15**). While the effect of radiation on PriGO8A cells was most marked at 5 and 10 Gy, there was a significant decrease in cell number even at 1 Gy compared to untreated cells. Furthermore, while the effect of radiation was seen after 24 hours of treatment, it was more pronounced after 48 hours.

The effect of radiation was also measured by cell counts in PriGO9A cells, the classical subtype, and PriGO17A cells, the mesenchymal cells, at 1, 3, 5, and 10 Gy after 48 hrs of treatment. PriGO9A cells also showed a dose dependent decrease in cell number but less than the PriGO8A cells (**Fig 16 A and B**). However, radiation did not have a significant impact on the cell number of PriGO17A cells even at the highest radiation dose (**Fig 16 C**). To determine if radiation exerted long lasting effects on the cell number of PriGO cells, cell number after radiation was determined for an extended period of 14 days. PriGO8A cells treated with radiation of 5 and 10 Gy did not increase in cell number even 2 weeks after treatment (**Fig 17**).

Although radiation caused a decrease in cell counts of PriGO8A and PriGO9A cells, the mechanism by which radiation cause decreased cell numbers remains unknown. Therefore, I next

Figure 15. Radiation causes cell death of classical PriGO8A cells.

PriGO8A cells ($1 \times 10^5/\text{ml}$) were cultured for 24 hrs following which they were irradiated with a dose of 1, 5, or 10 Gray (Gy). Cells were cultured for another 6, 24, or 48 hours following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are from single pilot experiment.

Fig 15:

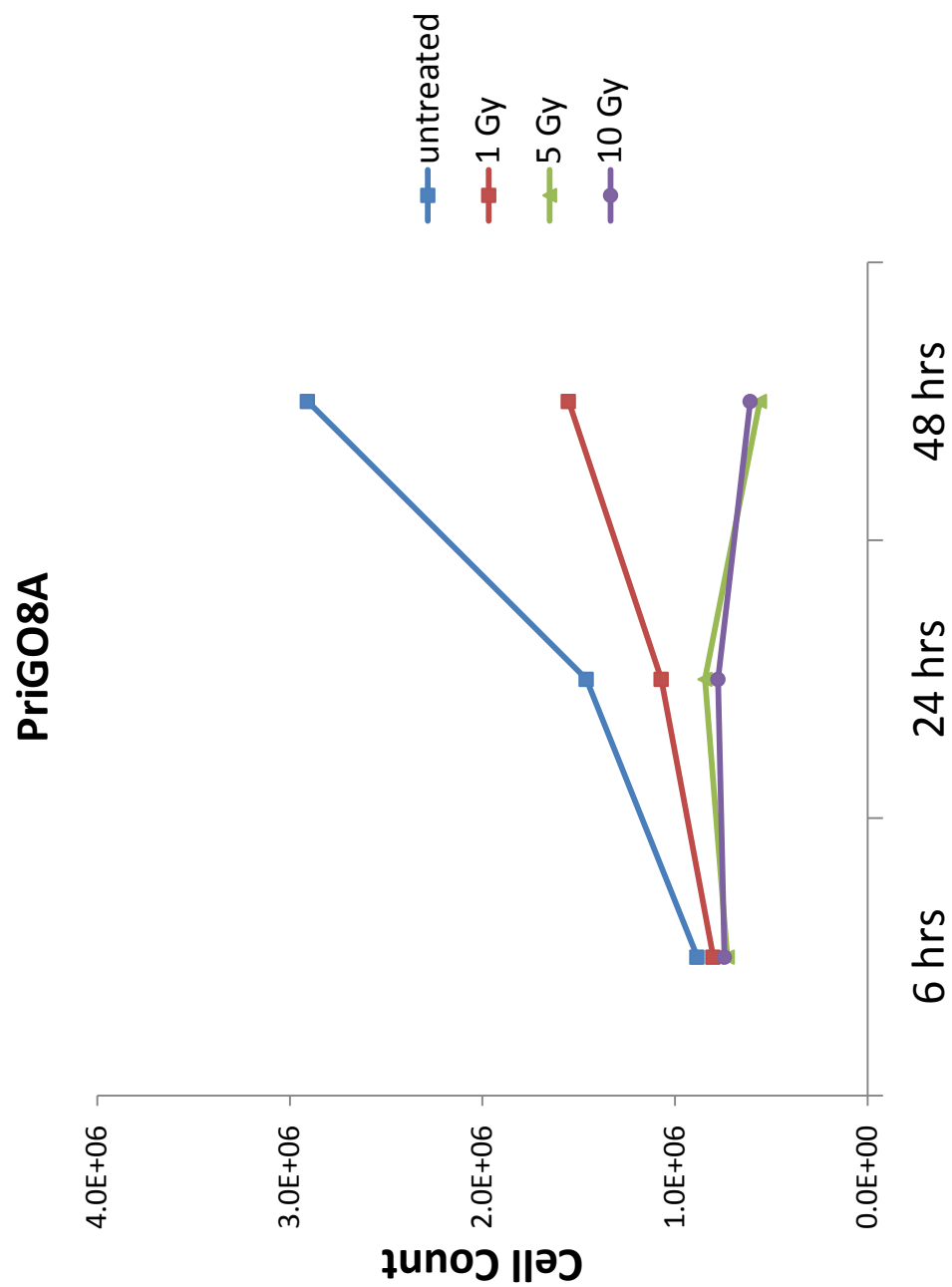
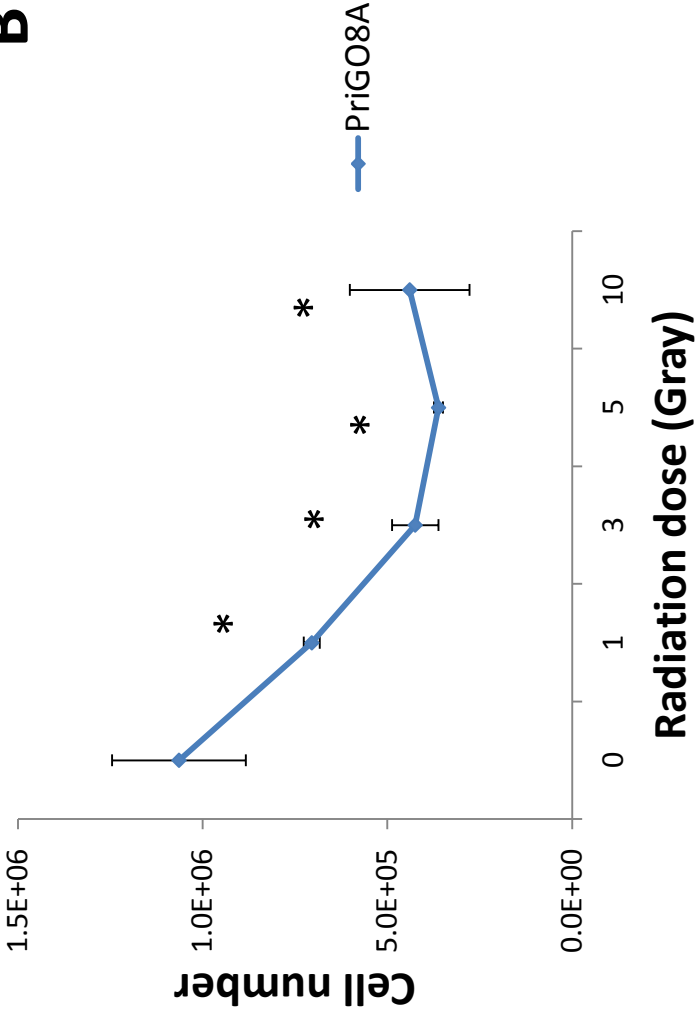


Figure 16. Radiation induces cell death of classical PriGO8A and 9A cells whereas mesenchymal PriGO17A cells are resistant to the cytotoxic effect of radiation.

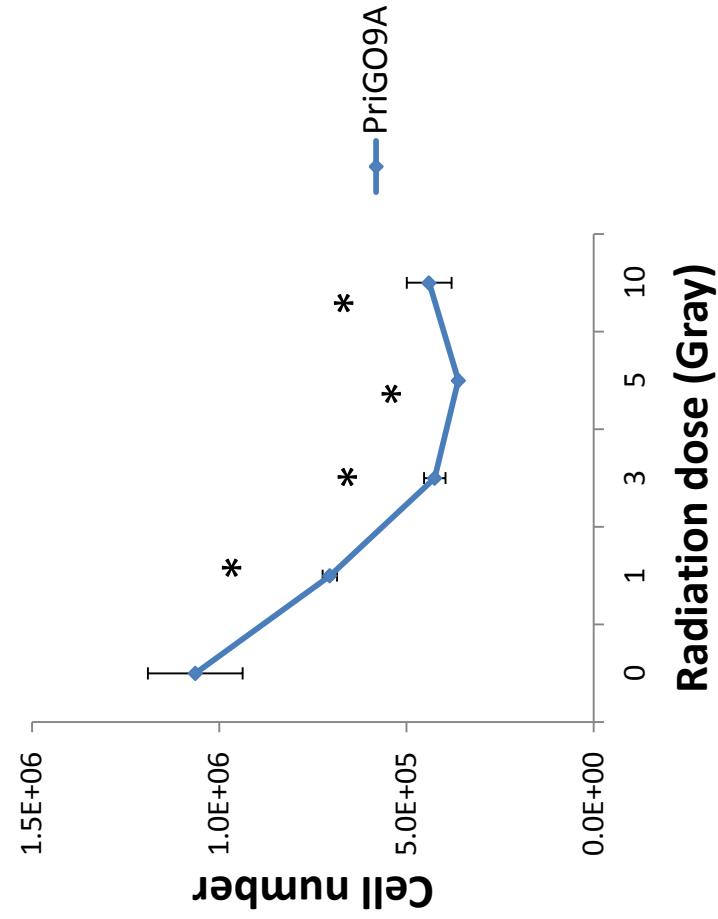
A) PriGO8A, (B) PriGO9A and (C) PriGO17A cells ($1 \times 10^5/\text{ml}$) were cultured for 24 hrs following which they were irradiated with a dose of 1, 3, 5, or 10 Gray (Gy). Cells were cultured for another 48 hours following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are a mean $\pm$ SD. (N=4). Statistical significance was determined using one way ANOVA. * indicates $p < 0.05$.

Fig 16:

A



B



C

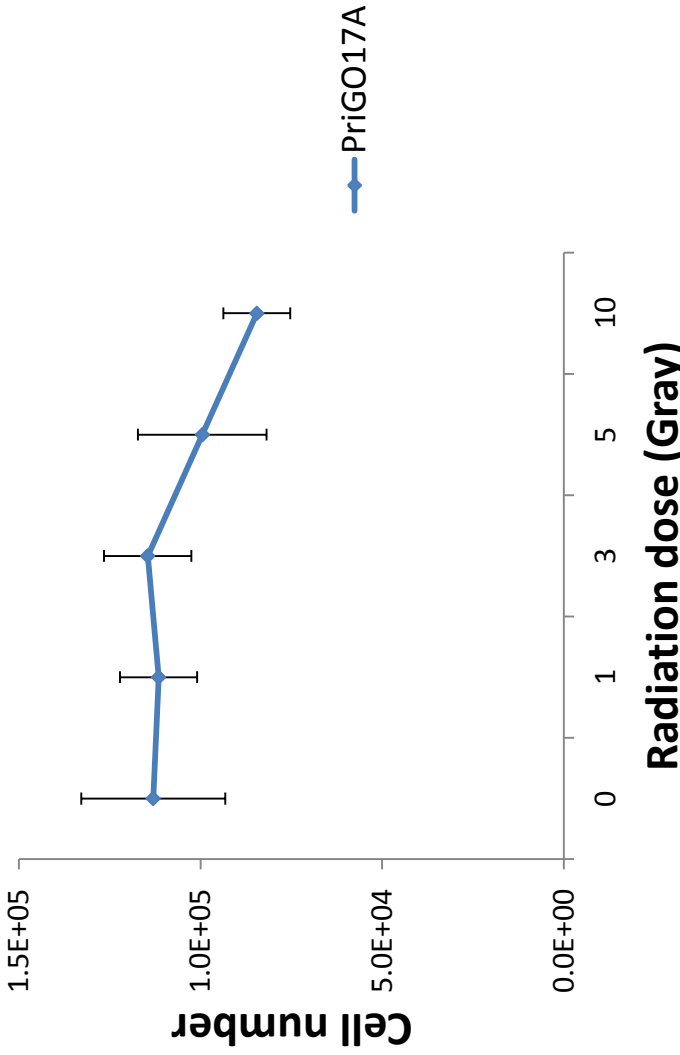
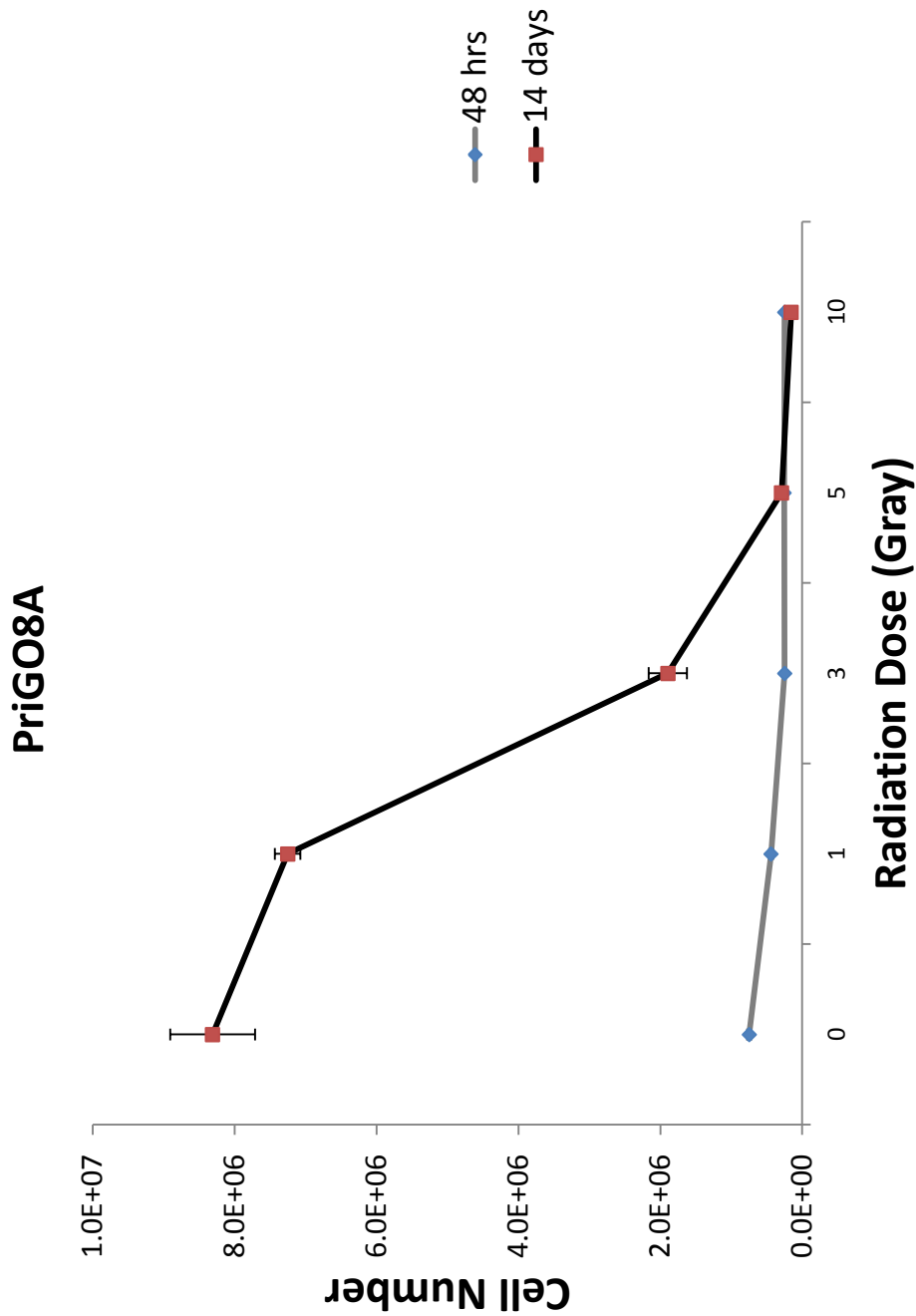


Figure 17. Radiation permanently inhibits proliferation in classical PriGO 8A cells: A) PriGO8A (1×10^5 /ml) were cultured for 24 hrs following which they were irradiated with a dose of either 1, 3, 5, or 10 Gray (Gy). Cells were cultured for another 48 hours or 14 days following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Statistical significance was determined using one way ANOVA. * indicates $p < 0.05$, $N=3$.

Fig 17:



determined whether the effect of radiation on decreasing cell counts in PriGO8A cells was due to apoptosis. To determine if apoptosis was the involved mechanism, markers of apoptosis such as Annexin V and cleavage of caspase 3 were evaluated in response to radiation. Annexin V staining of PriGO8A cells at 6, 24, and 48 hrs after irradiation at 10 Gy showed a dose dependant increase in the number of Annexin V positive cells (**Fig 18**). At 10 Gy, almost 50% of the cells were positive compared to less than 10% in untreated cells. PriGO9A cells irradiated at 1, 5, or 10 Gy demonstrated a similar dose dependant increase in Annexin V positivity (**Fig 19**). Although at 10 Gy PriGO9A cells demonstrated a lesser response than PriGO8A cells with 35% positivity compared to 50% respectively, it still indicated a significant amount of cell death.

Subsequently, I confirmed the cytotoxic effect of radiation on PriGO8A cells was due to apoptosis by analyzing caspase-3 activation measured after 6, 24, and 48 hrs following irradiation at 10 Gy by Western blot analysis. There was a significant increase in cleaved caspase 3 levels at 48 hrs while a less intense but still significant response was seen at 24 hrs (**Fig 20 A**). However, cleaved caspase 3 activity was not seen in PriGO8A cells treated with 1 or 5 Gy radiation after 48 hrs (**Fig 20 B**). In contrast, PriGO17A cells did not demonstrate cleaved caspase 3 activity even at 10 Gy after 48 hrs of irradiation (**Fig 20 C**). Thus, my results suggest that radiation caused significant decrease in cell number in PriGO8A cells that was attributed to apoptosis whereas PriGO17A cells were relatively resistant to radiation.

p21 is believed to be a marker of cell cycle arrest (270). To confirm that PriGO17A cells did not undergo cytostasis following irradiation, PriGO17A cells were further analyzed by determining the induction of p21 in response to radiation. An increase in p21 induction as detected by western blotting 48 hrs after irradiation in both PriGO8A and PriGO9A cells but there was no increase in p21 levels in PriGO17A cells, even at the highest dose of 10 Gy (**Fig 21**

Figure 18. Radiation induces apoptosis of PriGO8A cells cultured for various times as determined by Annexin-V staining.

(A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were irradiated with a dose of 10 Gray (Gy). Cells were cultured for another 6, 24, or 48 hours following which cells were stained with Annexin-V conjugated with BV711 fluorophore and analyzed for apoptosis by flow cytometry. Results shown are a representative of three experiments.

(B) Quantitation of Annexin-V staining. Results shown are a mean $\pm$ SD. N=3. Statistical significance was determined by one way ANOVA. * indicates $p < 0.05$.

Fig 18:

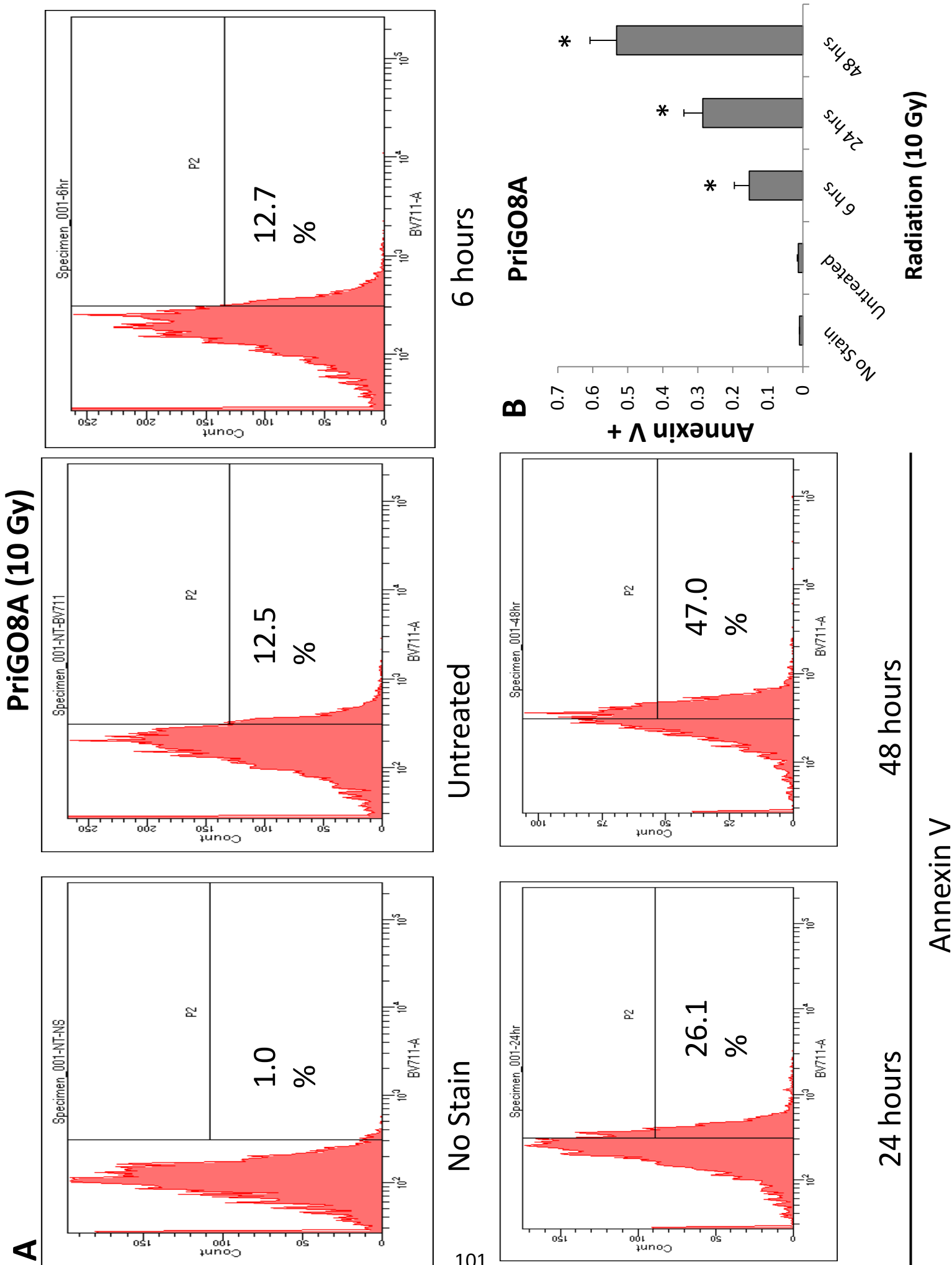


Figure 19. Radiation induces dose-dependent apoptosis of PriGO9A cells as determined by Annexin-V staining.

(A) PriGO9A cells (1×10^5 /ml) were cultured for 24 hrs following which they were irradiated with a dose of 1, 5 or 10 Gray (Gy). Cells were cultured for another 48 hours following which cells were stained with Annexin-V conjugated with BV711 fluorophore and analyzed for apoptosis by flow cytometry. Results shown are a representative of three experiments.

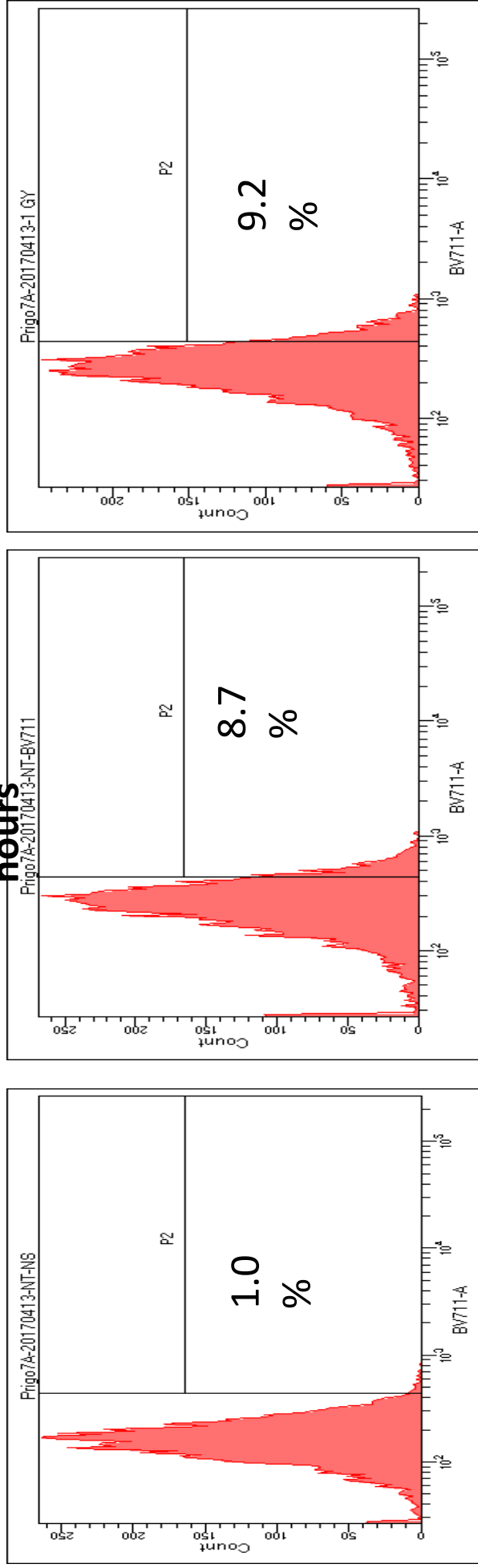
(B) Quantitation of Annexin-V staining. Results shown are a mean $\pm$ SD. N=3. Statistical significance was determined by one way ANOVA. * indicates $p < 0.05$.

Fig 19:

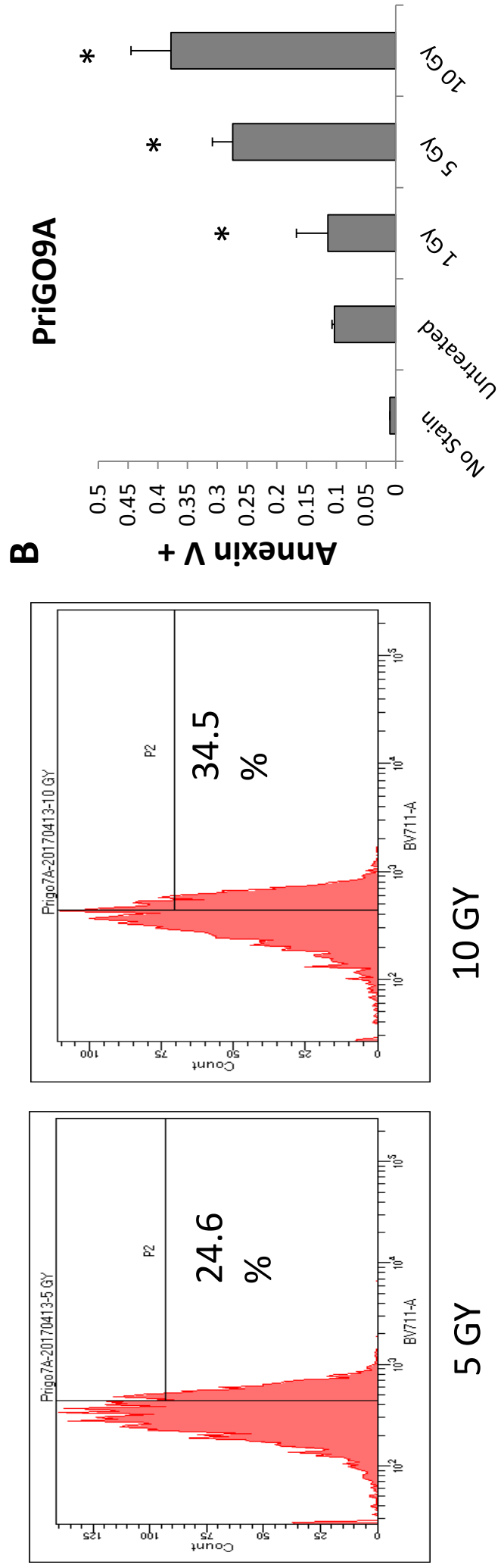
PriGO9A 48

hours

A



103



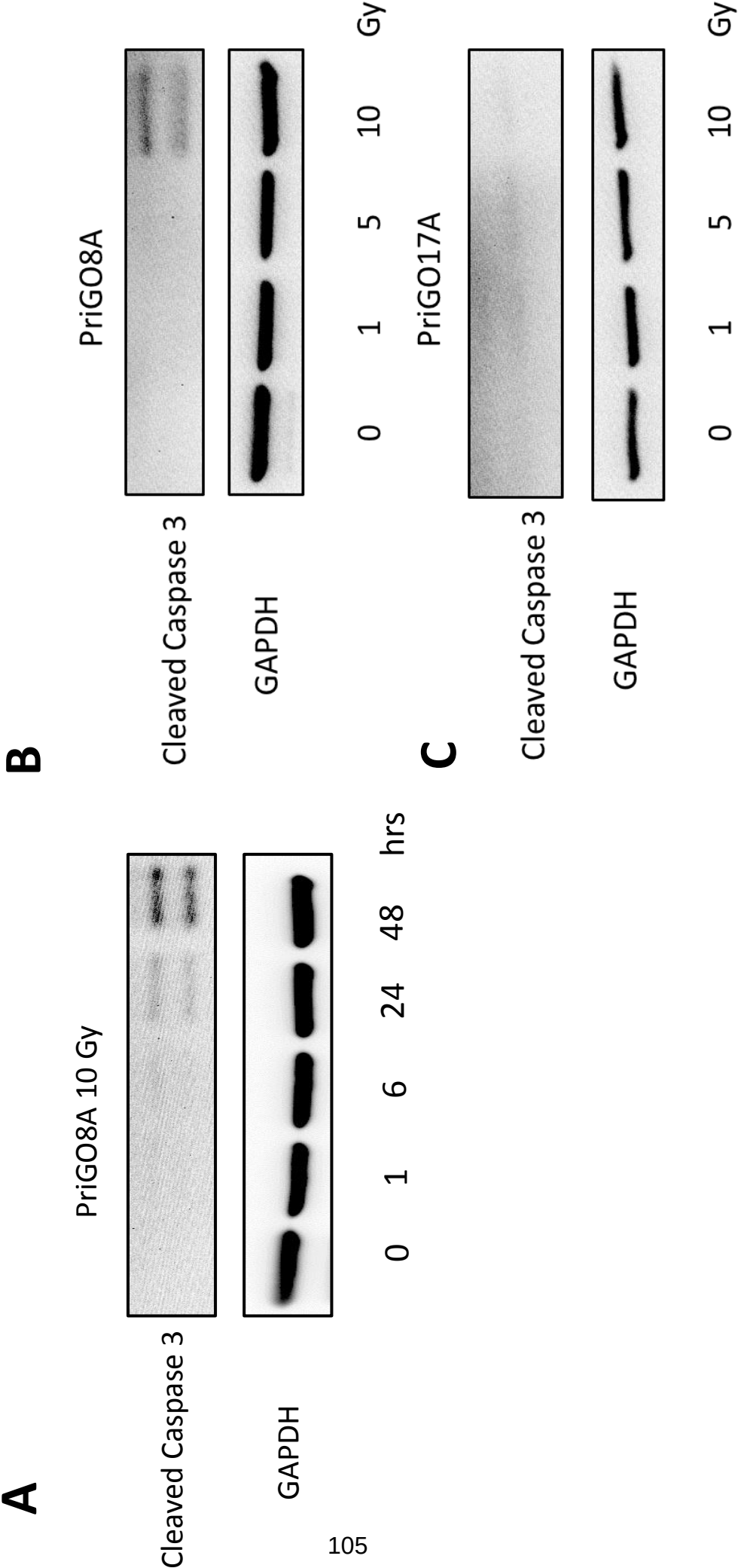
Annexin V

Figure 20. Radiation induces caspase-3 activation in classical PriGO8A and 9A cells whereas mesenchymal PriGO17A cells are resistant to caspase-3 activation following irradiation.

A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were irradiated with a dose of 10 Gray (Gy). Cells were cultured for another 1, 6, 24 or 48 hrs followed by the collection of cell lysates and Western Blot analysis for cleaved Caspase 3.

(B) PriGO9A and (C) PriGO17A cells (1×10^5 /ml) were cultured for 24 hrs following which they were irradiated with a dose of 1, 5 or 10 Gray (Gy). Cells were cultured for another 48 hrs followed by the collection of cell lysates and Western Blot analysis for cleaved Caspase 3. Results shown is a representative of two experiments

Fig 20:



A, B, and C). This again supports that PriGO17A cells were resistant to radiation as they were not responding to radiation in p21 mediated mechanisms responsible for senescence or apoptosis.

To confirm the above findings as valid, WI38 human fibroblasts were used as a positive control. WI38 cells were irradiated as before and analyzed for cell counts 48 hrs later and senescence 7 days later. WI38 cells responded with a 50% decrease in cell number at all doses of radiation (**Fig 22 A**). They demonstrated a significant increase in the total number of Sa β Gal positive cells as well as the percentage of Sa β Gal positive cells (**Fig 22 B**). Furthermore, WI38 cells were positive for other senescent markers following radiation treatment. For example, radiation dramatically caused an increase in p21 levels as detected by western blotting, even at low doses (**Fig 22 C**). Moreover, cells treated with radiation also demonstrated an increase in γ H2AX positivity signifying an active DNA damage repair response (**Fig 22 D**). These data indicate that WI38 fibroblasts are undergoing senescence rather than being selected for senescent cells in response to radiation.

Overall, my results show that radiation does not induce premature senescence in any of the PriGO cell lines, but rather selects for senescent cells in PriGO8A and PriGO9A cells by inducing cell death. This also suggests dsDNA damage may not be the mechanism of serum-induced senescence in PriGO cells. Furthermore, consistent with the literature (16) the mesenchymal cell line PriGO17A is resistant to ionizing radiation with respect to apoptosis, and novel to this study, senescence. Furthermore, PriGO17A cells are not only resistant to radiation-induced apoptosis and senescence but they are also resistant to serum-induced senescence. This is in contrast to the classical subtype of PriGO cells which are sensitive to radiation by undergoing cell death, a novel finding.

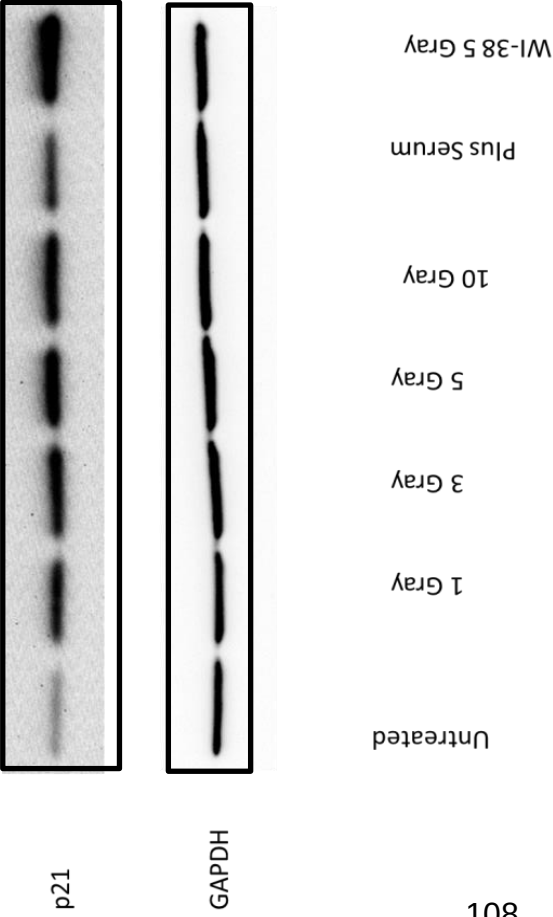
Figure 21. Radiation induces p21 activation in classical PriGO8A and 9A cells whereas mesenchymal PriGO17A cells are resistant to p21 induction following irradiation.

(A) PriGO8A, (B) PriGO9A, and (C) PriGO17A cells ($1 \times 10^5/\text{ml}$) were cultured for 24 hrs following which they were irradiated with a dose of 1, 3, 5 or 10 Gray (Gy). In parallel, WI-38 cells were cultured and irradiated with 5 Gray as a positive control. PriGO cells were also cultured in the presence of serum as another control. Following radiation, cells were cultured for another 48 hours. Subsequently, total cell lysates were analyzed by Western blotting for p21 induction. Results shown is a representative of two experiments

Fig 21:

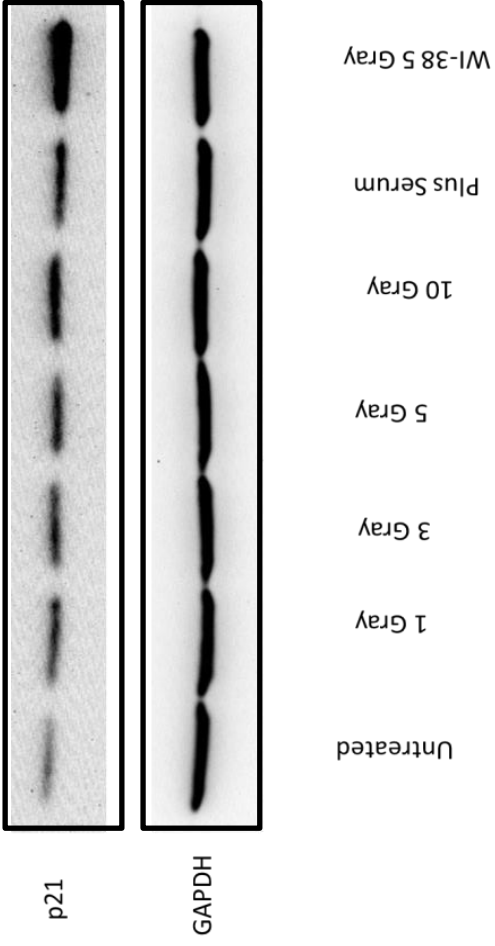
A

PriGO8A



B

PriGO9A



C

PriGO17

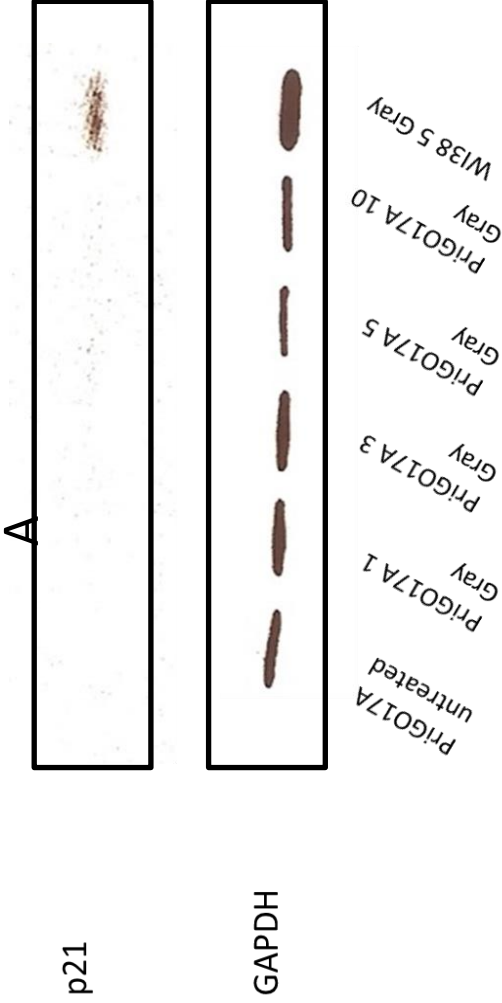


Figure 22. Radiation inhibits proliferation, causes senescence and induces p21 expression in WI38 human fibroblasts.

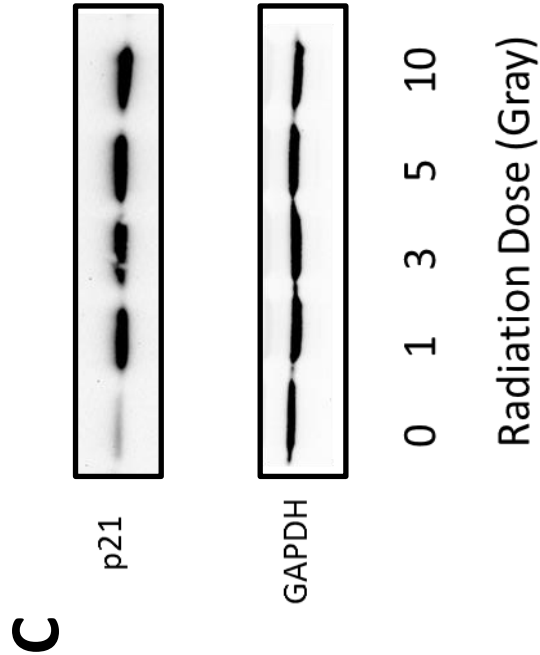
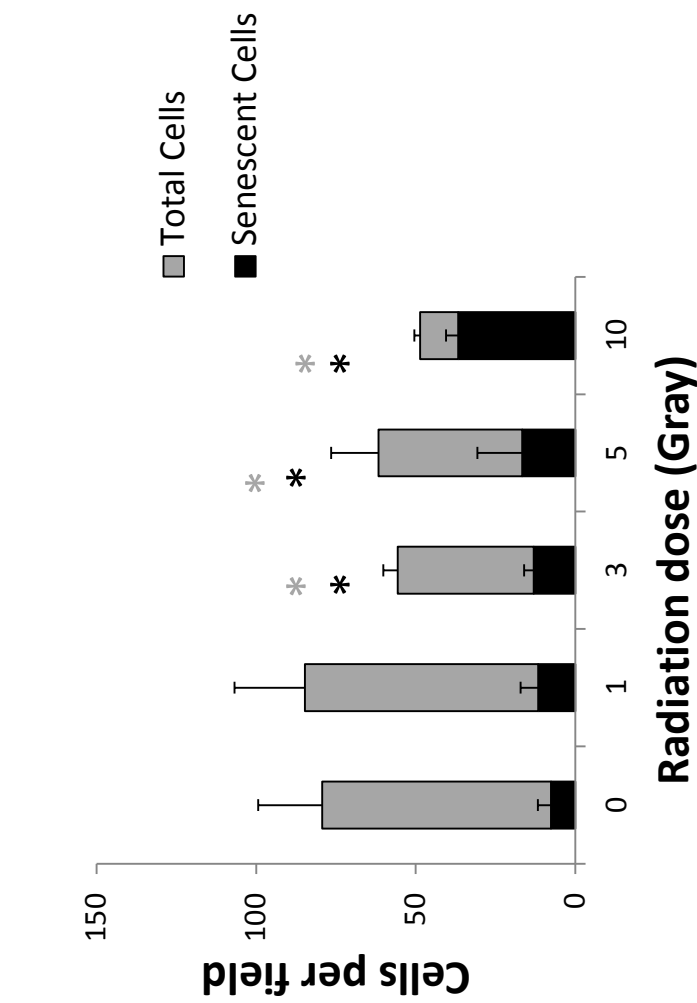
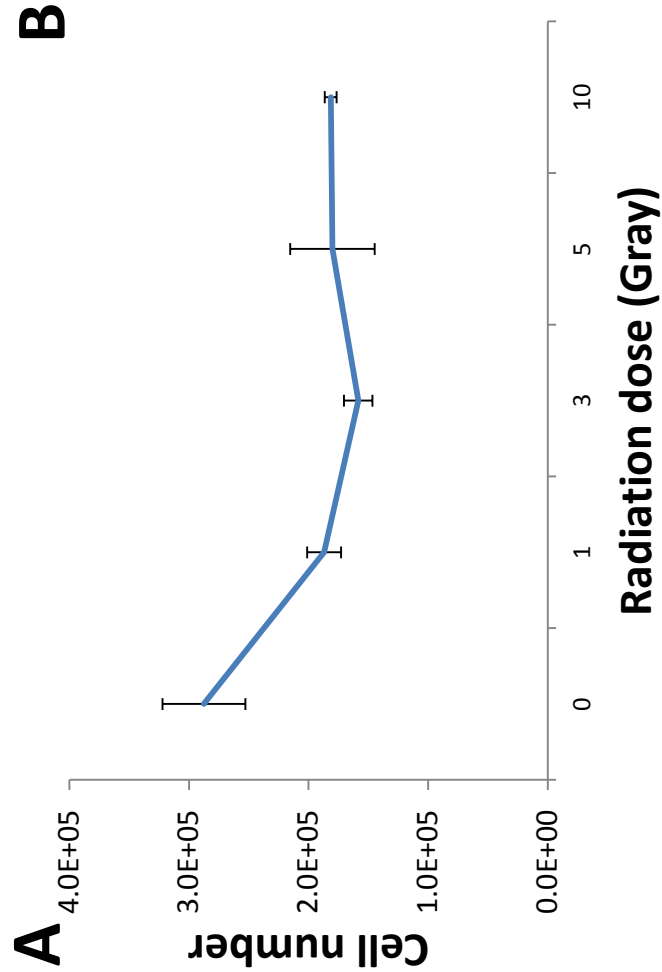
(A) WI-38 human fibroblasts (1×10^5 /ml) were irradiated with 1, 3, 5, and 10 Gy. Cells were cultured for another 48 hours following which cells were counted using the Vi-Cell cell counter using trypan blue exclusion. Statistical significance was determined using one way ANOVA. * indicates $p < 0.05$, $n = 3$.

(B) Cells (1×10^4 /ml) were irradiated as before. Cells were cultured for another 7 days following which cells were fixed and stained for SA β Gal. Data shown is from analysis of five random fields per condition and represented as mean $\pm$ SD. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, $n = 3$.

(C) Cells were treated with radiation as before. Cells were cultured for 48 hrs followed by the collection of cell lysates and detection of p21 by Western Blot analysis. Results shown are a representative of two experiments.

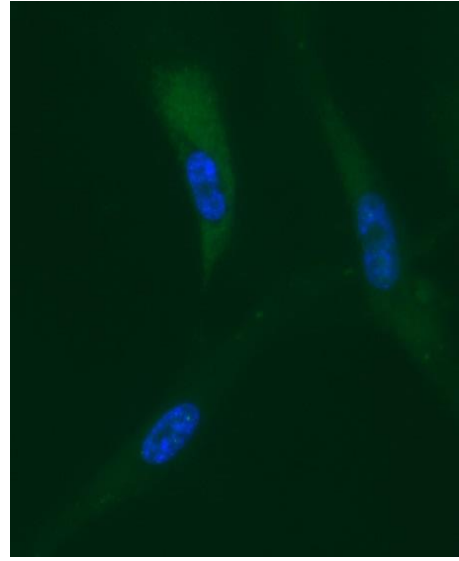
(D) Cells were treated with radiation as before. Cells were cultured for 7 days following which cells were fixed and stained for γ H2AX. Representative images of untreated cells and cells treated at 3 Gy are shown. Results shown are a representative of two experiments.

Fig 22:

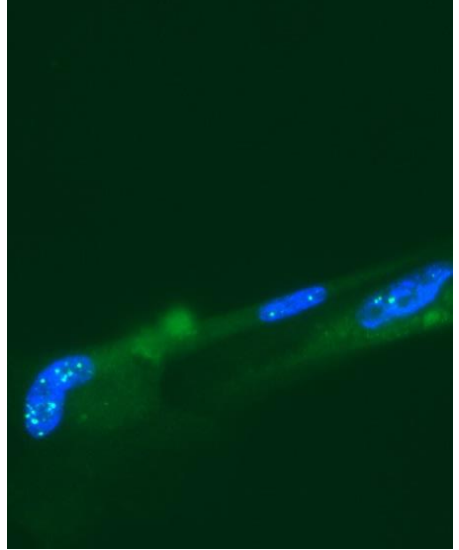


D

YH2AX



0 Gray



3 Gray

1.3.3: The induction of p21 is a non-specific marker of senescence:

The above results show that PriGO8A and PriGO9A cells do not undergo senescence in response to radiation but at the same time exhibit p21 induction. Furthermore, serum also induces p21 expression but causes senescence rather than cell death. Yet ectopic expression of p21 in cells has been shown to induce a senescent state (271). To clarify the role of p21 in senescence induction in PriGO cells, I next determined whether overexpression of p21 in PriGO cells would cause senescence. To elucidate the effect of p21 on PriGO8A cells, a lentiviral system with constitutive expression of p21 was transduced into PriGO8A cells. Increased expression of p21 led to a modest but significant decrease in cell number (**Fig 23 A**) and likewise a 20% increase in Sa β Gal positivity, much less than the effect of serum (50%) (**Fig 23 B**). PriGO9A cells demonstrated even less of a response to p21 expression than PriGO8A cells (**Fig 23 A and B**). Thus, p21 induction alone may not be sufficient to induce senescence in PriGO cells to appreciable quantity and p21 may be a non-specific marker of cell cycle arrest in PriGO cells.

1.3.4: Radiation does not induce differentiation in PriGO cells:

Given that p21 was seen to be a non-specific marker of cell cycle arrest and PriGO8A and PriGO9A cells did not undergo senescence despite demonstrating an increase in p21 with radiation treatment, it was questioned whether radiation induced other cytostatic processes such as differentiation in these cells. PriGO8A cells irradiated with 1, 3, 5, and 10 Gy did not show an increase in differentiation markers GFAP or TUJ1 compared to untreated cells, indicating they were not differentiating into either the astrocytic or neuronal lineage respectively (**Fig 24**).

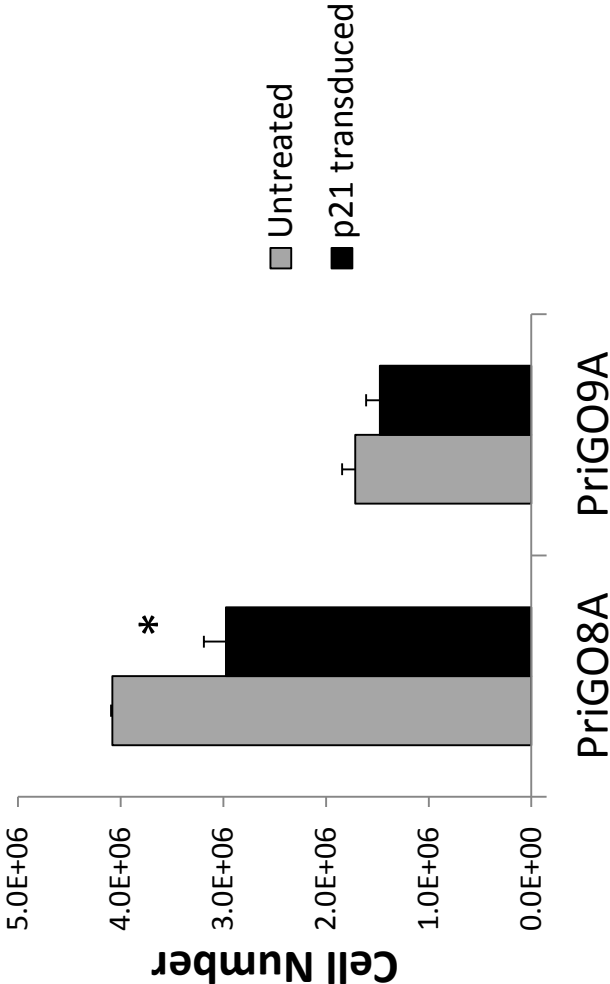
Figure 23. Constitutive expression of p21 induces senescence and inhibits proliferation in PriGO8A and PriGO9A cells.

A. PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were transduced with a lentivirus with constitutive expression of p21. Untreated and treated cells were cultured for 7 days following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are a mean $\pm$ SD. N=3. * indicates $p < 0.05$

(B) PriGO8A and PriGO9A cells were transduced with p21 as above. Cells were cultured for another 7 days following which cells were fixed and stained for SA β gal activity. Data shown are from analysis of five random fields per condition and represented as mean $\pm$ SD. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, N=3.

Fig 23:

A



B

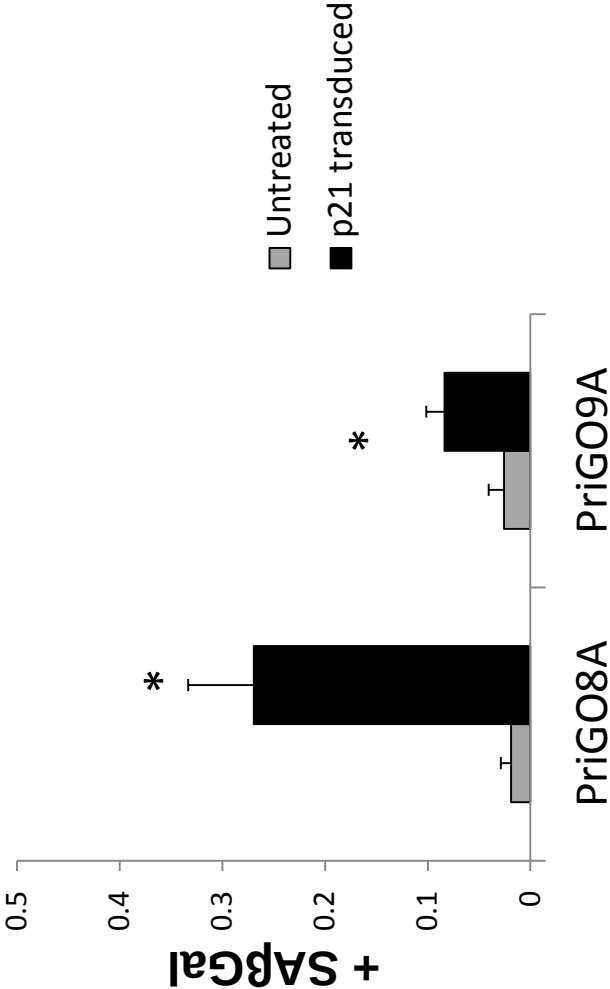
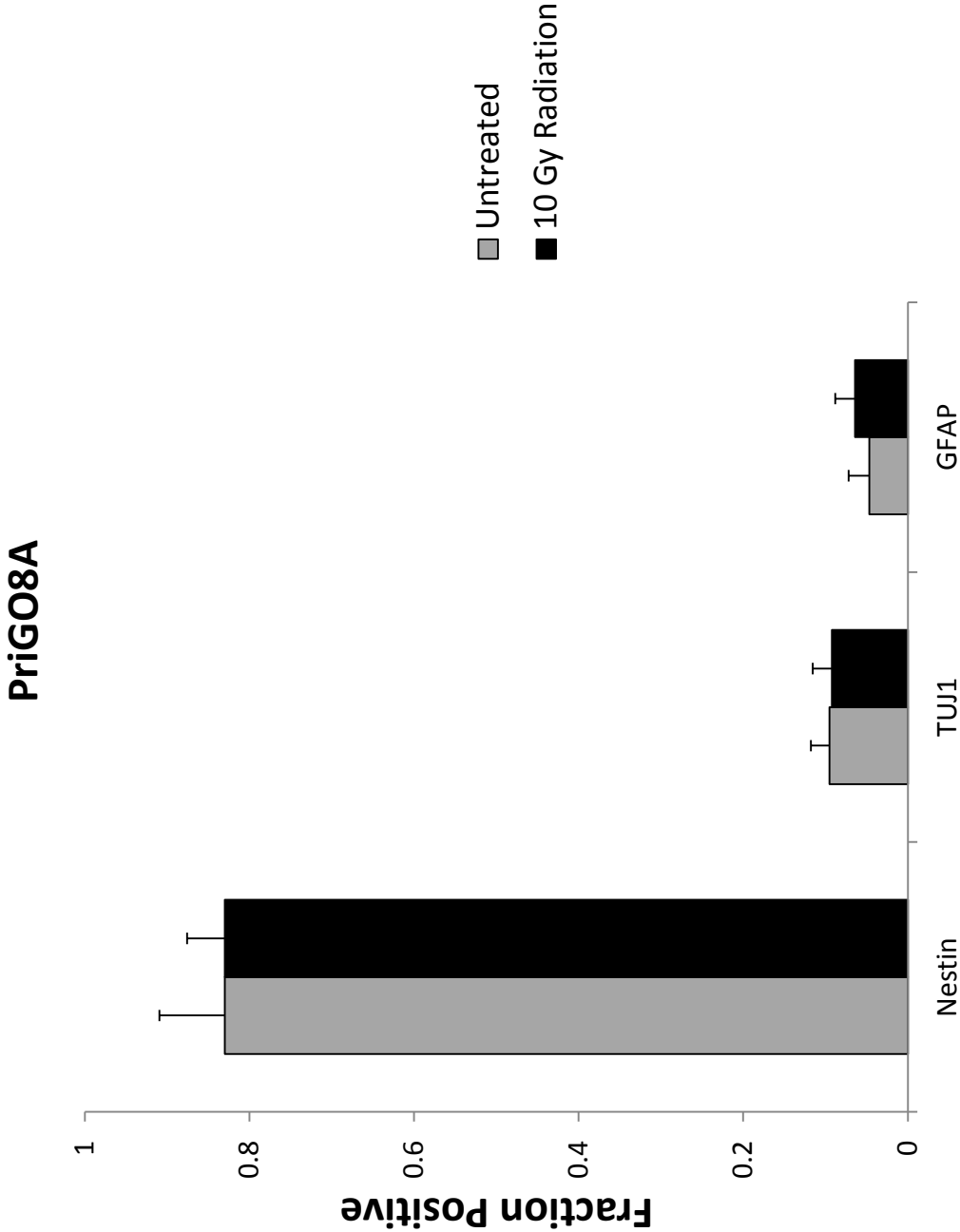


Figure 24. Radiation does not induce differentiation of PriGO8A cells.

PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were irradiated with a dose of 10 Gray (Gy). Cells were cultured for another 7 days following which cells were analyzed for Nestin, TUJ1, and GFAP expression. Data are from analysis of five random fields per condition. Results shown are a mean $\pm$ SD. Statistical significance was determined using one way ANOVA. * indicates $p < 0.05$, $N=3$.

Fig 24:



1.3.5: Differentiation of PriGO cells towards an astrocytic or neuronal lineage does not impact the effect of radiation on senescence and cell death:

PriGO cells have stem cell like features that may protect them from DNA damage (43). Bao *et al.* demonstrated that certain primary glioblastoma cells were better able to repair DNA damage by activating the DDR and inhibiting apoptosis due to their stem cell like properties (43). It was hypothesized that differentiation of PriGO cells may reduce the capability of these cells to repair DNA damage making them more susceptible to ionizing radiation. To test this hypothesis, PriGO8A cells were differentiated with BMP4, an agent known to differentiate PriGO cells into the astrocytic lineage (44). Subsequently, cells were treated with 1, 3, 5, and 10 Gy of radiation and analyzed by cell counts and Sa β Gal activity. Although BMP4 induced a cytostatic effect on its own, it did not lead to a significant difference in cell number upon irradiation at any of the doses (**Fig 25 A**). In addition, BMP4 treatment did not result in an increase in Sa β Gal positivity upon irradiation (**Fig 25 B**).

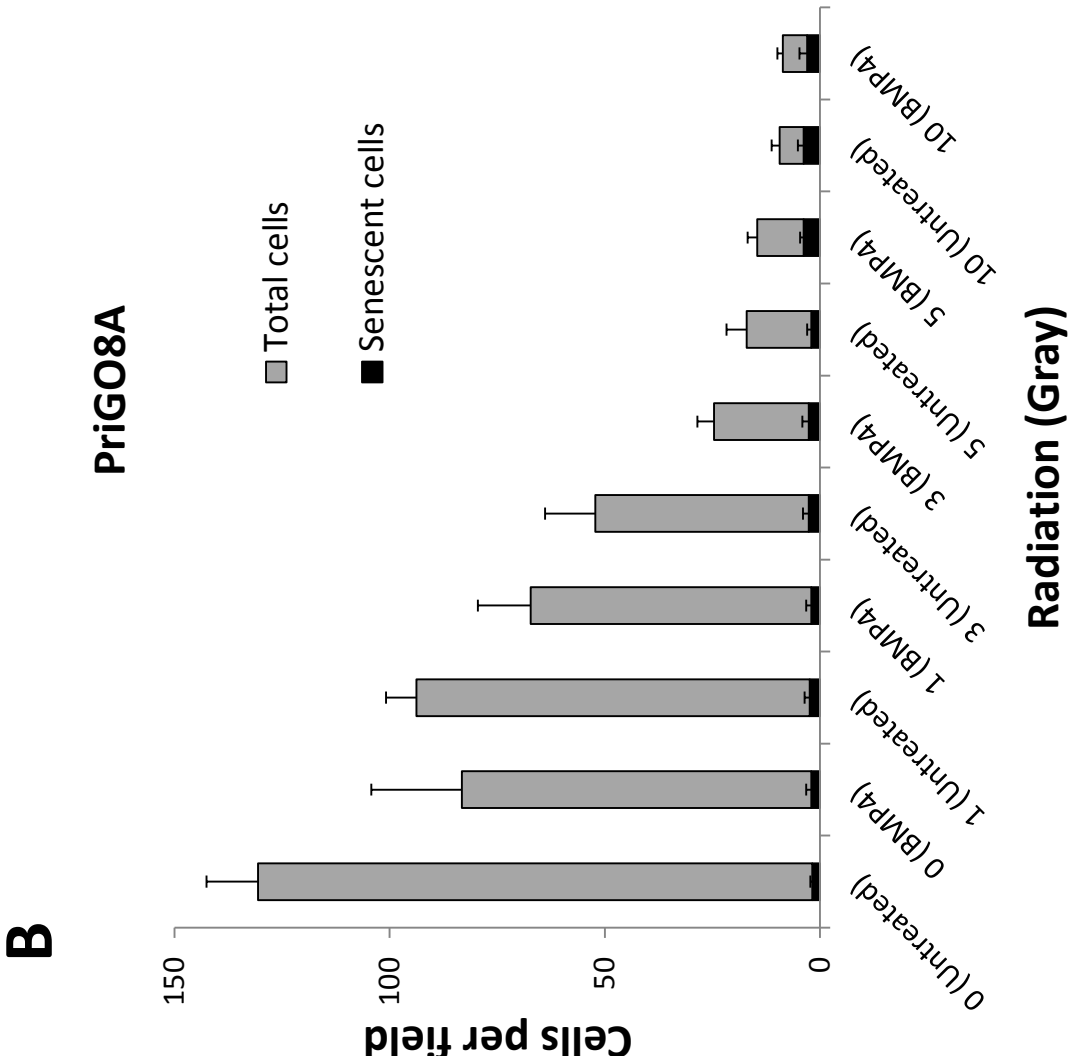
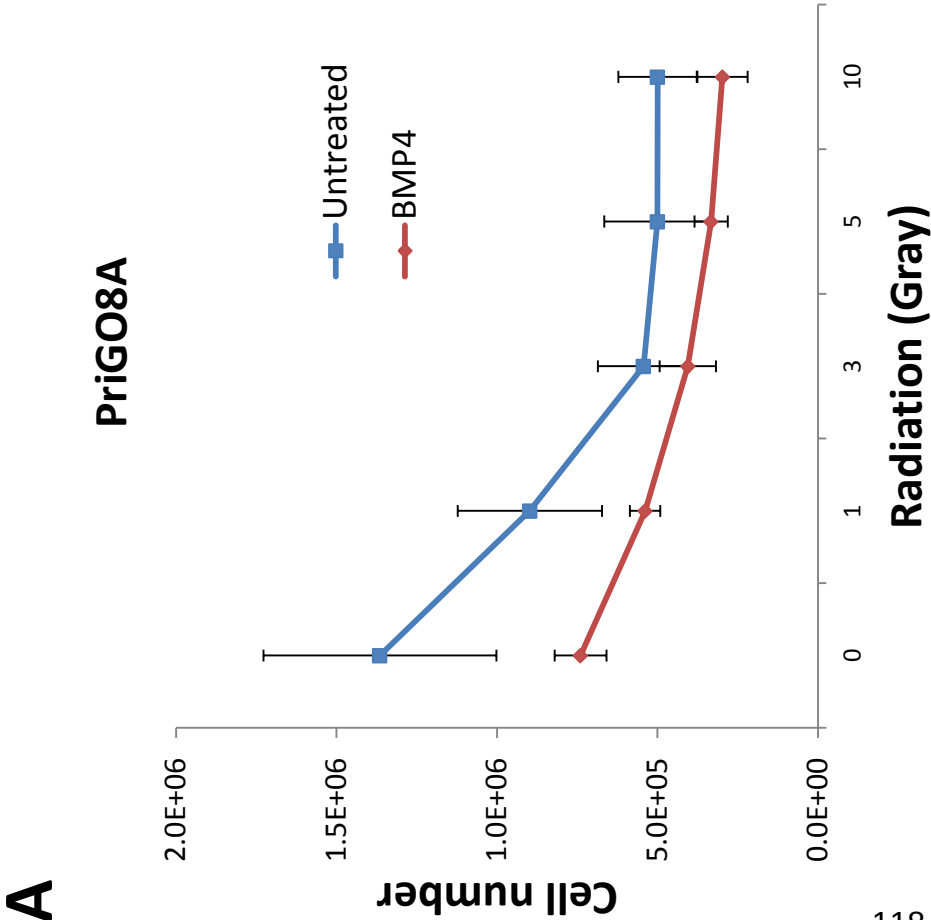
Although differentiation along the astrocytic lineage did not confer increased sensitivity to radiation in PriGO8A cells in terms of senescence or cell death, it was determined if differentiation along a neuronal lineage might have a greater effect. Lgl1 is a protein highly expressed in the mouse brain with known tumor suppressive effects. Gont *et al.* demonstrated that inactivation of Lgl1 by phosphorylation leads to a sustained undifferentiated population of PriGO cells (265). Likewise, abolishing Lgl phosphorylation by converting serine to alanine (Lgl3SA) at three codons (656, 600, 664) led to a significant differentiation of PriGO cells (including PriGO8A) towards a neuronal lineage (258). Using the same doxycycline inducible lentiviral vector for Lgl3SA as in the above mentioned experiments (258), PriGO8A cells were differentiated into the neuronal lineage. Subsequently, cells were irradiated as before and

Figure 25. Figure 13. Differentiation of PriGO8A cells along an astrocytic lineage does not enhance sensitivity to radiation

(A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were treated with BMP4 (100 ng/ml) towards an astrocytic lineage and cultured for 7 days. Cells were then irradiated with either 1, 3, 5 or 10 Gy. Cells were then cultured for another 48 hr following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are a mean $\pm$ SD. N=3.

(B) PriGO8A cells (1×10^4 /ml) were treated with BMP4 (100 ng/ml) as above and cultured for 7 days. Cells were then irradiated with 1, 3, 5 or 10 Gy. Cells were cultured for another 7 days following which cells were fixed and stained for SA β gal activity. Data shown are from analysis of five random fields per condition and represent mean $\pm$ SD. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, N=3.

Fig 25:



analyzed for cell counts and Sa β Gal activity. Differentiated cells towards the neuronal lineage did not demonstrate a decrease in cell number in response to irradiation nor did they show an increase in Sa β Gal positivity compared to undifferentiated cells (**Fig 26 A and B**). Hence, differentiation of PriGO8A cells **towards the astrocytic or neuronal lineage** does not sensitize them towards senescence or cell death following radiation treatment.

Objective 1.4: Determine the effect of inhibitors of ribonucleotide reductase on classical and mesenchymal PriGO subtypes with respect to senescence and cell death.

Classical PriGO cells are susceptible to Triapine (ribonucleotide reductase inhibitor) whereas the mesenchymal PriGO17A cells are resistant with respect to senescence and cell death.

Deoxyribonucleotides are essential in the replication of DNA during cell proliferation (272). Disruption of the availability of deoxyribonucleotides during replication leads to stalling of the replication fork, a process known as replicative stress (273). Replicative stress is another mechanism by which cells can undergo senescence (65). Ribonucleotide reductase (RR) is an enzyme that converts ribonucleotides into deoxyribonucleotides. Certain subtypes of RR such as RRM2 have been found to play an important role in cellular senescence (100). Knockdown of RRM2 in epithelial ovarian cancer cells triggers senescence (274). However, the role of RRM2 in glioblastoma with respect to senescence induction remains unknown.

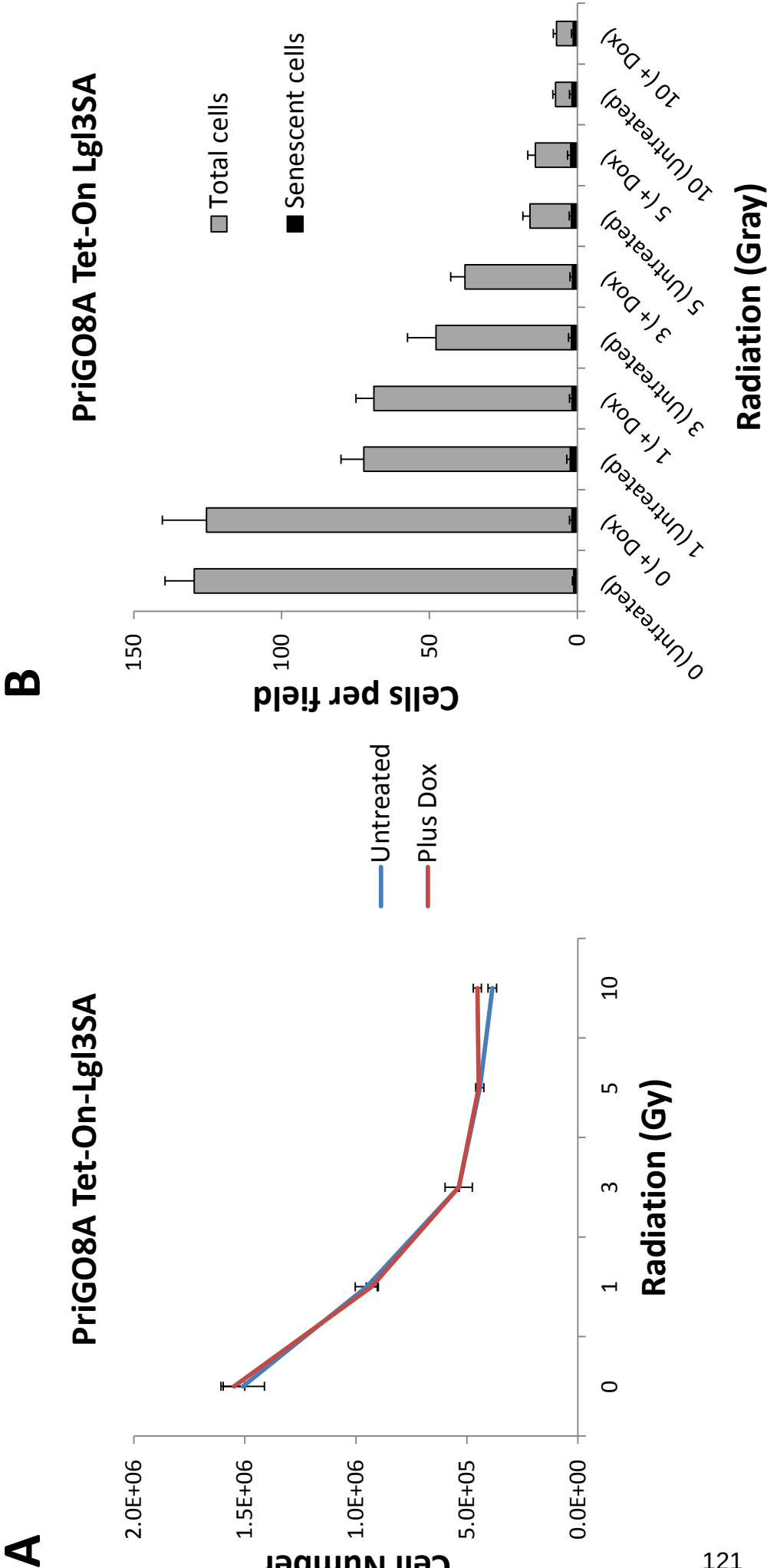
Triapine, an inhibitor of RRM2, is a potent chemotherapeutic shown to cause single strand DNA (ssDNA) damage (275). Given that dsDNA damage by means of ionizing radiation did not induce senescence in PriGO cells, it was questioned whether ssDNA damage via

Figure 26. Differentiation of PriGO8A cells along a neuronal lineage does not enhance sensitivity to radiation.

(A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were transduced with a lentivirus with doxycycline inducible expression of Lgl3SA leading to differentiation towards the neuronal lineage. Cells were cultured for 7 days following which they were radiated with 1, 3, 5 and 10 Gy of X-ray radiation. Cells were cultured for another 48 hrs following which live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are a mean $\pm$ SD. N=3.

(B) PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were transduced with a lentivirus with doxycycline inducible expression of Lgl3SA. Cells were cultured for 7 days following which they were radiated with 1, 3, 5 and 10 Gy. Cells were cultured for another 7 days following which cells were fixed and stained for SA β gal activity. Data shown are from analysis of five random fields per condition and represent mean $\pm$ SD. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, n=3.

Fig 26:



disruption of the replication fork by Triapine through inhibition of RRM2 might induce senescence in PriGO cells.

To determine whether Triapine induces senescence in PriGO cells, PriGO8A cells were treated with Triapine at 2.5, 5, 10, and 20 μ M and Sa β Gal was performed 7 days later. Similar to radiation treatment, Triapine treatment led to an increase in the percentage of Sa β Gal positive cells but not in the number of Sa β Gal positive cells (**Fig 27 A**). While PriGO9A cells responded similarly to PriGO8A cells (**Fig 27 B**), PriGO17A cells, as before with radiation, did not show an increase in either the percentage or the total number of Sa β Gal positive cells (**Fig 27 C**).

To determine if Triapine caused selection of senescent PriGO cells by inducing cell death, PriGO8A cells were treated with Triapine as before and viability was determined by cell counts 48 hours later. PriGO8A cells responded to Triapine with decreased viability in a dose dependent manner. There was only 30% viability of PriGO8A cells at 20 μ M compared to 90% viability of untreated cells (**Fig 28 A**). In addition, Propidium Iodide (PI) staining demonstrated over 40% positivity at 20 μ M compared to 5% in untreated cells (**Fig 29**). PriGO9A cells demonstrated a significant but less of a decrease in viability to Triapine treatment compared to PriGO8A cells (**Fig 28 B**). PriGO17A cells did not have significantly different viability at any dose of Triapine treatment compared to untreated cells (**Fig 28C**).

As with radiation treatment of PriGO8A and PriGO9A cells, Triapine selected for senescent cells rather than inducing senescence. Thus, ssDNA damage does not cause senescence in PriGO cells. Likewise, ssDNA damage is unlikely to be the mechanism of action of serum induced senescence in PriGO cells. In addition, the mesenchymal subtype of PriGO cells (PriGO17A) are not affected by ribonucleotide reductase inhibitors whereas the classical subtype (PriGO8A and PriGO9A) undergo cell death. Alongside being radioresistant, the mesenchymal

Figure 27. Triapine does not induce senescence in PriGO cells.

(A) PriGO8A, (B) PriGO9A, and (C) PriGO17A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with various concentrations of Triapine. Cells were cultured for 7 days following which they were fixed and stained for SA β gal activity. The graphs show total cells (light grey bars) and SA β gal-positive cells (dark black) per field. Data are from analysis of five random fields per condition. Results shown are a mean $\pm$ SD. N=3. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$.

Fig 27:

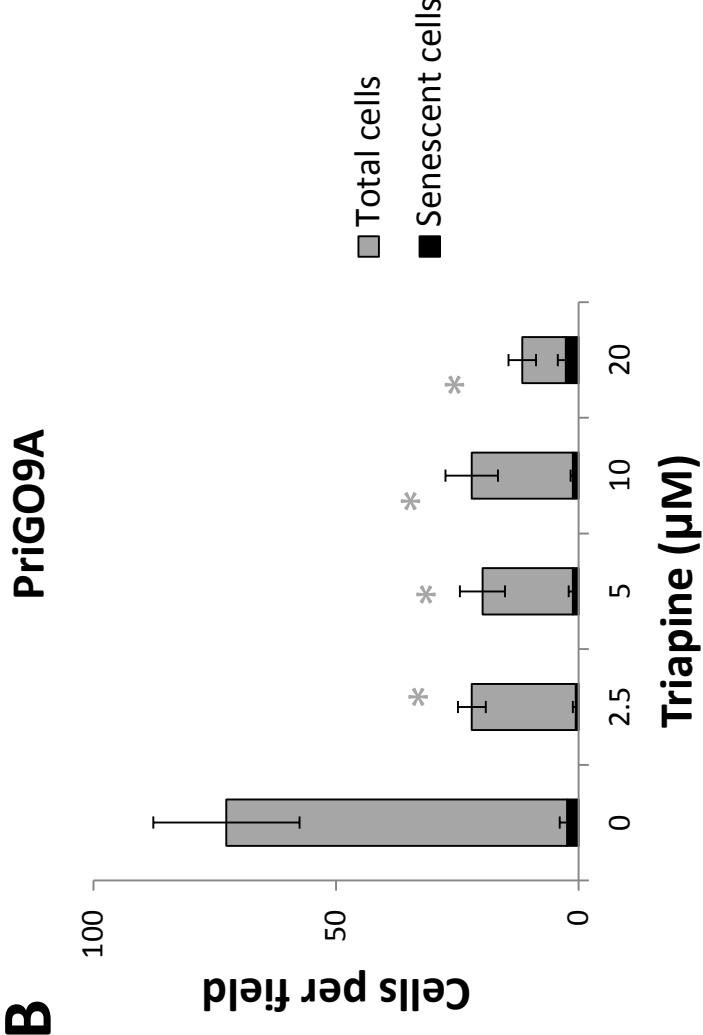
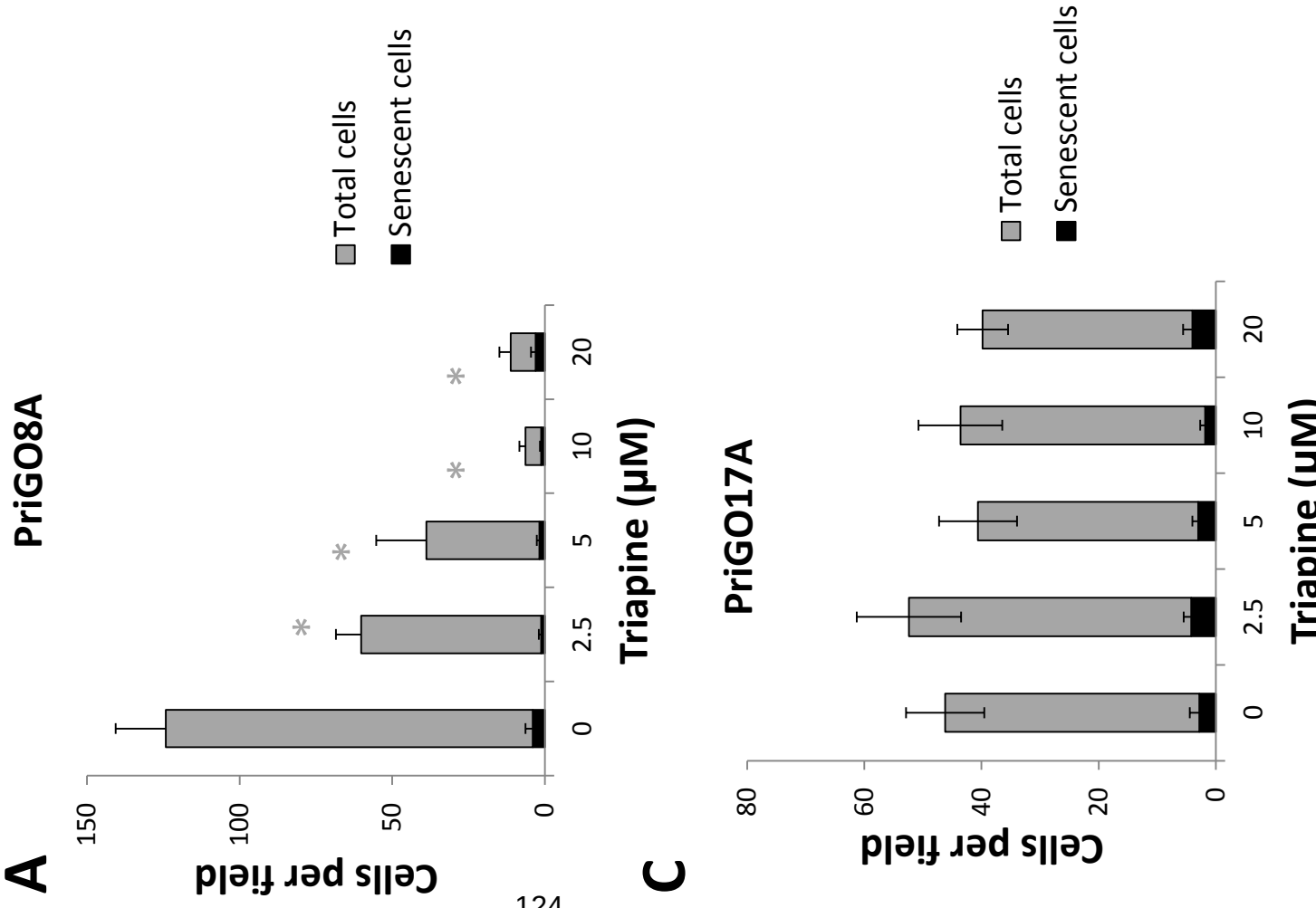
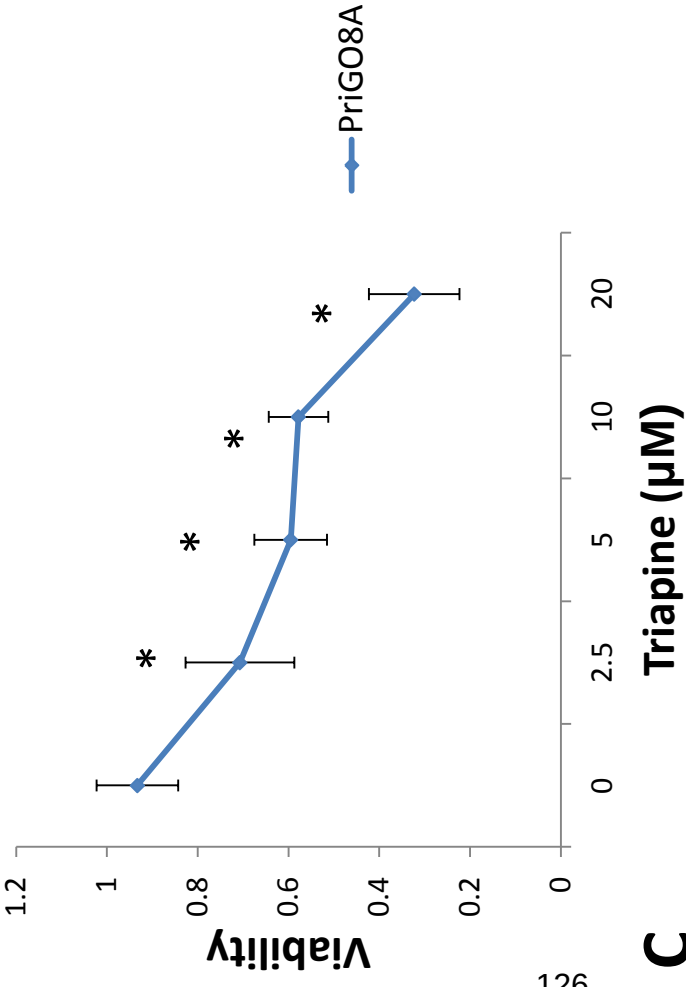


Figure 28. Classical PriGO cells are susceptible to Triapine whereas the mesenchymal PriGO cells are resistant with respect to cell death.

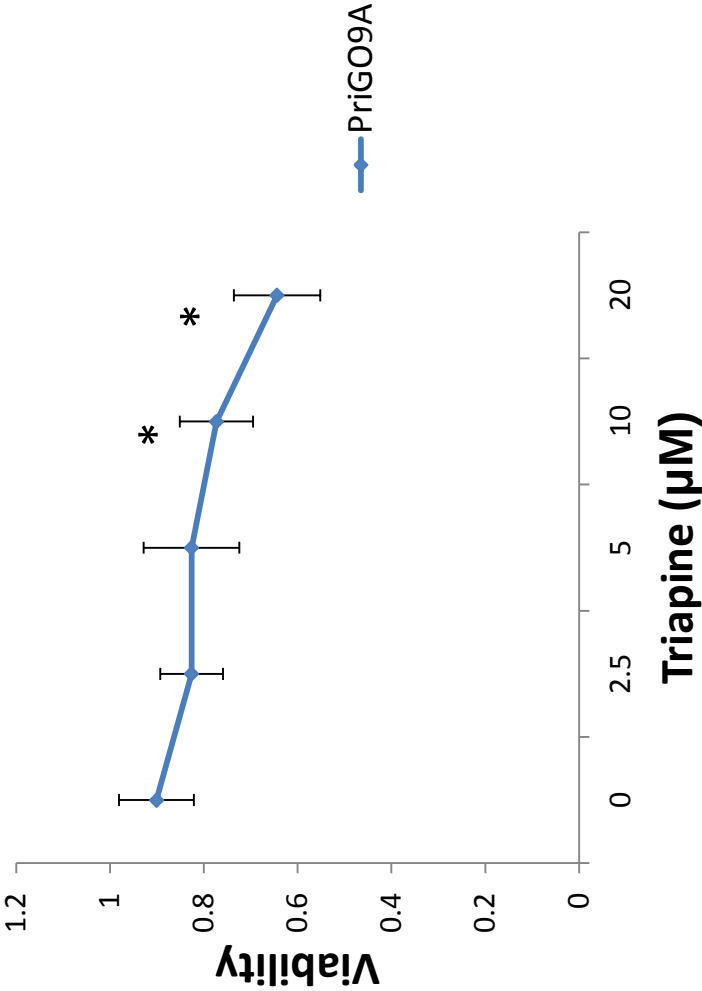
(A) PriGO8A, (B) PriGO9A and (C) PriGO17A cells ($1 \times 10^5/\text{ml}$) were cultured for 24 hr following which they were treated with 2.5, 5, 10, and 20 μM of Triapine. After 48 hrs, live cells were counted using the Vi-Cell cell counter by trypan blue exclusion. Results shown are a mean $\pm$ SD. Statistical significance was determined using one way ANOVA. * indicates $p < 0.05$, $N=4$.

Fig 28:

A



B



C

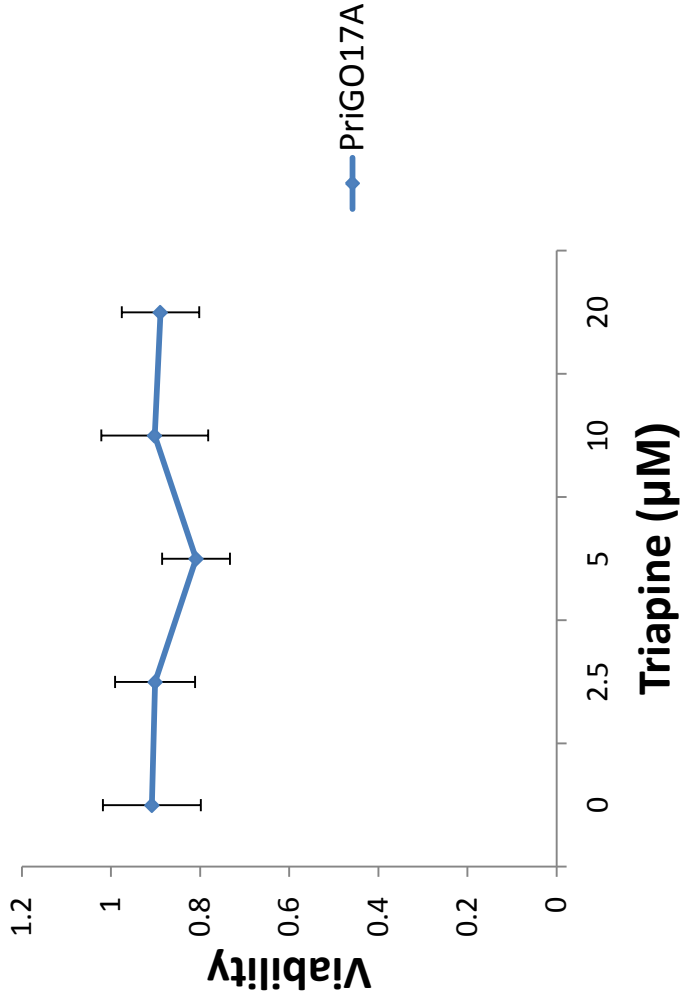


Figure 29. Classical PriGO cells undergo cell death following Triapine treatment.

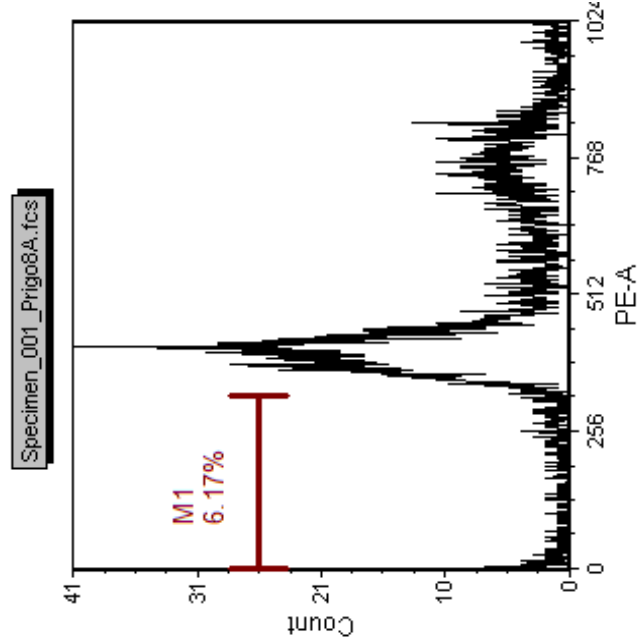
(A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hr following which they were treated with Triapine ($20 \mu\text{M}$) for 48 hrs. Cells were then stained with Propidium Iodide and analyzed for cell death by flow cytometry. Results shown are a representative of three experiments.

(B) Quantitation of PI staining. Results shown are a mean $\pm$ SD. Statistical significance was determined using two tailed t-test. * indicates $p < 0.05$, $N = 3$.

Fig 29:

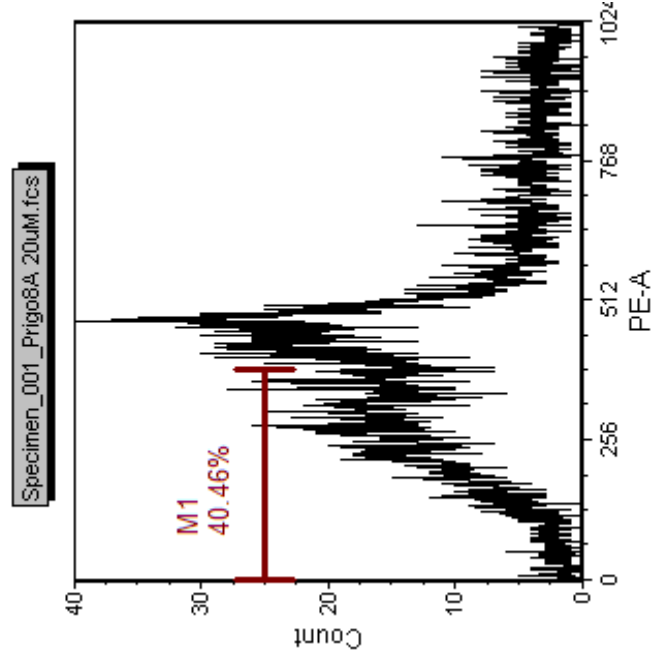
A

PriGO8A



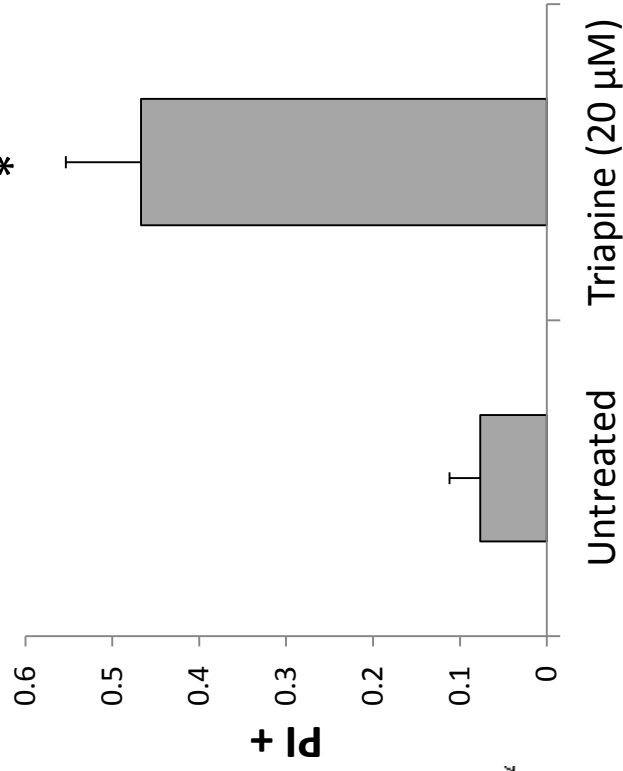
Untreated

B



Triapine 20 μ M

PriGO8A



subtype PriGO17A cells also appear to be chemoresistant. Finally, although the classical subtype is sensitive to Triapine in terms of cell death, the degree of sensitivity varies between cell lines.

Overall, I demonstrate for the first time that the classical subtype of primary glioblastoma cells are sensitive to senescence and apoptosis inducing stress agents, in contrast to the mesenchymal subtype PriGO17A cells.

Objective 2: To determine the molecular mechanism governing serum-induced senescence in classical PriGO cells.

Objective 2.1: Determination of serum induced gene expression changes in classical PriGO8A cells.

Focusing on the potential therapeutic value of senescence and that serum induces senescence in a subset of PriGO cells through unidentified molecular mechanisms, efforts were made to determine the signalling pathways activated by serum and identify the agent in serum responsible for inducing senescence. Lee *et al.* have published a detailed study on the effects of long-term culture in the presence of serum on glioblastoma cells isolated from patients (54). These included: altered morphology; altered growth kinetics; aberrant differentiation; transient loss of telomerase activity, loss of tumorigenic potential, altered gene expression profiles and genomic rearrangements (54). While that study described in detail the long-term consequences of serum exposure, the signalling pathways that drive this response to serum were not assessed. Hence, herein I have studied the short term responses of primary glioblastoma cells to serum by microarray analysis to identify targetable pathways.

2.1.1: Microarray gene expression analysis of primary glioblastoma cell senescence.

Microarray expression analysis was used to compare gene expression profiles between untreated PriGO8A cells and PriGO8A cells exposed to serum for 24 h or seven days. For the day seven data, 406 genes showed changes in gene expression greater than two-fold relative to the untreated control. The top 25 genes induced 7 days after serum treatment are shown in **Fig. 30A**. Pathway analysis with Enrichr and KEGG, or Wikipathways 2015 identified a strong extracellular matrix/focal adhesion/integrin-mediated cell adhesion pathway component in the senescent phenotype. There was no obvious senescence-associated secretory phenotype, with the mRNA expression of cytokines such as IL6 and IL8 being unchanged.

Analysis of expression changes after 24 h of serum exposure showed that the expression of 261 genes was increased by two-fold or more. The top 25 genes induced 24 hours after serum treatment are shown in **Fig. 30B**. Pathway analysis with multiple bioinformatics analysis methods (DAVID Bioinformatics Resources 6.7 and KEGG pathway analysis, Enrichr with Wikipathways 2015 or Biocarta 2015 indicated that TGF β pathway activation was a prominent signalling pathway activated with 24 h exposure to serum. Transcription factor analysis using Enrichr and ChEA31 identified SMAD2, SMAD3 and SMAD4 as candidate transcription factors mediating the changes in these 49 genes, consistent with TGF β pathway activation and transcriptional regulation by the canonical TGF β pathway. Previously, Nogueira *et al.* reported activation of the NF κ B pathway in primary glioblastoma cells induced to differentiate with serum (276). This was not evident in the analysis performed here. Microarray analysis of PriGO8A cells treated with serum for 24 hours also revealed an upregulation of GADD45 β , a gene previously shown to be involved in the premature senescence of mouse embryonic fibroblasts. Therefore the involvement of these two pathways was investigated further.

Figure 30. Gene expression profile in PriGO8A cells following long term (7 days) and short term (2 days) treatment with serum by microarray analysis.

PriGO8A cells were treated without or with 10% serum for either seven days (**A**) or 24 h (**B**). RNA was then isolated and microarray expression analysis performed. The 25 genes showing the largest increases in expression with serum are shown. In (**B**), genes that have previously been shown to be regulated by TGF β are highlighted in grey.

Fig 30:

A.

Gene	Log 2 fold change
GPNNMB	5.5182
SLN	5.1772
HLA-DRA	4.9786
TMOD1	4.254
C1QTNF1	3.8349
GRIK3	3.6852
ALPK2	3.6001
POSTN	3.5403
SLC16A9	3.5347
FAM198B	3.5117
SORL1	3.3329
HLA-DRB1	3.3044
OLFML3	3.2522
MYOF	3.1837
SCG2	3.1197
MCHR1	3.0814
ARID5B	3.0663
LYPD1	3.0157
CEMIP	2.9092
GRIN2B	2.9043
METTL7B	2.9009
LINC00707	2.8636
PGM2L1	2.8554
GLIPR1	2.8135
CAPN3	2.7929

B.

Gene	Log 2 fold change
THBS1	4.6099
SPP1	4.5454
SERPINE1	4.5194
SLN	3.9414
LEPREL1	3.5242
CYR61	3.5132
LYPD1	3.4991
CTGF	3.4199
TFPI2	3.3969
GLIPR1	3.3911
DOK5	3.2741
CLDN1	3.2339
GADD45B	3.2201
ARID5B	3.0895
ANGPT2	2.9674
ADAMTS15	2.9161
ITGA2	2.9015
PGM2L1	2.8554
LINC00707	2.8076
IDI	2.7973
KDR	2.7719
SMAD6	2.7651
CASC10	2.6839
SLC16A9	2.6485
GPC4	2.6105

Objective 2.2: Determination of the role of TGF β in serum-induced signalling in senescence induction in PriGO cells.

Serum-induced senescence in PriGO8A cells is mediated by TGF β :

Based on my microarray analysis as above (**Fig 30B**), I determined whether TGF β is a key agent in serum that induces senescence in PriGO8A cells. TGF β is known to induce the activation of SMAD2 by phosphorylation (277). Serum treated cells demonstrated increased expression of pSMAD2 without any change in total SMAD2 expression suggesting that serum-induced senescence in PriGO8A cells may be mediated by TGF β (**Fig 31A**). TGF β treatment of PriGO8A cells also led to a significant increase in TUJ1 and GFAP, as detected by immunofluorescence, compared to untreated cells (**Fig 31B**) suggesting that TGF β induces differentiation in PriGO8A cells towards the astrocytic and neuronal lineage. Treatment of PriGO8A cells with TGF β (20 ng/ml) led to a significant 50% increase in Sa β Gal positivity compared to untreated cells suggesting the involvement of TGF β in senescence (**Fig 31C**). PriGO8A cells treated with TGF β had significantly increased PML bodies compared to untreated cells (**Fig 31D**). TGF β also caused a decrease in PriGO8A cell proliferation as detected by EDU positivity (**Fig 31 E, F**). These data indicate TGF β reproduces many of the effects of serum in PriGO8A cells.

Pre-treatment of PriGO8A cells with the TGF β receptor inhibitor SB431542 (10 μ M) led to a significant 50% decrease in serum induced Sa β Gal positivity compared to serum only treated cells further confirming that serum induced senescence in PriGO8A cells may be mediated by TGF β (**Fig 32A**). Pre-treatment of PriGO8A cells with SB431542 followed by

Figure 31. TGF β induces differentiation and senescence in PriGO8A cells.

(A). PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs following which they were treated with 10% serum for the indicated period of time. Total cell lysates were then assayed for phosphorylated and total SMAD2 by Western blot analysis. A representative picture is shown

(B). PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with 20 ng/ml TGF β . Seven days later, cells were fixed and stained for immunofluorescence for GFAP and TUJ1 with appropriate antibodies as in Figure 13. The bar graph shows quantification of GFAP+ and TUJ1+ cells.

(C). PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with indicated concentrations of TGF β . Seven days later, cells were assayed for SA β gal activity as in Figure 2.

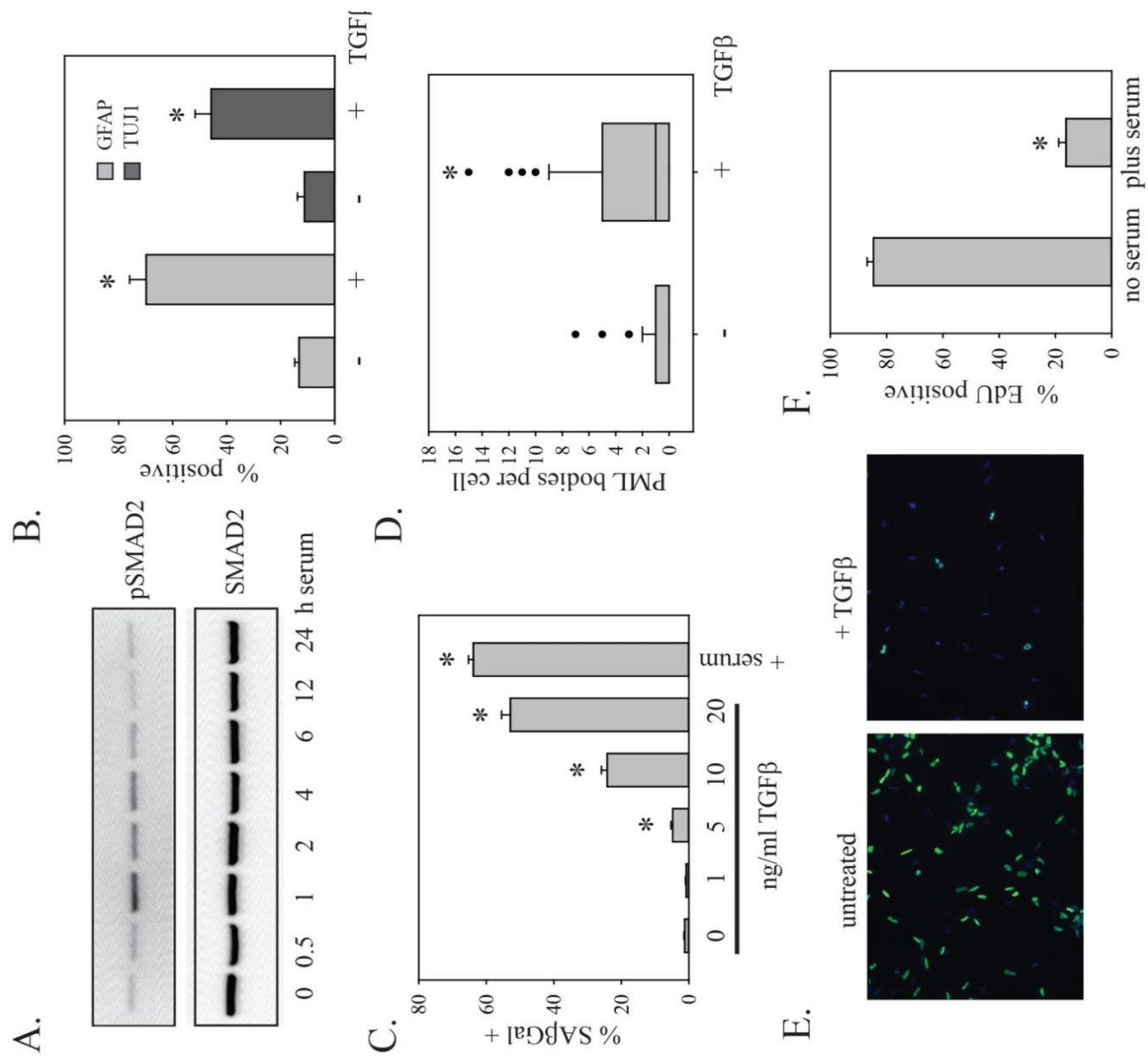
(D). PriGO8A cells were treated with 20 ng/ml of TGF β as in C. Seven days later they were fixed and analysed for PML bodies by immunofluorescence as in Figure 3. Data are from 100 cells per condition. Whiskers show 10th and 90th percentiles.

(E). PriGO8A cells were treated with 20 ng/ml of TGF β as in C. On day 6 they were labelled for 24 hrs with EdU. Cells with EDU incorporation were detected using the Click-IT EdU detection kit from Thermo Fisher. A representative picture of EdU incorporation in the presence and in the absence of TGF β is shown

(F): Quantitation of EDU incorporation, data are from five randomly chosen fields.

For B and C, statistical significance was determined with two-tailed t-tests. For D and F, statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$ in comparison with the no TGF β control data. Results shown are a mean $\pm$ SD. N=3.

Fig 31:



serum treatment also led to a decrease in pSMAD2 level without any concomitant change in total SMAD2 (**Fig 32B**).

Objective 2.3: The role of THBS1 in serum induced senescence in PriGO cells.

2.3.1: Senescence induction in PriGO8A cells: THBS1 is required for TGF β induced senescence in PriGO8A cells

Interestingly, the gene that had the greatest increase in expression in serum treated PriGO8A cells was THBS1 (**Fig 30 B**). In addition to activating latent TGF β , THBS1 is also a TGF β -regulated gene (278). THBS1 levels were measured by ELISA for all four cell lines (**Fig 33**). PriGO8A cells express THBS1 basally and THBS1 levels increased significantly in response to serum. This provides a potential mechanism of THBS1 mediated activation of latent TGF β in serum with a positive feedback mechanism for further activation of latent TGF β .

To show serum induced senescence in PriGO8A cells depends on THBS1 activation of latent TGF β , PriGO8A cells were first pretreated with a peptide inhibitor of THBS1 (LSKL at 50 μ M). A decrease in the level of pSMAD2 in pretreated PriGO8A cells compared to serum only treated cells was observed (**Fig 34A**). There were no changes seen in the total SMAD2 level. Subsequently, treatment with serum in LSKL pretreated cells decreased Sa β Gal positivity significantly by 50% (**Fig 34B**). To further confirm these findings, THBS1 mRNA was knocked down by two siRNAs for THBS1 and the decrease in THBS1 levels were confirmed by ELISA (**Fig 34C**). Sa β Gal analysis of PriGO8A cells with THBS1 knockdown followed by serum treatment demonstrated a significant 70% decrease compared to serum only treated cells (**Fig 34D**). THBS1 knockdown in PriGO8A cells also reduced the level of pSMAD2 in response to

Figure 32. TGF- β mediates serum-induced senescence in PriGO8A cells.

(A) Blockage of TGF- β receptor reduces serum-induced senescence in PriGO8A cells.

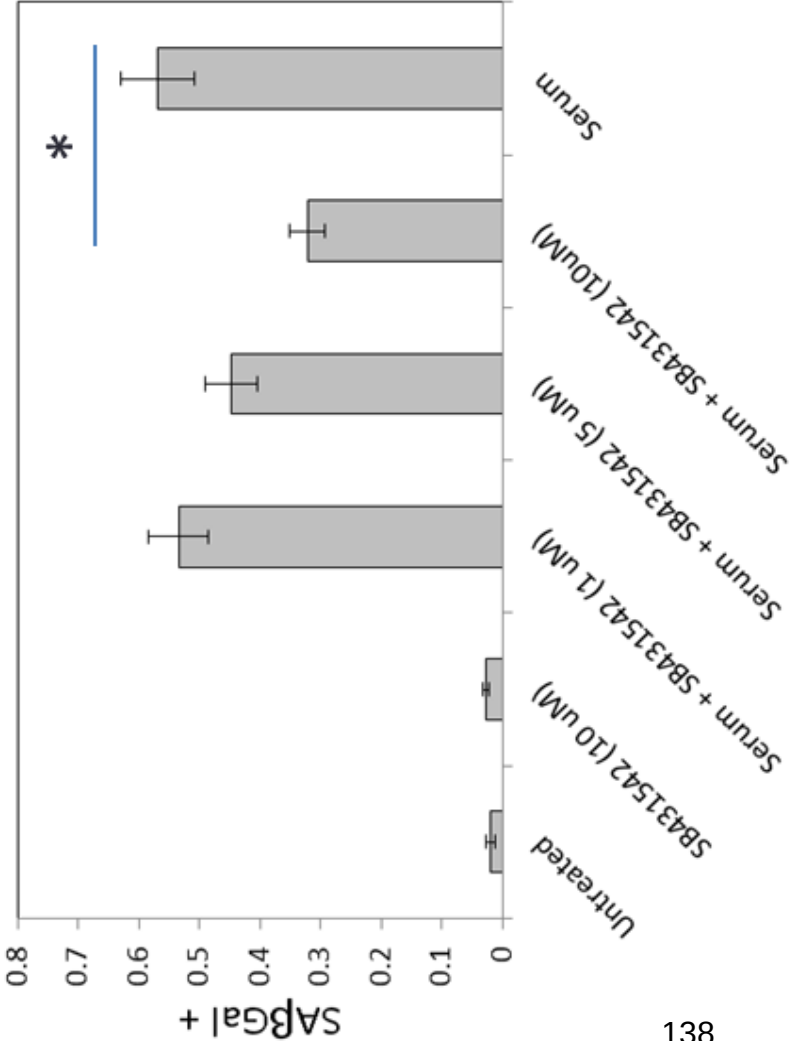
PriGO8A cells were pretreated with the TGF β receptor inhibitor SB431542 (10 μ M) for 24 hours. Cells were then treated with 10% serum for 7 days. Cells were fixed and stained for SA β Gal induction. Data are from analysis of five random fields per condition and represents mean $\pm$ SD. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, $n = 3$.

(B) Blockage of TGF- β receptor reduces serum-induced SMAD-2 phosphorylation in

PriGO8A cells. PriGO8A cells were pretreated with the TGF β receptor inhibitor SB431542 (10 μ M) for 24 hours. Cells were then treated with 10% serum. Cell lysates were collected 1 hr later and analyzed for SMAD2 phosphorylation. The figure shown is a representative of three experiments.

Fig 32:

A



B

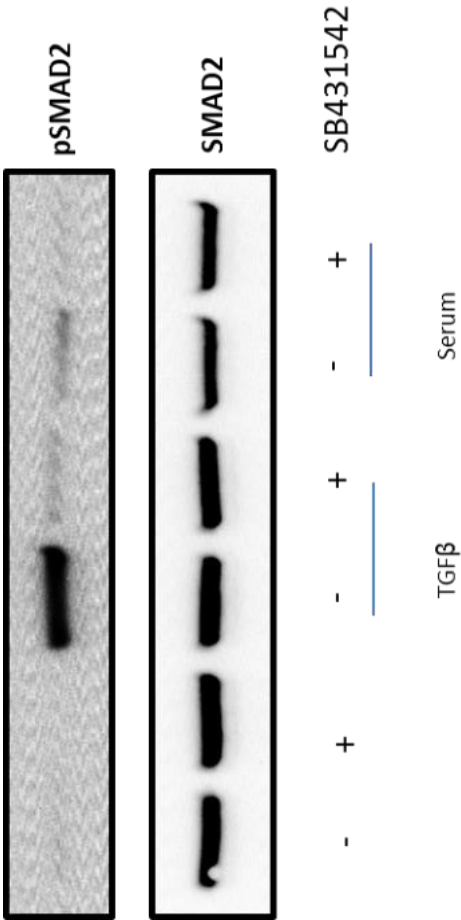


Figure 33. Analysis of THBS1 levels in PriGO cells.

PriGO7A, PriGO8A, PriGO9A, and PriGO17A cells ($1 \times 10^5/\text{ml}$) were cultured for 24 hrs following which they were treated with 10% serum for another 24 h. the supernatants were collected and analyzed for levels of thrombospondin by ELISA. Data shown are mean $\pm$ SD for triplicate analyses for each condition and are corrected for background of either media alone or media plus serum, as well as corrected for cell number at the time of collection of conditioned media. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, $n = 3$.

Fig 33:

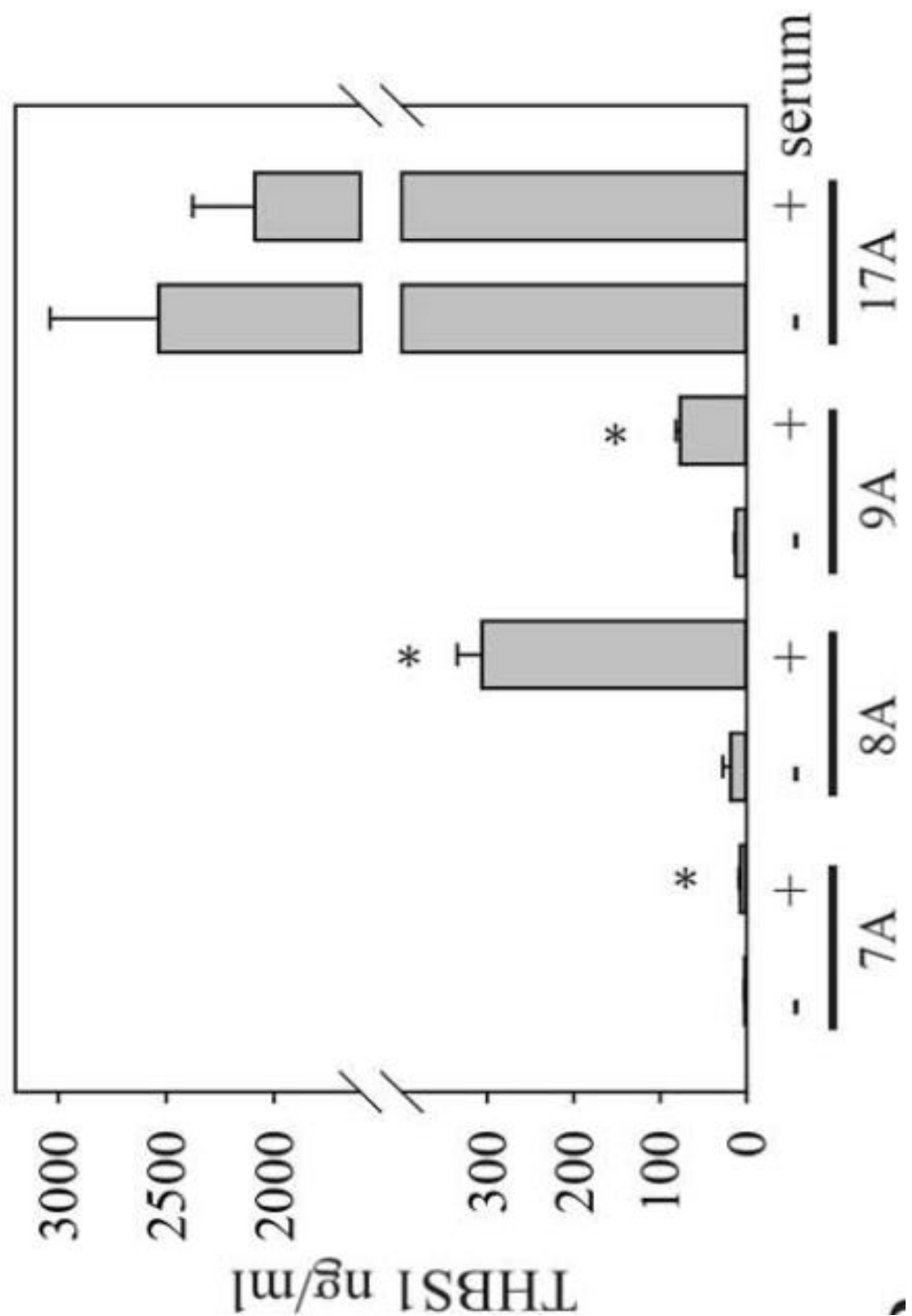


Figure 34. Thrombospondin is required for activation of the TGF β pathway by serum in PriGO8A cells.

(A). PriGO8A cells (1×10^5 /ml) were pretreated for 1 h without or with the thrombospondin inhibitor peptide LSKL as indicated. Cells were then treated with media without or with 10% serum for one hour. Total cell lysates were analyzed for phosphorylated and total SMAD2 levels by Western blotting. Representative results from one of three independent experiments are shown.

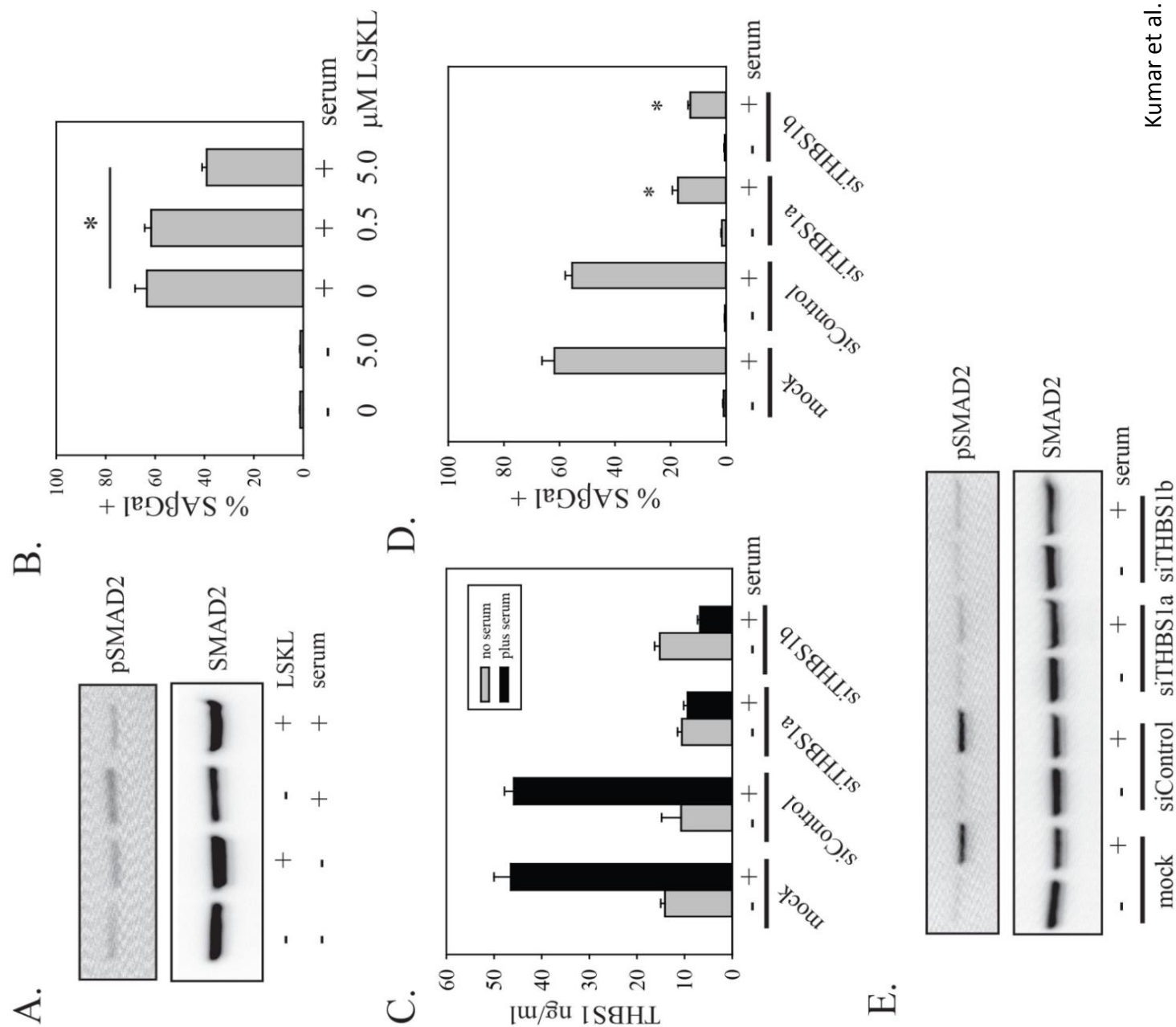
(B). PriGO8A cells (1×10^4 /ml) were treated with or without serum plus the indicated concentrations of LSKL peptide. Media with or without peptide was replaced every two days and SA β gal activity was determined after seven days.

(C). PriGO8A cells (1×10^5 /ml) were mock transfected, or transfected with control siRNA or two different siRNA targeting thrombospondin 1. 48 h after transfection, media was removed and replaced with media either without or with serum. Conditioned media samples were collected after 24 h and assayed for thrombospondin levels by ELISA.

(D). PriGO8A cells (1×10^4 /ml) were transfected or transfected with control siRNA or two different siRNA targeting thrombospondin 1 as in C. Two days later cells were treated without or with serum as indicated. SA β gal assays were performed 7 days after the initiation of serum treatment.

(E). PriGO8A cells (1×10^5 /ml) were transfected as in C. 48 h later media was removed and replaced with fresh media without or with serum. One hour later total cell lysates were collected and analyzed for phosphorylated and total SMAD2 by Western blot. Representative results from one of three independent experiments are shown.

Fig 34:



serum without any changes in total SMAD2 (**Fig 34E**). These results suggest that serum-induced senescence is mediated by TGF β and THBS1 is required for TGF- β -induced senescence in PriGO8A cells.

2.3.2: Senescence induction in PriGO7A cells: Unresponsiveness of PriGO7A cells to serum induced senescence is due to its inability to upregulate THBS1 and to activate latent TGF β :

Serum-induced senescence was not observed in PriGO7A cells (**Fig 35A**). To confirm the role of THBS1 in serum induced senescence, the effect of THBS1 activation of latent TGF β in serum was assessed in the other PriGO cell lines. THBS1 was minimally expressed in PriGO7A cells, and although serum caused a significant increase in THBS1, the levels detected were minimal (**Fig 33**). The inability of serum to induce senescence in PriGO7A cells (**Fig 35A**) might be due to a lack of its ability to significantly upregulate THBS1 expression to a level required to activate latent TGF β . This was confirmed by treating cells with active TGF β . The treatment of PriGO7A cells with active TGF β bypassed the need for activation of latent TGF β by serum and resulted in significantly higher levels of Sa β Gal positivity compared to untreated cells (**Fig 35B**).

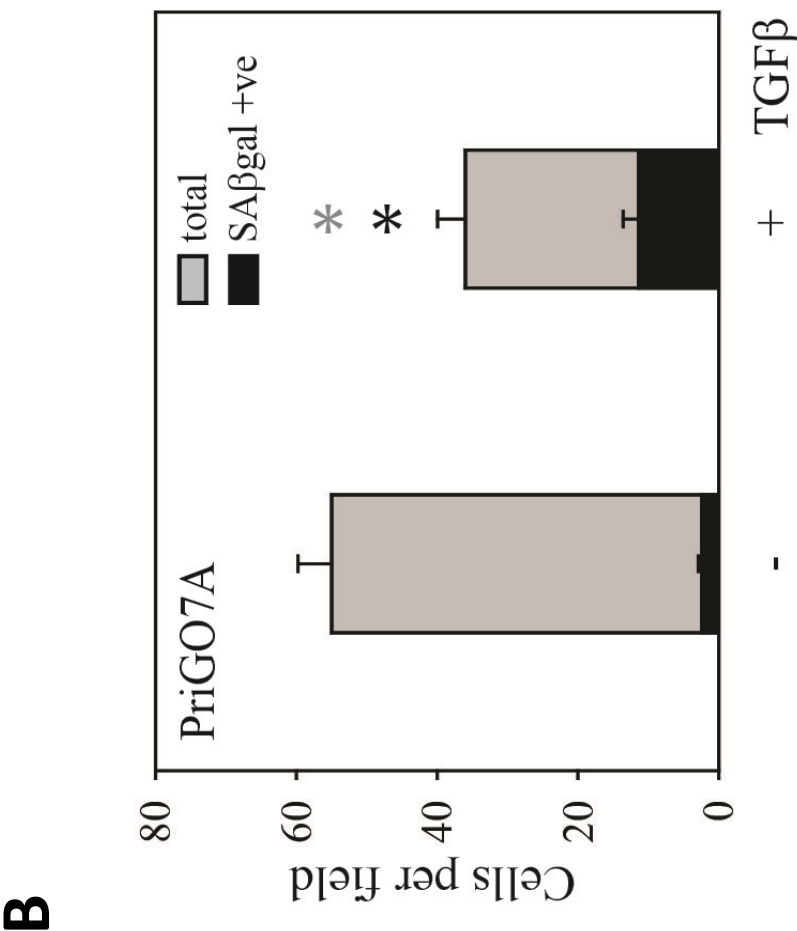
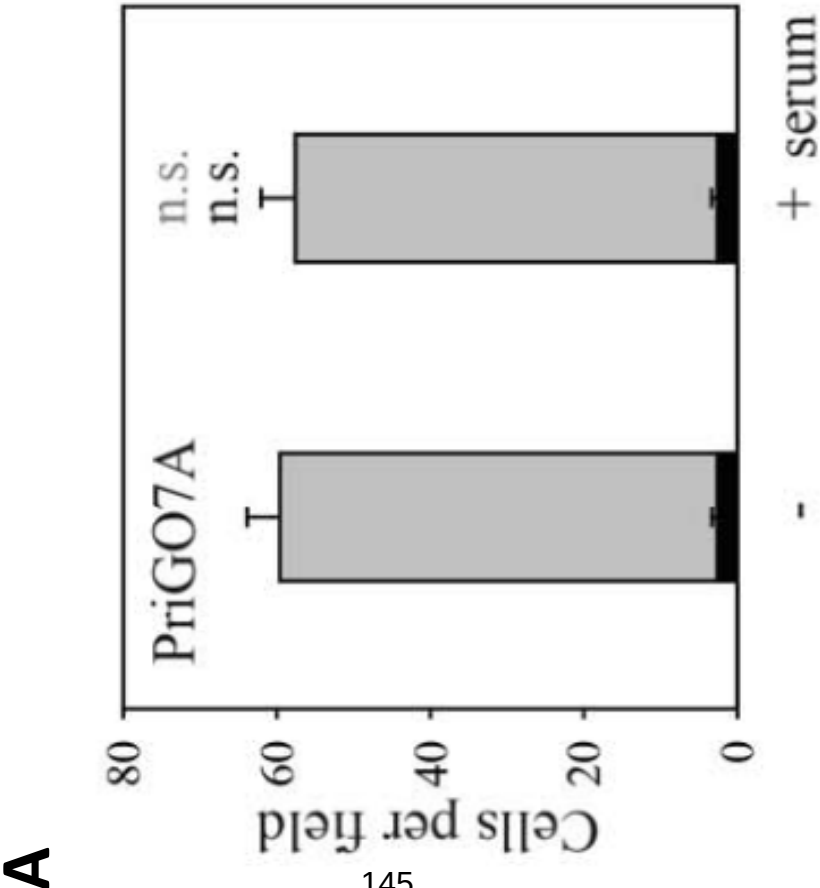
2.3.3: Senescence induction in PriGO17A cells: Unresponsiveness of PriGO17A cells to serum and TGF β induced senescence is due to the interaction of the Ras pathway with TGF β signalling.

PriGO17A cells were found to be unresponsive to serum induced senescence (**Fig 8 A, B, C**). Serum also did not cause proliferation of PriGO17A cells as determined by increase in cell number (**Fig 8 B**) and cell proliferation as determined by EdU incorporation (**Fig 9 A, B**). I next

Figure 35. PriGO7A cells undergo senescence in response to TGF β but fail to induce senescence in response to serum.

A: PriGO7A cells (1×10^4 /ml) were cultured for 24 hrs following which they were treated with either 10% serum (A) or with 20 ng/ml of TGF β (B). Seven days later, cells were assayed for SA β Gal activity as in Figure 2. Data shown are from analysis of five random fields per condition and represented as mean $\pm$ SD. N=3. Statistical significance was determined using two-tailed t-tests. * indicates $p < 0.05$ and n.s. indicates not significant.

Fig 35:



determined the molecular mechanism responsible for the unresponsiveness of PriGO17A cells to serum induced senescence. I found that in contrast to PriGO7A and PriGO8A, PriGO17A cells produced a large amount of THBS1 basally without any significant increase in response to serum (**Fig 33**). The mRNA expression data of THBS1 in The Cancer Genome Atlas (TCGA) correlates with significantly higher THBS1 amounts in the mesenchymal subtype compared to the other subtypes in glioblastoma, consistent with my findings (**Fig 36A**). High THBS1 levels may be driven by Ras pathway overactivity as THBS1 mRNA positively correlated with phosphorylated MEK in the TCGA database (S217) ($p=1.4 \times 10^{-3}$). Ras pathway overactivity may be caused by a high frequency of NF1 mutations (a negative regulator of Ras signalling) in the mesenchymal subtype (13). I hypothesized that high THBS1 levels were being driven by the Ras pathway. Treatment of PriGO17A cells with the MEK inhibitor U0126 (20 μ M) resulted in a significant decrease in THBS1 levels as measured by ELISA (**Fig 36B**), indicating the high THBS1 levels were indeed driven by the Ras pathway.

Ras pathway overactivity also repressed TGF β pathway activation in PriGO17A cells. TGF β did not induce senescence in PriGO17A cells (**Fig 37A**). Cells pretreated with U0126 with subsequent treatment with TGF β showed significantly higher Sa β Gal positivity compared to cells treated with TGF β alone (**Fig 37A**). In addition, TGF β treatment led to increased pSMAD2 levels in cells pretreated with U0126 compared to TGF β treated cells alone (**Fig 37B**). In contrast, U0126 treatment did not have an effect on the ability of PriGO8A cells to undergo TGF β mediated senescence or pSMAD2 induction (**Fig 37 C, D**). These results suggest that unresponsiveness of PriGO17A cells to serum and TGF β induced senescence is due to the hyperactivity of the Ras pathway and high basal levels of THBS1, features characteristic of the mesenchymal subtype.

Figure 36 A. Thrombospondin-1 levels are significantly increased in mesenchymal subtype of PriGO cells

TCGA database analysis of thrombospondin 1 mRNA expression was performed using cBioportal and the TCGA glioblastoma 2013 database. Thrombospondin 1 mRNA expression data are plotted with respect to molecular subtype. Statistical significance was determined using the All Pairwise Multiple Comparison Procedures (Dunn's Method). * indicates $p < 0.05$.

Figure 36 B. THBS1 expression is regulated by the Ras pathway in PriGO17A cells.

Inhibition of Ras pathway inhibits THBS-1 levels in PriGO17A cells. PriGO17A cells (1×10^5 /ml) were cultured for 24 hrs following which the media was changed and cells were treated with the inhibitor, 50 μ M of U0126, for 24 h. Supernatant was collected and assayed for levels of thrombospondin 1 by ELISA. Statistical significance was determined using the Mann-Whitney Rank Sum test. * indicates $p < 0.05$, N=3.

Fig 36:

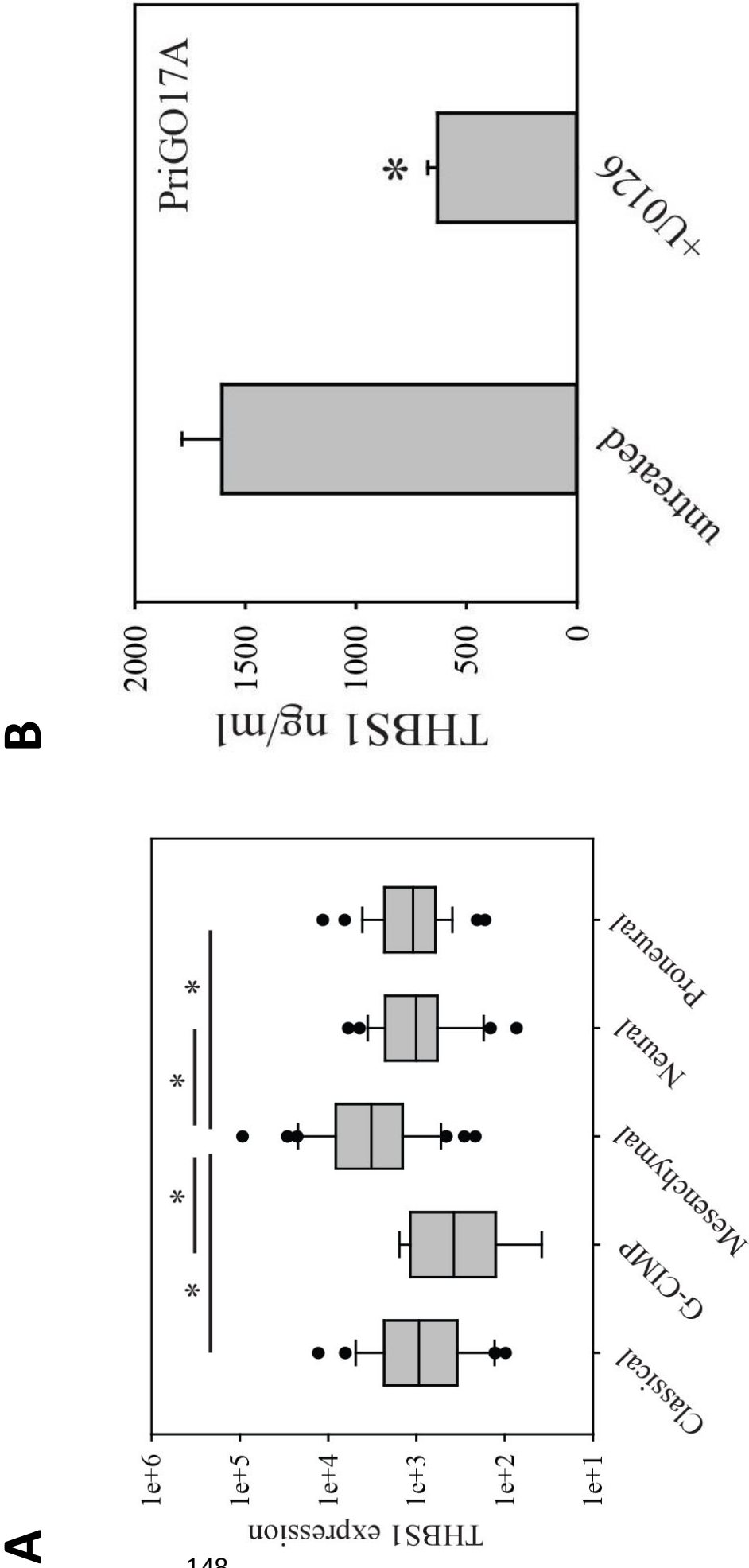


Figure 37: The Ras pathway overactivity inhibits TGF β signalling in PriGO17A cells.

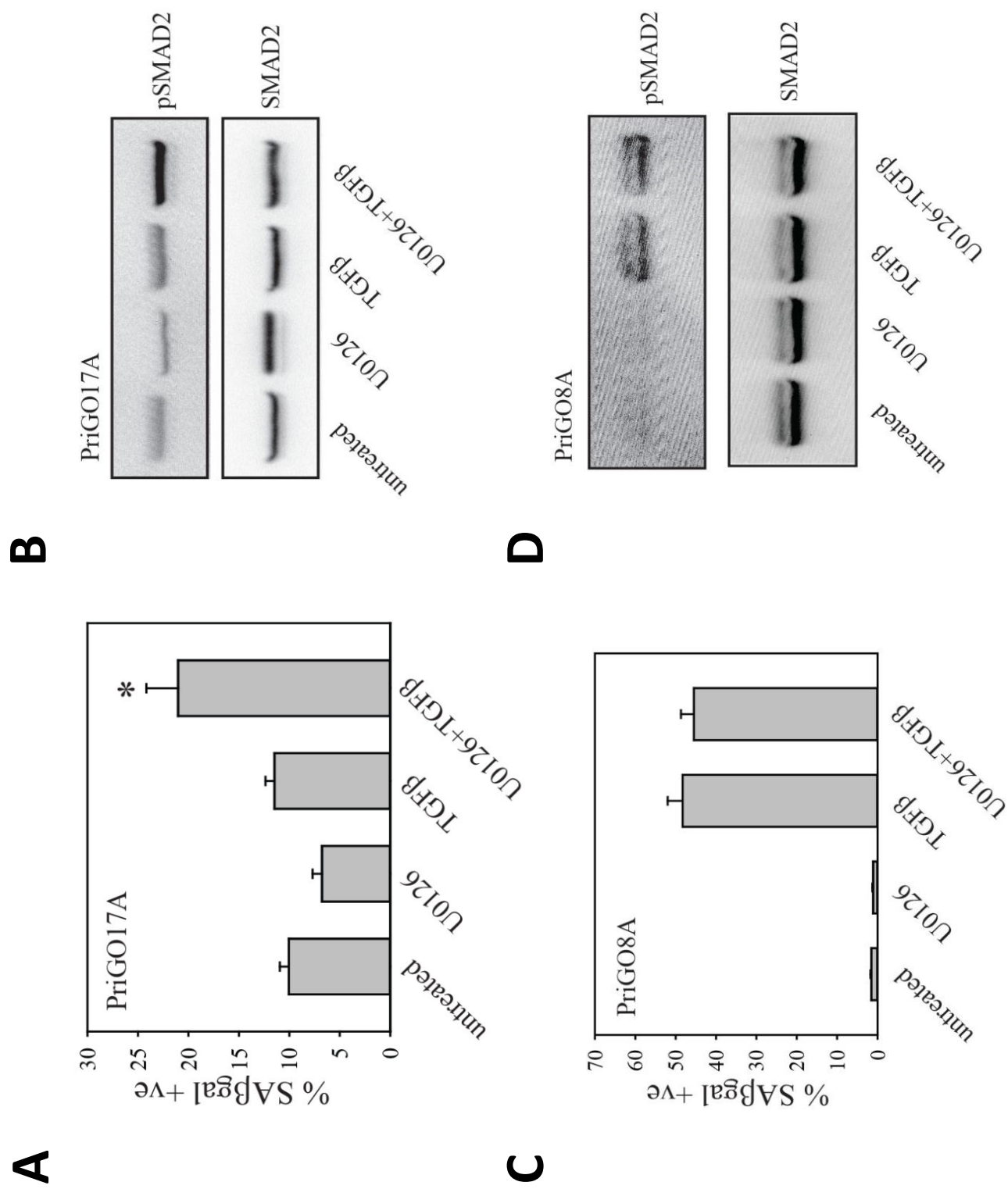
(A): PriGO17A cells were pretreated for 24 h with 50 μ M U0126 as indicated. Cells were then treated with 20 ng/ml TGF β . Seven days later, cells were fixed and assayed for SA β Gal activity.

(B): PriGO17A cells were treated with U0126 and TGF β as in A and total cell lysates were collected 1 h later. Levels of phosphorylated and total SMAD2 were analyzed by Western blotting.

(C): PriGO8A cells were treated and analyzed for SA β Gal activity as in A.

(D): PriGO8A cells were treated and analyzed for SMAD2 phosphorylation as in B. Error bars show the standard error. Statistical significance was determined using All Pairwise Multiple Comparison Procedures (Holm-Sidak method). * indicates a $p \leq 0.05$ compared to all other groups.

Fig 37:



In conclusion, serum activates the TGF β pathway to induce senescence in PriGO cells. However, the ability of TGF β to do so depends not only on its conversion from its latent to active form by THBS1 but also by Ras pathway activity.

Objective 2.4: Determine the role of GADD45B in serum induced senescence in PriGO cells.

GADD45B is not involved in serum induced senescence in PriGO cells.

As per the microarray analysis, another highly expressed gene in response to serum treatment of PriGO8A cells was GADD45B (**Fig 30**). GADD45 family of proteins are known to play a diverse role in the cellular stress response, including promoting survival or inducing apoptosis (35). This depends on the nature and intensity of the stimulus as well as the specific cell type. GADD45B has also been shown to be involved in senescence (266). It was found to inhibit premature senescence in primary mouse embryonic fibroblasts induced by oxidative stress (266). However, the role of GADD45B in PriGO cells in terms of senescence induction is unknown and was investigated in this study.

Oxidative stress, through cellular metabolism or agents such as TGF β , is known to damage DNA and induce senescence in a variety of cell types (59). Although serum did not demonstrate a traditional DNA damage response through dsDNA breaks, oxidative stress induced DNA damage through serum mediated TGF β signalling in PriGO8A cells may still occur and cause an increase in GADD45B levels as a compensatory mechanism to improve survival. In such context, decreasing GADD45B in the presence of oxidative stress may cause an increase in cellular senescence.

To determine the role of GADD45B in serum induced senescence in PriGO8A cells, GADD45B was knocked down with two siRNA duplexes as confirmed with RT-PCR (**Fig 38 A**). SA β Gal analysis of cells with GADD45B knocked down did not show an increase in senescence (**Fig 38 B**). Furthermore, GADD45B knockdown did not result in changes in proliferation as assessed by EdU incorporation nor did it cause an increase in PML body detection (**Fig 39A and B**).

The role of GADD45B was alternatively investigated by overexpressing GADD45B in PriGO8A cells. To test whether upregulation of GADD45B could alter the response of PriGO8A cells to serum, a doxycycline inducible GADD45B lentiviral system was developed. Doxycycline treatment resulted in an increase in GADD45B levels but failed to demonstrate a change in SA β Gal positivity with serum treatment (**Fig 40 A and B**).

GADD45B was not shown to be involved in the senescence response of serum in PriGO8A cells. However, as mentioned before serum has a pleiotropic effect on PriGO cells that includes differentiation. Hence, it was hypothesized that GADD45B may be involved in differentiation of PriGO8A cells in response to serum.

PriGO8A cells were initially treated with two different siRNA for GADD45B followed by serum treatment and evaluation of differentiation markers by immunofluorescence 1 week later. GADD45B knockdown did not affect the ability of serum to differentiate PriGO8A cells along either the astrocytic or neuronal lineage as determined by TUJI and GFAP induction, respectively (**Fig 41 A**).

Similar to GADD45B knockdown experiments, overexpression of GADD45B via doxycycline treatment of PriGO8A tet on GADD45B cells prior to serum treatment also did not alter the cells ability to differentiate into the astrocytic or neuronal lineage (**Fig 41 B**). Hence, the

Figure 38. Knockdown of GADD45 β does not affect serum-induced senescence in PriGO8A cells.

(A) PriGO8A cells (1×10^5 /ml) were cultured for 24 hrs after which cells were mock transfected, or transfected with control siRNA or two different siRNA targeting GADD45 β . After 48 hrs of transfection, cells were treated with 10% serum. Cells were cultured for another 24 hrs following which total RNA was extracted using the QIAGEN RNeasy kit. RNA was transcribed to cDNA and qPCR was performed using primers for GADD45 β . Results shown are a mean $\pm$ SD. N=3.

(B) PriGO8A cells (1×10^4 /ml) were transfected as in A. Two days later cells were treated without or with serum as indicated. SA β Gal assays were performed 7 days after the initiation of serum treatment. Results shown are a mean $\pm$ SD. N=3. Statistical significance was determined using one way ANOVA. n.s. indicates not significant.

Fig 38:

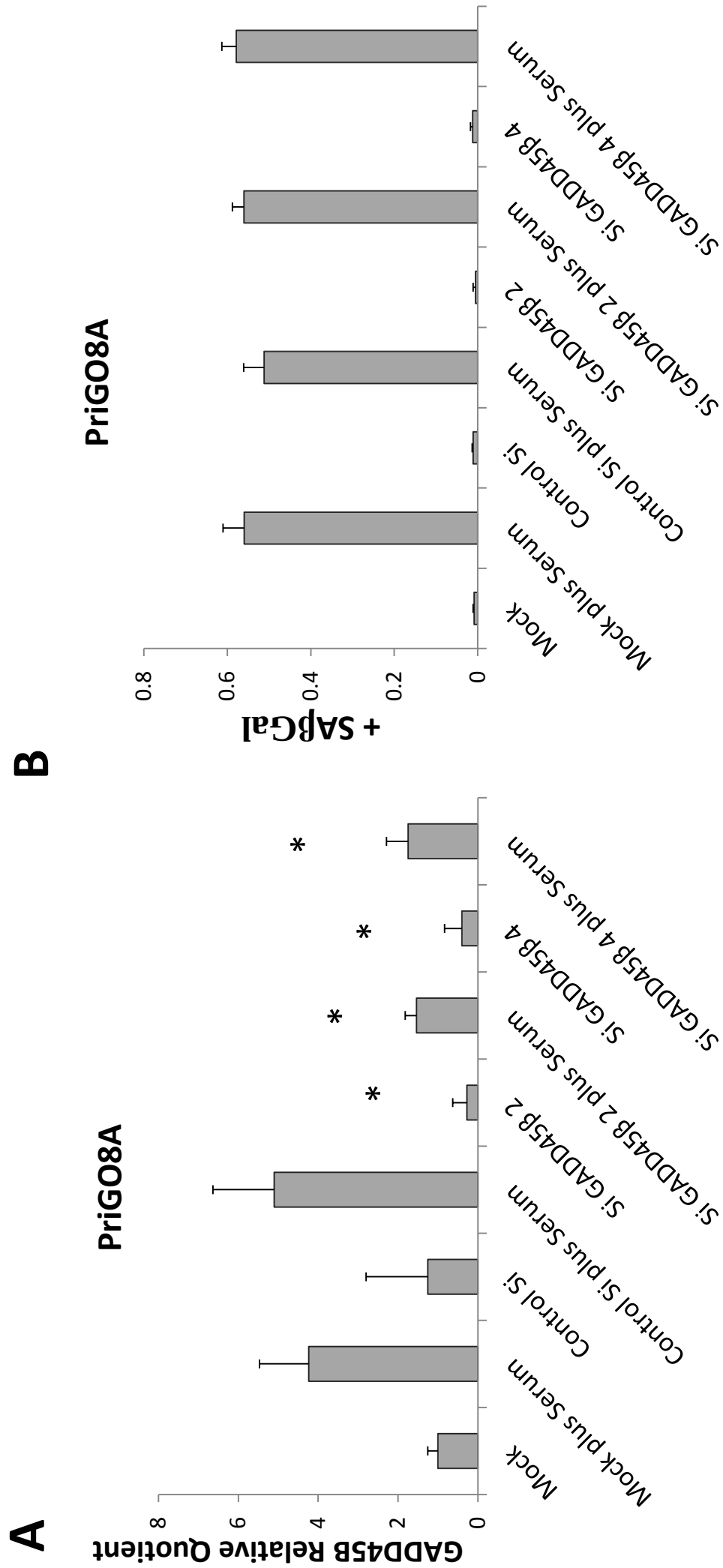


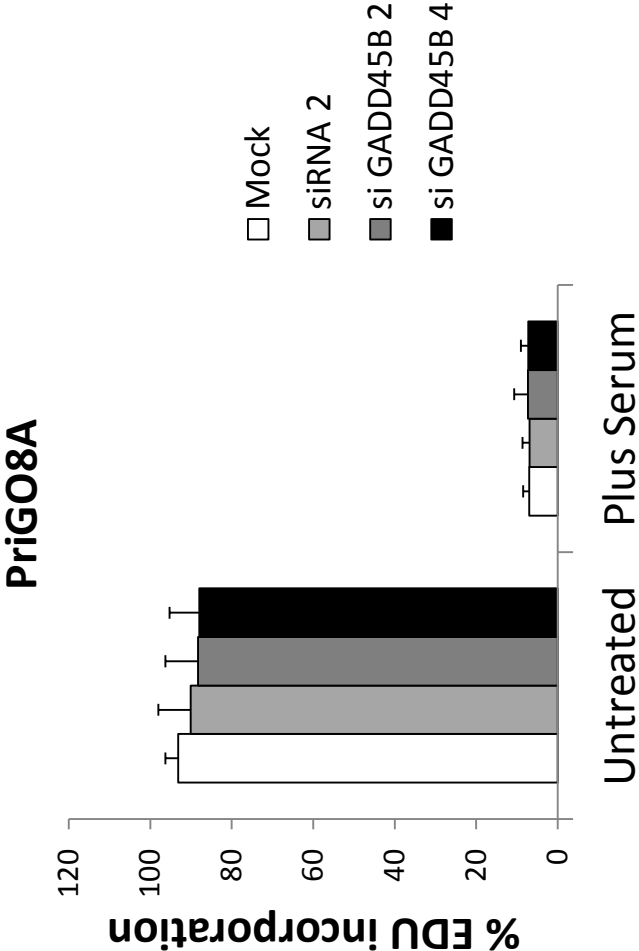
Figure 39. Knockdown of GADD45 β does not affect cell proliferation as determined by EdU incorporation in PriGO8A cells.

(A) PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs after which cells were mock transfected, or transfected with control siRNA or two different siRNA targeting GADD45 β . After 48 hrs of transfection, cells were treated with 10% serum. After six days, cells were labelled with EdU for 24 hrs. Cells with EdU incorporation were detected using the Click-IT EDU detection kit from Thermo Fisher. Results shown are a mean $\pm$ SD. N=3.

(B) PriGO8A cells were transfected with siRNA for GADD45 β as in Figure 38. Cells were fixed and stained for PML bodies 7 days later. Results shown are a mean $\pm$ SD. N=3.

Fig 39:

A



B

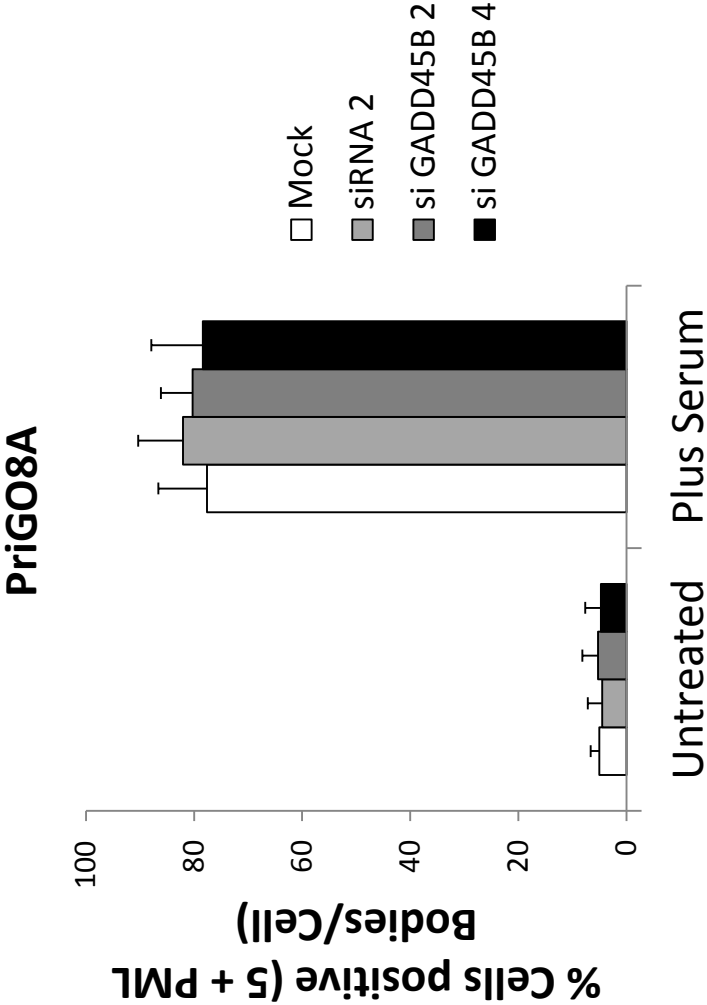


Figure 40. Overexpression of GADD45 β does not modulate serum-induced senescence in PriGO8A cells.

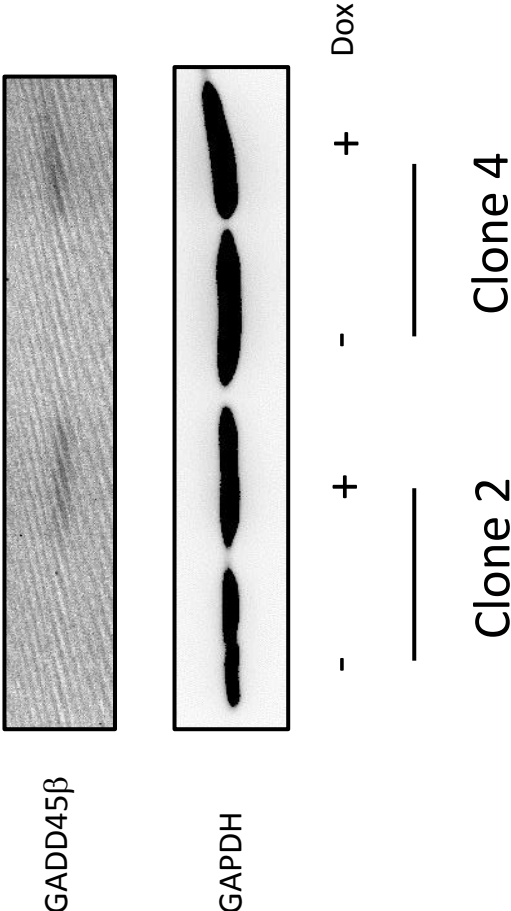
(A) PriGO8A cells (1×10^5 /ml) were transduced with a lentivirus with doxycycline inducible expression of GADD45 β . Cells were treated with Dox for 24 hours and total cell extracts were collected and analyzed for GADD45 β by Western Blotting. Representative results from one of three independent experiments are shown.

(B) PriGO8A cells (1×10^4 /ml) were transduced with inducible GADD45 β as in A and treated with Dox for 24 hours. Cells were then treated with 10% Serum then fixed and stained for Sa β Gal 7 days later. Results shown are a mean $\pm$ SD. N=3.

Fig 40:

A

PriGO8A Tet on GADD45 β



B

PriGO8A

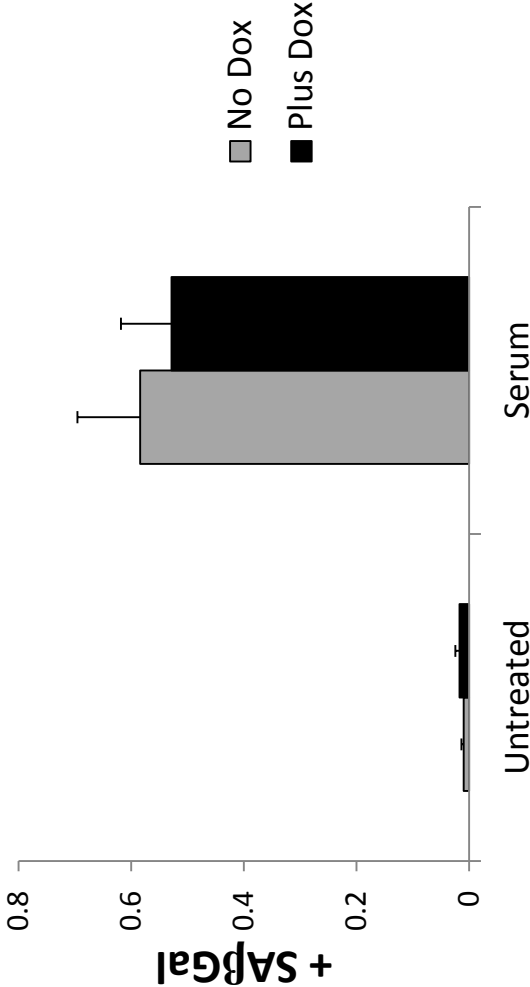


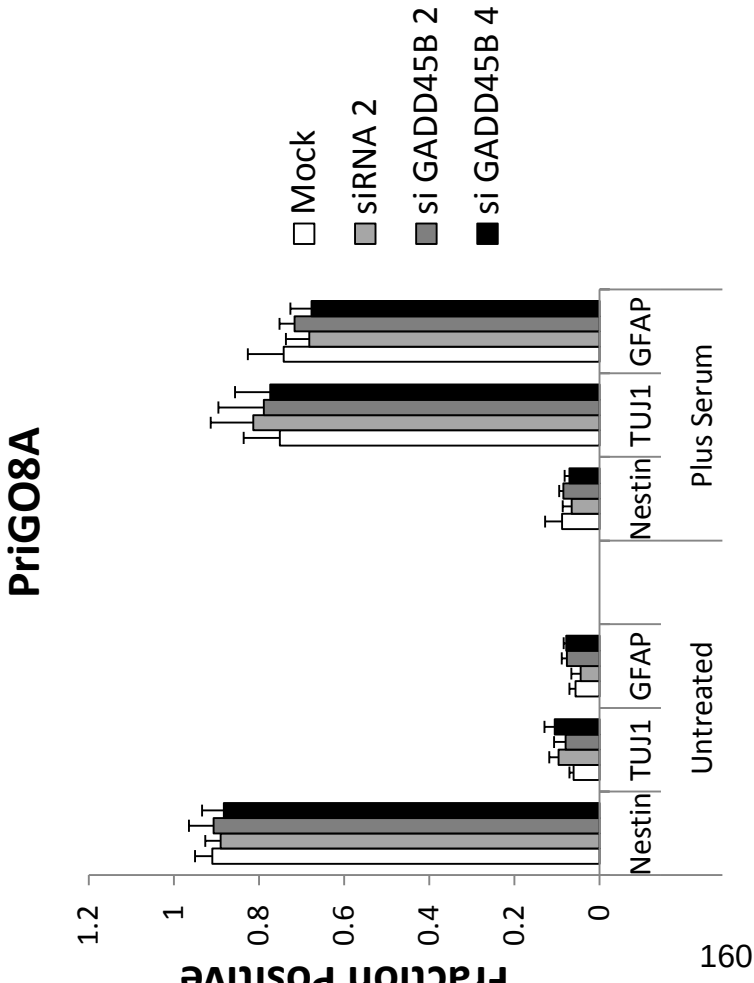
Figure 41. Knockdown of GADD45 β (A) or overexpression of GADD45 β (B) does not modulate serum-induced differentiation in PriGO8A cells.

(A) PriGO8A cells (1×10^4 /ml) were cultured for 24 hrs after which cells were mock transfected, or transfected with control siRNA or two different siRNA targeting GADD45 β . After 48 hrs of transfection, cells were treated with 10% serum. Seven days after serum treatment, cells were fixed and analyzed for the expression of Nestin, TUJ1, and GFAP by immunofluorescence. Results shown are a mean $\pm$ SD. N=3.

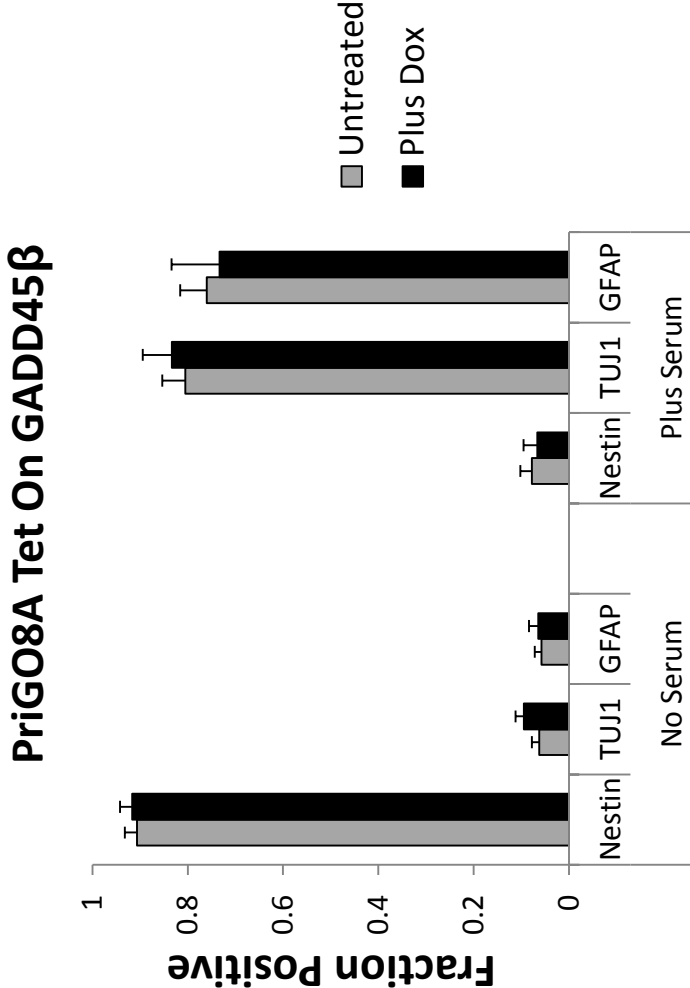
(B) PriGO8A cells (1×10^4 /ml) were transduced with inducible GADD45 β as in Figure 41 A and treated with Dox for 24 hours. Cells were then treated with 10% Serum then fixed and analyzed for the expression of Nestin, TUJ1, and GFAP by immunofluorescence. Results shown are a mean $\pm$ SD. N=3.

Fig 41:

A



B



function of GADD45B is still unknown, but it does not appear to play a role in senescence induction or differentiation in response to serum treatment.

DISCUSSION

Glioblastoma is the most common adult primary brain tumor. Despite maximum therapy consisting of surgical debulking followed by adjuvant radiation and chemotherapy, median survival is only 14 months after diagnosis (254). Resistance to therapy is thought to be due to extensive heterogeneity present in GBM. Alongside histological heterogeneity, considerable heterogeneity exists at the molecular level in GBM. Gene expression profiling of GBM has shown four main subtypes including proneural, classical, mesenchymal, and neural (13). Furthermore, considerable heterogeneity exists at the single cell level with multiple subtypes present in the same GBM cell. Of note, the proneural subtype was present to some degree in all tumors (17). Furthermore, evidence shows dynamic transition between the various molecular subtypes in GBM (16). However, despite the wealth of molecular data greatly expanding the biological understanding of GBM, the clinical significance of heterogeneity in GBM is largely unknown. This is especially true regarding the different response to therapeutics of the various molecular subtypes and warrants investigation.

Primary glioblastoma cells harvested from human patients with GBM have become an important model of study for glioblastoma. They have shown to be more representative of the parent disease than traditional cultured cell lines. Hence, further insight into the biology of treatment response and resistance of primary glioblastoma cells may prove to be beneficial towards the development of novel and efficacious therapy against GBM.

Senescence is defined as irreversible growth arrest with continued metabolic activity (56). It is characterized by morphological changes, increased lysosomal compartments as detected by SA β GaL, markers of cell cycle arrest such as p21 as well as other markers like PML

bodies (119,279). Senescence has recently been shown to be a novel mechanism to inhibit tumorigenesis. Induction of senescence in certain tumors has led to activation of the immune system resulting in tumor clearance (135). Hence, senescence is now being investigated as a novel cancer therapeutic.

Glioblastomas have high frequency of mutations in the TERT promoter and CDKN2A (p16) locus that are expected to render them resistant to both replicative and oncogene-induced senescence (280,281). Nonetheless, established GBM cell lines such as U87MG have been shown to undergo senescence, rather than apoptosis, in response to ionizing radiation (147,269). In addition, induction of p16 in U87MG cells has also been shown to cause senescence (282). However, the ability of primary glioblastoma cells, a more accurate model of GBM behavior, to undergo premature senescence is not known. Investigation of premature senescence in primary glioblastoma cells may lead to novel therapeutics for the treatment of GBM.

Firstly, I determined that primary glioblastoma cells are capable of undergoing premature senescence. Serum is an agent known to induce aberrant differentiation in these cells (54). It has also been shown to cause senescence in both MEFs and human fibrosarcoma cells (112,270). Serum treatment of our PriGO cells induced aberrant differentiation and in a subset of PriGO cells also induced senescence. However, the senescence response was not linked with differentiation. Interestingly, it was also seen without an obvious DNA damage repair response. In addition, agents that caused dsDNA and ssDNA damage such as ionizing radiation and Triapine, respectively, also did not induce senescence in any of the PriGO cells.

The second aim of my project was to determine the molecular mechanisms governing premature senescence in PriGO cells. Microarray analysis suggested serum treatment of

PriGO8A cells led to induction of the TGF β pathway. Subsequently, I confirmed that serum-induced senescence was at least in part mediated by TGF β . In addition, the response of PriGO8A cells to serum was dependent on basal expression of the TGF β activator protein thrombospondin. Other primary glioblastoma cells showed a variable ability to undergo senescence in response to serum. PriGO7A cells, also of the classical subtype, lacked basal thrombospondin expression and did not undergo senescence in response to serum, but did in response to TGF β . PriGO17A cells only underwent senescence in response to TGF β when Ras pathway activity was blocked. Hence, the signaling machinery required to carry out the senescence program appears to be maintained in PriGO cells.

Finally, it was also observed that the response of the various PriGO cells to the stress inducing agents varied widely. The classical cell lines responded by undergoing senescence or cell death, albeit to different degrees, whereas the mesenchymal cell line was resistant. This further supports the notion that response to therapy in GBM is heavily influenced by its heterogeneity.

Senescence induction in PriGO cells:

Serum: Serum has been frequently used to demonstrate the multilineage differentiation potential of primary GBM cells. I show here that this differentiation can be aberrant, in that many cells are positive for markers of both astrocytic and neuronal lineages. This is in agreement with previous findings (54). I show here that these cells that have undergone “aberrant differentiation” and show multiple markers of senescence. A challenge in the field is that no one marker, or even one set of markers, appears to conclusively identify a cell as senescent. Herein, decreased growth and EdU incorporation, increased SA β gal activity, morphological changes, increased p21 and an

increase in PML bodies supported the conclusion that these cells were senescent. Treatment of PriGO8A cells with BMP4 induced them to undergo differentiation along the astrocytic lineage that was not aberrant in so far as there was no co-expression of neuronal markers. However, BMP4 treatment did not cause a rise in SA β gal activity, showing that this is not a feature of differentiation *per se*. Moreover, it shows that differentiation and senescence are not linked. It has been shown previously that PriGO8A cells can also be induced to differentiate preferentially along the neuronal lineage by expression of PTEN or a constitutively active version of the tumour-suppressor protein Lgl (258). Thus, although PriGO8A cells are capable of selectively undergoing differentiation along astrocytic or neuronal lineages, serum induces an alternate state that more closely resembles senescence. Therefore, some caution should be used in the use of serum as a reagent to induce “differentiation” of primary glioblastoma cells.

The fact that PriGO8A primary GBM cells underwent senescence in response to serum exposure is a novel finding. However, mouse embryonic stem cells have been shown to undergo senescence in response to serum (112). In this paper, mouse embryo cells exposed to serum underwent a growth crisis, which was followed by the appearance of established aneuploid cells. This is similar to the response to serum of primary glioblastoma cells described by Lee *et al.*, where in response to serum exposure, these cells underwent a growth delay followed by the appearance of cells with altered chromosome numbers (54). Many of the permanent GBM cell lines currently in use may be cells that have escaped senescence imposed by serum exposure. Demidenko and Blagosklonny have also described induction of senescence by serum in human fibrosarcoma cells that are cell cycle arrested by artificial expression of p21 (267). They suggest that signals that enhance cell growth (*i.e.* an increase in cell mass) in a cell cycle-arrested context promote senescence.

We did not detect an active DNA damage response in PriGO8A cells that had undergone serum-induced senescence, as assessed by immunofluorescence for γ H2AX foci. The DNA damage response has been proposed to be an essential mediator of both replicative and oncogene-induced senescence (82,95). However there are multiple reports of DNA damage response-independent (or, more precisely, DNA double strand break-independent) senescence (118,261,283-286). Recent evidence suggests that the DNA double strand break initiation of senescence may be restricted to fibroblasts, while epithelial cells undergo senescence in response to sustained DNA single strand breaks (77). This mechanism involves downregulation of PARP1 at the mRNA level; this is not evident in our microarray expression analysis, suggesting that this may not be the mechanism by which primary glioblastoma cells undergo senescence in response to serum.

Radiation

Radiation induces senescence by initiating dsDNA damage, activating the DDR (65). PriGO8A cells did not undergo senescence in response to ionizing radiation. This resistance to radiation-induced senescence makes the finding of senescence induction by serum more intriguing. As serum induced senescence lacked DNA damage markers such as γ H2AX, commonly seen in dsDNA damage, it fits with the inability of dsDNA damage to induce senescence in PriGO cells.

My results showed that radiation, however, did induce cell death in a subset of PriGO cells. Radiation induced cell death in both PriGO8A and PriGO9A cells was mostly due to apoptosis as shown by Annexin V positivity as well as Caspase-3 activation. I also demonstrated that radiation had a lasting inhibitory effect on proliferation as PriGO8A cells did not increase in cell number even 2 weeks after radiation treatment. However, PriGO17A cells were resistant to

radiation. Even at the highest single dose of 10 Gy, PriGO17A cells did not respond with changes to cell number as determined by cell counts nor did they show markers of apoptosis such as caspase-3 activation. Further evidence that PriGO17A cells did not undergo a cytostatic response to radiation was the lack of an increase in p21 which was seen in irradiated PriGO8A and PriGO9A cells.

The molecular mechanism underlying resistance to apoptosis in response to radiation in PriGO17A cells is not clear. This may be attributed to the stem cell-like features in PriGO cells that may protect them from DNA damage and radiation (43). Bao *et al.* found that only GBM cells that had stem cell properties, specifically those that were CD133+, were able to repair DNA damage and inhibit apoptosis induced by radiation. However, in this study I observed that despite all the tested PriGO cells possessing criteria established in the literature for stem cells, such as Nestin positivity, only PriGO17A cells were resistant to radiation. The CD133 status of PriGO cells was not determined in this study. While it is plausible that PriGO17A cells may have been resistant to radiation by being CD133+ and the other PriGO cells may be CD133-, the more likely reason for PriGO17A cells being resistant to radiation is their molecular subtype being predominantly mesenchymal. Bhat *et al.* convincingly demonstrated that their mesenchymal GBM cell lines tested were resistant to ionizing radiation, in contrast to their proneural GBM cells (16).

Since Bao *et al.* suggested PriGO cells with stem cell properties had increased capability of DNA damage repair (43), I hypothesized that differentiation of PriGO cells may reduce the capability of PriGO8A cells to repair DNA damage making them more susceptible to ionizing radiation. My results show that differentiation of PriGO cells with BMP4 did not impact the effect of radiation on cell death in PriGO8A cells. Likewise, differentiation along the neuronal

lineage by expressing a non-phosphorylatable version of Lgl, Lgl3SA, also did not result in an increase in radiation sensitivity. This phenomenon might be due to PriGO8A cells already being sensitive to radiation thus further sensitization not having a drastic effect. Future studies looking at the effect of differentiation of PriGO17A cells to increase radiation sensitivity might prove to be more impactful.

Triapine

Ribonucleotide reductase inhibitors induce ssDNA damage by depleting the available pool of deoxyribonucleotides during DNA replication and stalling the replication fork (273,287). ssDNA damage is unlikely to be the mechanism of serum induced senescence in PriGO cells as a potent inhibitor of ribonucleotide reductase, Triapine, itself did not induce senescence in PriGO cells. It did cause PriGO8A and PriGO9A cells to undergo cell death as shown by a decrease in viability. PI staining in PriGO8A cells confirmed cell death in response to Triapine. PriGO17A cells demonstrated resistance to Triapine, as with radiation and serum, proving to be an especially malignant cell line.

TGF β : A mediator of serum-induced senescence in PriGO cells

In my study, microarray expression analysis suggested that the response to serum was due to activation of the TGF β pathway in PriGO8A cells. This was supported independently by both gene ontology analyses and transcription factor data. The finding that serum exposure induced the activation of SMAD2, a key downstream mediator in the TGF β pathway, confirmed this, as did the finding that TGF β was able to induce senescence in PriGO8A cells. In addition, TGF β -induced senescence has been reported for other cell types (288). TGF β has a complex role in cancer pathogenesis, exhibiting both cancer repressive and cancer promoting activities (278).

Cancer repressive activities include the ability of TGF β to induce cytostasis or apoptosis in some cells. Cancer promoting activities include the ability of TGF β to promote invasion by inducing an epithelial-to-mesenchymal transition and the ability of TGF β to induce an immunosuppressive state in tissue-resident macrophages. An important consideration in TGF β pathway signalling is that differences in the strength and duration of signals can result in completely different cellular responses (289,290). In these experiments, it is a robust activation of the TGF β pathway that leads to senescence. A mechanism for TGF β receptor stabilization in glioblastoma has been described (291). This could potentially enhance the effects of TGF β pathway activation in these cells.

As mentioned earlier, PriGO cells did not display a typical DNA damage repair response. PriGO8A cells are p16 null. The gene locus that codes for p16 (*CDKN2A*) also expresses ARF and is commonly lost in GBM (281). Loss of ARF can abrogate the p53 response including p21 expression. In the absence of fully functional p53/p16-dependent pathways, senescence induction may be mediated in part by non-p53-dependent mechanisms of p21 induction. TGF β is known to mediate p21 upregulation independent of p53 through the FOXO transcription factors (120,292). Senturk *et al.* described TGF β mediated premature senescence in human hepatocellular carcinoma cells through p21 and p15 upregulation independent of p53 and p16 (293).

PriGO8A cells also did not demonstrate a SASP response to serum treatment as determined by microarray expression analysis. It is worth noting that SASP is not universal in cells that have undergone senescence. Rodier *et al.* demonstrated that SASP occurred as a response to dsDNA damage that is separable from the growth arrest of senescent cells (294). Furthermore, Rapisarda *et al.* demonstrated TGF β mediated senescence in human primary

fibroblasts without observing dsDNA damage or the DDR (191). Hence, dsDNA damage or SASP are not critical to TGF β mediated senescence.

p21 is known to be a non-specific marker of cell cycle arrest in multiple cancer cells. This was also evident in PriGO cells as p21 levels increased in response to serum and radiation but only serum induced senescence. Ectopic expression of p21 has been shown to induce a senescence like state, one without a DNA damage repair response or SASP (270). Ectopic expression of p21 produced a significant increase in senescence in PriGO8A cells and to a lesser degree in PriGO9A cells. However, it did not match the level of senescence induction of serum in either of these cell lines.

Involvement of TGF β signaling in senescence induction has previously been described to involve DNA damage through the production of mitochondrial generated oxidative stress with subsequent activation of the p38 MAPK pathway (80). Future investigations of DNA damage and the DNA damage repair response in serum and TGF β mediated senescence in PriGO cells are needed to investigate the presence of mitochondrial dysfunction, generation of oxidative stress, and involvement of the p38 MAPK pathway.

The activation of the TGF β pathway in PriGO8A cells was dependent on the basal expression of thrombospondin 1. Thrombospondin 1 is well-known as an activator of latent TGF β , the form of TGF β that is present in serum. The requirement for thrombospondin 1 was supported by the findings that a peptide inhibitor of thrombospondin 1 could block TGF β pathway activation by serum, as could siRNA to thrombospondin 1. In addition, analysis of two additional patient glioblastoma cultures (PriGO7A and PriGO9A) showed that their susceptibility to senescence induction by serum roughly corresponded to their basal levels of

thrombospondin 1 expression. In PriGO7A cells, which express barely detectable levels of thrombospondin 1 basally, serum did not induce senescence. The requirement for thrombospondin 1 in serum-induced senescence could be bypassed by treating these cells directly with active TGF β .

Although senescence induction in PriGO7A, PriGO8A and PriGO9A cells by serum corresponded to basal levels of thrombospondin expression, this was not the case in PriGO17A cells. PriGO17A cells showed very high basal expression of thrombospondin 1, but were completely resistant to senescence induction by serum. Microarray expression analysis of PriGO17A cells showed that they had a substantial mesenchymal type gene expression signature, which was absent in the cultures from other patients, which were predominantly classical. High thrombospondin 1 expression appears to be a general feature of the mesenchymal subtype in glioblastoma, based on analysis of the TCGA database. The mesenchymal subtype often has inactivating mutations in NF1, a negative regulator of Ras pathway signaling. The correlation of high thrombospondin levels with phosphorylated Raf1 and MEK1 in the TCGA database, along with the effects of U0126 on thrombospondin levels in PriGO17A cells, show that the high levels of thrombospondin are being driven by Ras pathway activation in these cells, rather than by TGF β signalling. Ras pathway signaling also repressed activation of SMAD2 in these cells. Shifts in the migration of phosphorylated and total SMAD2 on SDS-PAGE suggest that this may involve direct modification of SMAD2. Inhibition of SMAD2 function by ERK phosphorylation has been described previously (295,296). Inhibition of Ras pathway signaling restored the ability of these cells to undergo TGF β -induced senescence, presumably because of the restoration of strong SMAD2 activation.

Cancer cells develop mechanisms to overcome senescence in order to perpetuate their malignant phenotype. In glioblastoma, over 80% of cases have mutations in the *TERT* promoter that enhance telomerase expression, which confers resistance to replicative senescence (280,281). About 60% of glioblastomas have inactivation of *CDKN2A* by homozygous deletion and the microarray expression analyses done here are consistent with PriGO7A and PriGO8A cells having *CDKN2A* deletions (281). This generally compromises both Rb and p53 function and would therefore be predicted to confer resistance to oncogene-induced senescence. This study shows that, in spite of these resistance mechanisms, primary glioblastoma cells retain the capacity to undergo senescence in response to activation of the TGF β pathway. This pathway was common to glioblastoma cells isolated from four different patients. However the mechanisms of upstream signaling varied significantly between patients. Further study of the mechanism of this senescence induction pathway and comparison with normal brain cell behaviour may identify common glioblastoma cell-selective mediators of senescence induction that could serve as therapeutic targets.

It should also be noted that TGF β elicited a different response in our cells than what has been described in the literature. Many papers have described the negative impact of TGF β in GBM. In one study, TGF β was found to increase primary glioblastoma cell proliferation (215). Others have shown TGF β expression in GBM correlates with poor survival and trials have been conducted looking at the effect of TGF β 1 inhibitors, with encouraging results (297,298).

The reality is that pleiotropic cytokines such as TGF β have context specific effects that vary greatly based on cell type and have multiple interactions with other signaling pathways such as the Ras pathway (296). In the study conducted by Penuelas *et al*, proliferation of primary glioblastoma cells in response to TGF β depended on LIF production (215). Cells transcribing

LIF through the STAT signaling mechanism underwent proliferation whereas those with suppression of LIF did not. Furthermore, epigenetics plays a large role in TGF β signaling, just as in BMP4 signaling. Methylation of PDGFB suppressed the proliferative effects of TGF β in primary glioblastoma cells (298). Hence, the effects of a complex cytokine as TGF β can vary tremendously based on the cellular conditions.

PriGO cells in our study also widely varied in response to TGF β , with only 1 cell line showing a clear response. While PriGO8A cells demonstrated a robust response to TGF β , PriGO7A cells responded mildly. PriGO17A cells were not affected by TGF β treatment at all. Only with concomitant Ras pathway inhibition were some effects of TGF β observed. This also highlights the interactions of TGF β with multiple pathways leading to extensive cross talk. Finally, this also reaffirms the notion that responses of primary glioblastoma cells cannot be generalized and must be taken into consideration within the specific context.

Targeting the TGF β pathway for therapeutic purposes is known to be challenging. Although TGF β induced senescence in PriGO cells, thereby reducing their proliferation, the pleiotropic effects of a complex cytokine such as TGF β limit its use as a therapeutic in GBM (299). Although these effects are generally seen with systemic delivery, local delivery of TGF β could be a possibility at the time of surgical debulking to maneuver around this obstacle. However, the effects of TGF β in PriGO cells *in vivo* has not yet been determined. It is possible that *in vivo*, GBM cells may secrete agents that trap TGF β such as Gremlins trap BMP4 to negate its effect (300). Furthermore, the effect of TGF β was determined in only a few different PriGO cell lines. BMP4 induced a pronounced cytostatic effect on GBM cells when initially tested but subsequent investigations determined that a significant number of cells actually underwent proliferation in response to BMP4 (44,301). This was attributed to methylation of the

BMP receptor 1B in resistant cells leading to BMP4 having mitotic effects rather than inducing differentiation (301). It remains to be seen whether the effect of TGF β is similarly limited or broad. Hence, this study does not necessarily suggest TGF β as a therapeutic option for GBM.

Alongside the limitations of TGF β as a therapeutic for GBM, this study also demonstrates the limitation of senescence induction as a potential treatment of GBM. Only 50% of PriGO8A cells treated with serum, the cell line showing the strongest response to serum, underwent senescence. It is likely that the rest escaped senescence and continued proliferation. This was evident in the study published by Fine *et al.* where serum treated primary glioblastoma cells initially underwent a growth crisis but then re-established proliferation with a different phenotype, likely having escaped senescence (54). Importantly, in our study PriGO8A cells treated with serum did not mount a SASP. SASP, while having the potential to be tumor promoting, has the capacity to induce senescence in neighboring non-senescent cells as well as facilitating immune clearance. Hence, lack of an effective SASP response diminishes the therapeutic capacity of senescence induction in PriGO cells.

GADD45B in PriGO cells

GADD45B has been shown to play a role in senescence induction in MEFs. GADD45B null MEFs underwent senescence more readily than wild type MEFs in response to oxidative stress. Thus, it was hypothesized GADD45B upregulation in response to serum treatment in PriGO8A cells reduced the magnitude of serum induced senescence. However, knockdown of GADD45B in PriGO8A cells did not increase serum induced senescence. Overexpression of GADD45B also did not impact the capacity of serum to induce senescence in PriGO8A cells. Similarly, knockdown or over expression of GADD45B also did not influence differentiation in serum

treated PriGO8A cells. Hence, the function of GADD45B in PriGO cells is still unidentified. It is possible that it may be involved in other processes than those investigated in this study. However, it is also plausible that the techniques utilized may not have been efficient enough to detect changes in phenotype and GADD45B may still be involved in senescence induction or differentiation in PriGO cells.

Heterogeneity in PriGO cells results in varied responses to stress inducing agents

GBM has a wealth of molecular data available to researchers through various portals, chief among them the TCGA. Mining through this data has yielded important insights into the biology of GBM. Although heterogeneity in GBM was previously appreciated at the histological level, it is now seen to be significant at the molecular level as well. For example, primary and secondary GBM are thought to be different diseases due to their molecular landscape. Furthermore, primary GBM has not only been shown to exhibit heterogeneity by having subclasses based on expression data, but also by having multiple subtypes present in individual cells.

The clinical relevance of heterogeneity in GBM is still not well known. Correlational studies conducted by Veerhak *et al.* suggested patients with the mesenchymal subtype responded better to more intensive therapy (defined as concurrent radiation and chemotherapy) with a reduced mortality rate compared to the proneural subtype (13). However, other evidence suggests the mesenchymal subtype is associated with a worse prognosis (302). Furthermore, Bhat *et al.* demonstrated radioresistance of mesenchymal GBM cells compared to proneural cells (16). The mesenchymal subtype also has rich expression of inflammatory genes contributing to increased necrosis, a negative prognostic indicator (13). However, little is known about the

response of the other subtypes to therapy, such as the classical subtype, and its subsequent prognostic implications. Hence, the clinical significance of the molecular heterogeneity in GBM requires elucidation especially the influence of heterogeneity, in terms of molecular subtype, on therapeutic responses of stress inducing agents with respect to senescence and cell death.

In my study, there was a clear influence of heterogeneity in the capability of PriGO cells to respond to various stress inducing agents. Cell lines within the same classical molecular subtype demonstrated different degrees of senescence/cell death in response to serum, radiation, and Triapine. PriGO8A and PriGO9A cells readily underwent senescence in response to serum and PriGO7A cells underwent senescence in response to TGF β but not to serum. PriGO8A and PriGO9A cells also underwent cell death in response to ionizing radiation and the chemotherapeutic agent Triapine. Hence, my data presents a correlation of the classical subtype of PriGO cells with responsiveness to multiple modalities of stress inducing agents, despite the level of response varying between cell lines.

Response to stressors also varied between subtypes in PriGO cells. PriGO17A cells, which were of the mesenchymal subtype, were resistant to all three modalities in terms of both senescence and cell death. Therapeutic resistance may be a feature of the mesenchymal subtype in general. PriGO17A cells were consistent with the previous report linking the mesenchymal signature with radioresistance. In addition, the resistance of PriGO17A cells to serum and TGF β mediated senescence was attributed to TGF β pathway repression due to Ras pathway overactivity, itself a common feature of the mesenchymal subtype due to frequent mutations in NF1. However, validation of the generalizability of the mesenchymal subtype to therapeutic resistance will require further investigation of more cell lines with the mesenchymal signature.

CONCLUSIONS AND FUTURE STUDIES

The major conclusion from this work is that primary glioblastoma cells retain the capacity to undergo premature senescence. Despite the loss of tumor suppressive elements such as p16, the signaling machinery to carry out the senescence program remains intact. In our cells, acute activation of the TGF β signaling pathway induced senescence. Interestingly, there was an absence of the traditional DNA damage repair response with senescence induction. Specifically, markers of dsDNA such as γ H2AX were not detected. Consistent with this, ionizing radiation did not induce senescence. Evidence of ssDNA damage was also absent, such as a lack of PARP1 induction seen in the microarray analysis as well as lack of senescence induction by the ssDNA damaging agent Triapine.

Another conclusion is heterogeneity of GBM causes significant variations in response to stress inducing agents. This was seen within a molecular subtype as senescence induction varied between three different cell lines in the classical subtype. This was based on the basal level of THBS1, an activator of TGF β . Furthermore, the response to stress inducing agents also varied across subtypes. Mesenchymal PriGO cells were resistant to TGF β induced senescence due to repression of TGF β signaling by the Ras pathway with restoration of TGF β mediated senescence upon Ras pathway inhibition. In addition, PriGO cells of predominantly classical subtype were sensitive to radiation and Triapine whereas the sole mesenchymal PriGO cell line was resistant to both.

Finally, my study has also demonstrated the importance of culture conditions of primary glioblastoma cells. Serum has multiple effects on primary glioblastoma cells including aberrant differentiation as well as senescence. Serum induced senescence is not linked with differentiation

and does not occur in all primary glioblastoma cells. Nonetheless, caution should be used in culturing primary cells in the presence of serum.

Future Work

Although dsDNA and ssDNA damage was not detected in our cells that underwent senescence, further evaluation of DNA damage is warranted. TGF β signaling in senescence induction has been linked with mitochondrial dysfunction and oxidative stress resulting in DNA damage and activation of the p38 MAPK pathway (80). Subsequent investigations will study the role of reactive oxygen species and p38 MAPK in TGF β mediated senescence in PriGO cells.

Heterogeneity in GBM leads to varied responses to stress inducing agents. Elucidation of the mechanisms responsible for resistance to these agents between cells may give insight into novel therapeutic strategies. PriGO17A cells proved to be resistant to all three stress inducing agents. However, Ras pathway inhibition facilitated TGF β mediated senescence in PriGO17A cells. Further studies will aim to determine if the Ras pathway plays a similar role in radiation and chemotherapeutic resistance in these cells.

The NF- κ B pathway activation correlates with radioresistance of mesenchymal primary glioblastoma cells (16). Similarly, it may have an involvement in resistance of these cells to other therapeutic modalities such as ssDNA damage. To test this, NF- κ B pathway activation in PriGO17A cells would be first determined. Presuming downstream effectors of the NF- κ B pathway are active in PriGO17A cells, subsequent experiments could be performed examining the effect of Triapine or TGF β on PriGO17A cells with inhibition of these factors.

PriGO17A cells may be resistant to therapy due to having greater stem cell properties relative to the other PriGO cells. This may impart a greater ability to repair DNA damage. To test this

hypothesis, PriGO17A cells could be differentiated by either BMP4 treatment or Lgl3SA transduction and subsequently treated with radiation or Triapine.

PriGO17A cells may be resistant to therapy because of a general association between the mesenchymal gene expression signature and therapy resistance. To elucidate this, more primary glioblastoma cells would be harvested in an attempt to isolate more PriGO cell lines with the mesenchymal expression pattern and test their response to the three stress inducing agents used in this study. Another means to test this hypothesis would be to convert other PriGO subtypes into mesenchymal ones. TNF- α has been shown to convert the proneural subtype into mesenchymal and this could be attempted in the established classical PriGO cells such as PriGO7A, PriGO8A, and PriGO9A (16).

Finally, GADD45B was not found to play a role in serum mediated senescence or differentiation. However, these may have been due to technical issues. GADD45B knockdown by siRNA may not have been efficient enough to elicit a difference in the effect of serum. Other techniques such as CRISPR knockout of GADD45B may be more impactful. Likewise, a more effective increase in GADD45B expression could be attempted by using a lentivirus constitutively expressing GADD45B and subsequently testing its effect on serum mediated responses such as senescence or differentiation.

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RITESH KUMAR

M.D., Ph.D.

Education and Qualifications

Postgraduate	Postgraduate year 5, Neurosurgery Residency training.	University of Ottawa, Canada	2009-Present
	Ph.D. Thesis title "Induction of premature senescence in primary glioblastoma cells."	University of Ottawa, Canada	2018
	M.D.	University of Ottawa, Canada	2005-2009
Undergraduate	B.Sc. Biochemistry, Honours	University of Ottawa, Canada	2000-2004

Awards

Brien G. Benoit Research Award (\$1000 CDN)	University of Ottawa, Canada	2018
W.E. Collins Department of Surgery Research Prize (\$500 CDN)	University of Ottawa, Canada	2018
Biochemistry Ph.D. seminar prize (3 rd place)	University of Ottawa, Canada	2017
Brien G. Benoit Research Award (\$1000 CDN)	University of Ottawa, Canada	2016
W.E. Collins Department of Surgery Research Prize (\$500 CDN)	University of Ottawa, Canada	2015
Brien G. Benoit Research Award (\$1000)	University of Ottawa, Canada	2015
Michael T. Richard Scholarship (\$140,000 CDN)	University of Ottawa, Canada	2015-2017
University of Ottawa Ph.D. Admission Scholarship (\$36,000 CDN)	University of Ottawa, Canada	2013-2017
Brien G. Benoit Research Award (\$500 CDN)	University of Ottawa, Canada	2012
University of Ottawa Medical Student Research Bursary (\$5000 CDN)	University of Ottawa, Canada	2006
University of Ottawa M.Sc. Admission Scholarship (\$10 000 CDN)	University of Ottawa, Canada	2005
National Science And Engineering Research Council (NSERC) Research Scholarship (\$6000 CDN)	University of Ottawa, Canada	2003

National Science And Engineering Research Council (NSERC) Research Scholarship (\$6500 CDN)	University of Ottawa, Canada	2002
Heart and Stroke Foundation Research Scholarship (\$3500 CDN)	University of Ottawa, Canada	May-July 2001
University of Ottawa B.Sc. Admission Scholarship (\$10 000 CDN)	University of Ottawa, Canada	2000-2004

Publications

Peer reviewed publications	Kumar R, Gont A, Lorimer IA. Differential response of the molecular subtypes of glioblastoma to various therapeutics. Manuscript in preparation. Kumar R, Gont A, Perkins T, Hanson J, Lorimer IA. Induction of senescence in primary glioblastoma cells by serum and TGFβ. Scientific Reports. 2017. 7(1): 2156 Restall IJ, Parolin DA, Daneshmand M, Hanson JE, Simard MA, Fitzpatrick ME, Kumar R, Lavictoire SJ, Lorimer IA. PKCα Depletion Initiates Mitotic Slippage-Induced Senescence In Glioblastoma. Cell Cycle. 2015. 14(18): 2938-2948 Faller E, Kakal J, Kumar R, Kryworuchko M, Macpherson P. The HIV tat protein acts synergistically with IL-7 to down regulate CD127 expression on the surface of CD8+ T cells. International Immunology. 2009. 21(3):203-216. Creery D, Weiss W, Graziani-Bowering G, Kumar R, Aziz Z, Angel J, Kumar A. Differential Regulation of CXCR4 and CCR5 Expression by Interleukin (IL)-4 and IL-13 Is Associated with Inhibition of Chemotaxis and Human Immunodeficiency Virus (HIV) Type 1 Replication But Not HIV Entry into Human Monocytes. Viral Immunology. Fall 2006, 19(3): 409-423.
Book Chapter	Kumar R, Sinclair J. Dural Arteriovenous Fistulas. CyberKnife Stereotactic Radiosurgery: Brain. Volume 1. 2014. pp205-212

Oral Presentations

National	Kumar R, Tsai E. The role of Vertebroplasty and Kyphoplasty in the treatment of Osteoporotic Spine Fractures. Canadian Neurological Sciences Federation. Ottawa. June 2012. Kakal J, Kumar R, MacPherson, P. Transcription of the CD127 gene is down regulated by IL-7 in CD8+ T cells. OHTN Research Conference. Toronto. November 27-28, 2006.
Regional	Kumar R, Gont A, Lorimer I. Induction of senescence in primary glioblastoma cells by serum and TGFβ. University of Ottawa Department of medicine Resident Research Day. June 2016. Kumar R, Lorimer I. Induction of senescence in primary glioblastoma cells by serum and TGFβ. University of Ottawa Collins Research Day. May 2016.

Kumar R, Lorimer I. Proliferative Inhibition of Glioblastoma Tumor Initiating Cells (GTICs) with Ribonucleotide Reductase Inhibitors. University of Ottawa Collins Research Day. May 2015.

Kumar R, Lorimer I. The role of YAP1 in Glioblastoma Tumor Initiating Cells (GTICs). University of Ottawa Collins Research Day. May 2014.

Poster Presentations

International	Kumar R , Lorimer I. Induction of senescence in primary glioblastoma cells by serum and TGFβ. European Association of Cancer Research. Manchester. July 2016.
	Kumar R , Lorimer I. Proliferative Inhibition of Glioblastoma Tumor Initiating Cells (GTICs) with Ribonucleotide Reductase Inhibitors. Congress of Neurological Surgeons. New Orleans. September 2015.
	Kumar R , Kassam A. Analysis of surgical biometrics in the resection of intracranial neoplasms. Congress of Neurological Surgeons. San Francisco October 2013.
National	Kumar R , Lorimer I. Induction of senescence in primary glioblastoma cells by serum and TGFβ. Canadian Neurological Sciences Federation. Quebec City. June 2016.
Regional	Kumar R , Kassam A. Analysis of surgical biometrics in the resection of intracranial neoplasms. University of Ottawa Collins Research Day. May 2012.

Research Experience

“Investigating induction of premature senescence in primary glioblastoma cells as a novel mechanism of inhibiting glioblastoma tumor progression.” Research in the laboratory of Dr. Ian Lorimer at the Department of Biochemistry, Microbiology, and Immunology, Ottawa, Canada.	2013-2017
“Investigating the role of the Tat protein in HIV pathogenesis of CD8+ T cells.” Research in the laboratory of Dr. Paul Macpherson at the Department of Infectious Disease, Ottawa, Canada.	June-August 2006
“Development of a vaccine against <i>Haemophilus Ducreyi</i> .” Research in the laboratory of Dr William Cameron at the Department of Infectious Diseases, Ottawa, Canada.	2003-2004
“Studying the role of the tyrosine phosphatase SHP1 in haematopoiesis.” Research in the laboratory of Dr. Maya Kozlowski at Health Canada, Ottawa, Canada.	May-August 2002
“Studying the role of the ryanodine receptor in skeletal muscle development.” Research in the laboratory of Dr. Balwant Tuana at the Department of Cellular and Molecular Medicine, Ottawa, Canada.	May-July 2001

Teaching Experience

Instructor for the Surgical Exploration And Discovery (SEAD) program for first year medical students.	2018
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Instructor for the Neurology Case Based Learning (CBL) course for second year medical students.	2017
Instructor for the Physician Skills and Development course for second year medical students.	2017
Instructor for the Surgical Exploration And Discovery (SEAD) program for first year medical students.	2016
Instructor for the Neurology Case Based Learning (CBL) course for second year medical students.	2016
Instructor for the Physician Skills and Development course for second year medical students.	2016
Instructor for the Surgical Exploration And Discovery (SEAD) program for first year medical students.	2015
Instructor for the suturing skills session of the link block for third year medical students.	2015
Instructor for the Surgery Boot Camp for first year surgical residents.	2015
Instructor for the Neurology Case Based Learning (CBL) course for second year medical students.	2015
Instructor for the Surgical Exploration And Discovery (SEAD) program for first year medical students.	2014
Instructor for the neurosurgery component of the clerkship course for third year Medical Students.	2014

Courses

Seventh annual world course in NeuroOncology	2018
Advanced Trauma Life Support	2014
Crucial Conversations	2012
Advanced Cardiac Life Support	2008

Volunteer Experience

Let's Talk Science	Demonstrated molecular biology techniques in cancer research to high school students as part of a national education program.	2014
Think First Program	Visited elementary schools to encourage usage of helmets while cycling to prevent head injury.	2011

References available on request