Implications of the Endothelial Cell Response in Glioblastoma to Stimulation by Mesenchymal Stem Cells and Ionizing Radiation

by

Tansy Y. Zhao

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institution of Medical Science
University of Toronto

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Abstract

Heightened angiogenesis is both the pathophysiologic hallmark and the potential cause of therapy resistance for glioblastoma (GBM), a deadly brain tumor. It is thought that mesenchymal stem cells (MSCs) play important roles in neovascularization and tumor progression. We postulated that MSCs protect ECs against radiotherapy, which subsequently enhances tumor angiogenesis, and promotes GBM tumor recurrence following therapy. We therefore sought to establish the in-vitro endothelial cell response to stimulation by MSC condition media and ionizing radiation (IR) treatment. We established the gene expression profiles of endothelial cells in response to IR, MSCs and the combination of both. Within the same gene profiles, we identified a unique gene signature that was highly predictive of response to Bevacizumab for GBM patients. We also demonstrated that MSC increased the viability of ECs in response to IR. Protein analysis in ECs suggested MSC-mediated cell cycle arrest as a mechanism for radio-resistance in ECs.
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Contributions

The Angiogenesis array used in this project was developed by Dr. Julie Metcalf (a scientific associate) in the lab.

The bioinformatics (hierarchical clustering and spectral clustering) algorithms were developed by Dr. Maimaitijiang Yasheng (a scientific associate) in the lab.
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List of Abbreviations

AATx: Anti-angiogenic treatment
Akt: Protein kinase b
ATCC: American type culture collection
BBB: Blood brain barrier
BM-MSCs: Bone marrow-derived mesenchymal stem cells
BMDCs: Bone marrow-derived cells
BSA: Bovine serum albumin
CNS: Central nerve system
CXCL5: C-X-C Motif Chemokine Ligand 5
CXCL10: C-X-C Motif Chemokine Ligand 10
CXCR3: C-X-C motif chemokine receptor 3
DMEM: Dulbecco's Modified Eagle's medium
DSBs: Double-stranded DNA breaks
EBM: Endothelial basal media
ECM: Extracellular matrix
ECs: Endothelial cells
EGFR: Epidermal growth factor receptor
EGM: Endothelial growth media
ERK: Extracellular signal-regulated kinases
FBS: Fetal bovine serum
FGF1: Fibroblast growth factor 1
GBM: Glioblastoma
GSCs: Glioma stem cells
HC: Hierarchical clustering
HGF: Hepatocyte growth factor
HIF: Hypoxia induced factors
HLA-DR: Leukocyte antigen-antigen D related
hMSCs: Human mesenchymal stem cells
HR: Homologous recombination
HUVECs: Human umbilical-derived vascular endothelial cells
ICAM-1: Intracellular adhesion molecule-1
IGF-1: Insulin-like growth factor 1
IL: Interleukin
IR: Ionizing radiation
MAPK: Mitogen-activated protein kinase
MMP-9: Matrix metallopeptidase 9
MSCCM: Mesenchymal stem cell condition media
NSCs: Neural stem cells
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
PECAM-1/CD31: Platelet endothelial cell adhesion molecule
ROS: Reactive oxygen species
RT-PCR: Real time polymerase chain reaction
SC: Spectral clustering
SERPINE1: Serpin Family E Member 1
SDF1α: Stromal cell-derived factor-1α
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNF: Similarity network fusion
SSBs: Single-stranded DNA breaks
TAM: Tumor-associated macrophages
TC: Tissue culture
TF: Tissue factor
TGF: Transforming growth factor
TME: Tumor microenvironment
VCAM-1: Vascular cell adhesion protein-1
VEGF-A: Vascular endothelial growth factor-A
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1. Background of Mesenchymal Stem Cells in Glioblastoma

1.1 Glioblastoma

1.1.1 The clinical and histopathological background of GBM

**Epidemiology**: GBM is the most aggressive and most common primary brain tumor in adults. The incidence of GBM within North America is 3-4 per 100,000. GBM affects patients of all ages with peak incidence between 50-75 years of age. The vast majority of GBM is sporadic, and rarely it may occur in p53 related syndromes such as Li-Fraumeni Syndrome and Neurofibromatosis-1 (Louis et al., 2007).

**Pathology**: GBMs may form anywhere within the brain parenchyma with particular predilection for the subcortical white matter in the frontal, parietal, and temporal lobes. GBM are typically infiltrative lesions with poor margins. Microscopically, they are characterized by pleomorphic astrocytes with increased cellularity, nuclear atypical and mitotic activity. The hallmarks of GBM include neovascular proliferation and extensive necrosis (Fidoamore et al., 2016). The extent of neovascularization is very pronounced in many adult solid tumors especially for GBM (Arrillaga-Romany, Reardon, & Wen, 2014; Kioi et al., 2010; R. Wang et al., 2010).

**Conventional classification**: Classification of astrocytoma has evolved over the past decades. Recent large scale genomic analyses focus on IDH-mutation status as important and pivotal factors in classification of astroctyomas. IDH1 mutation is more likely to be found in tumors of young GBM patients. It is thought that these tumors likely arise from low-grade gliomas that progress into GBM via sequential genetic aberrations such as RB1 loss, INK4A loss, CDK amplification, EGFR amplification, PDGFRA amplification and PTEN loss. Patients with IDH-mutant GBM (secondary GBM) in general have a more favorable prognosis to their wild-type counterparts. Primary GBM is more likely to be found in older patients, and makes up approximately 85% of all GBM. Primary GBM often lack IDH1 mutation and instead usually exhibits combinations of genetic and epigenetic abnormalities include RTK amplification, NF1 loss, NFK1B loss, PIK2R1/PIK3CA mutation, PTEN loss, TP53 loss of function CDKN2A/B loss, RB1 mutation and CDK4 amplification. (Colman & Aldape, 2008; Louis et al., 2007; Reifenberger, Wirsching, Knobbe-Thomsen, & Weller, 2016) There are 4 subtypes of primary GBM: proneural, neural, classical, mesenchymal.(Agnihotri et al., 2013) The proneural subtype is depicted by \( P53, PIK3R1, PTEN, \) or \( IDH1/2 \) mutations; HIF1a, SOX2, and...
neural stem cell markers expressions; PDGFRa amplification and the deletion of CDKN2A. The neural subtype is characterized by mutations in \(p53\), \(PTEN\), \(EGFR\), \(NF1\); gene expressions of ERBB2, MBP and FBXO3; amplification of EGFR, and deletion of CDKN2A. The classical subtype is depicted by mutations in EGFRvIII; gene expression of AKT2, NES, and FGFR3, amplifications of EGFR and CDK6 and the deletions of CDKN2A or PTEN. The mesenchymal subtype is characterized by mutations in \(NF1\), \(RB\), \(EGFR\) or \(PTEN\); gene expression of TRADD, RELB, CHI3L1, and CD44; the amplification of MET and deletions of CDKN2A, NF1 and PTEN. Among the 4 subtypes, the proneural subtype is thought to have the best response to treatment and prognosis whereas the mesenchymal subtype exhibits the worst response to treatment and prognosis. (Sandmann et al., 2015; Thomas, Di Stefano, & Ducray, 2013) Interestingly, secondary GBM mostly resembles the genetic aberrations of proneural subtype, though some show additional epigenetic anomalies such as MGMT methylation and hypermethylation on the CpG islands. (Guan et al., 2014; He et al., 2011)

1.1.2 Conventional treatment strategies of GBM

1.1.2.1 First line-treatment

The mainstay for treatment of newly diagnosed GBM includes maximal safe resection of tumor followed by adjuvant chemoradiation treatment. This protocol for treatment was established in 2005 in multi-institutional randomized trial which showed that median survival of newly diagnosed GBM patients with 14.6 months with radiation + chemotherapy versus 12.1 months with radiation alone. (Figure 1.1.2.1) Despite this multipronged surgical-chemo-radiation treatment protocol, the two year overall survival rates of GBM patients remains extremely low at 26.5% (Stupp et al., 2005).
1.1.2.2 Angiogenesis and second line-treatment with Bevacizumab

Angiogenesis refers to the remodeling and extension of vasculature from an existing vascular network. It is different from vasculogenesis, which represents the development of hematopoietic precursors into vascular cells and the assembly of these cells into primitive vascular plexus (Carmeliet & Jain, 2011; Janeczek Portalska et al., 2012). Endothelium is strategically involved in modulating a myriad of vital physiological activities such as vasomotor actions, inflammation (i.e. leucocyte trafficking, innate and acquired immunity), hemostasis regulation and thrombotic reactions.(Kazmi, Boyce, & Lwaleed, 2015)

Vascuulature-dependent tumor growth and progression are thus of the common features for solid tumors like GBM (Holash et al., 1999a; Leenders, Küsters, & de Waal, 2002). Furthermore,
generation of new blood vessels (or mechanisms that provide blood support) is necessary for any solid tumors mass that grew beyond 1 mm$^3$ (Folkman, 1971). As a basic building block for new vasculature, ECs are quiescent in normal physiological state during adulthood with the exceptions of pregnancy, menstrual cycle, and tissue injury. However, ECs may become activated in response to toxins, ionizing radiation, as well as pathologic conditions such as tumor microenvironment, etc. (Hardee & Zagzag, 2012; Verheij, Dewit, & van Mourik, 1995; J. Wang, Boerma, Fu, & Hauer-Jensen, 2007). The tumor microenvironment employs a complex molecular cascade that can result in recruitment of new blood vessels to the tumor site and subsequently the formation of independent vascular network that is vital for both tumor growth (Bergers & Benjamin, 2003; Folkman, 1971; Holash et al., 1999b). In contrast to normal brain vasculature, tumor vasculatures lack hierarchically organized vessel types and branches, efficient surface area to volume ratio, relatively stable structure and the ability to establish functional blood brain barrier (Sitohy, Nagy, & Dvorak, 2012).

There has been a plethora of research focusing on understanding the mechanisms of tumor angiogenesis. Vascular endothelial growth factor-A (VEGF-A) is the most well-established pro-angiogenic factors studied in GBM angiogenesis and has been found to trigger the differentiation of endothelial cell (EC) precursors, promoting EC survival, and forming tubal structures (Ellis, Rosen, & Gordon, 2006; Jain et al., 2007; Rubenstein et al., n.d.). Since the first pre-clinical study that showed the inhibition of the pro-angiogenic factor VEGFA could suppress GBM tumor growth, many animal studies and clinical trials have been conducted in an attempt to utilize anti-VEGF antibodies as a therapeutic strategy in GBM (K. J. Kim et al., 1993). Bevacizumab (©) is a humanized anti-VEGF monoclonal antibody and is one of the most widely used drug against angiogenesis in solid tumors (MICHA et al., 2007; Muller et al., 1998; Shih & Lindley, 2006). Bevacizumab has been approved for treatment of recurrent glioblastoma as monotherapy or in combination in multiple countries (Jain, Duda, Clark, & Loeffler, 2006; Jayson, Hicklin, & Ellis, 2012; Kazazi-Hyseni, Beijnen, & Schellens, 2010). Two recent phase III clinical trials, RTOG-0825 and AVAglio, were set to evaluate the efficacy of combining Bevacizumab with conventional treatment for newly diagnosed GBM (Chinot et al., 2011; Sandmann et al., 2015). Both trials demonstrated that the addition of Bevacizumab resulted in longer progression-free survival that unfortunately did not translate into improved overall survival (Chinot et al., 2011; Gilbert et al., 2014). Therefore, at present, Bevacizumab is not used as first-line treatment for the treatment of GBM. Given the limited success yield by AATx, many have started to investigate vascular
normalization as a potential treatment strategy with the philosophy of supporting physiological angiogenesis in hopes to suppress pathological angiogenesis and reducing adverse reactions secondary to AATx such as hypoxia, edema. (Jain, 2001; Jain et al., 2007, 2006; Winkler et al., 2004) However, the effectiveness of vascular normalization as a treatment strategy for GBM has yet to be supported by clinical trials (Batchelor et al., 2010; Christine Lu-Emerson et al., 2015). It fact, it is possible that the limited effectiveness of anti-angiogenic and conventional therapy for GBM may be mediated by the heterogeneous nature of the tumor microenvironment.

1.1.3 GBM tumor microenvironment (TME)

Tumor cells often coexist in an environment with a cocktail of other non-cancerous cell types, such as stromal cells. This environment and the interactions that consist between all cells are termed the TME. GBM TME is distinct in that it contains CNS specific cell types and a distinct extracellular matrix with a tightly regulated immune system (Engelhardt, Vajkoczy, & Weller, 2017; MEDAWAR, 1948). The ECM of brain tissue has unique protein constituents that can cultivate cancer cell niches. For example, proteoglycan, glycosaminoglycan, heparin sulfate proteoglycans and hyaluronic acid are macromolecules commonly found around the neural stem cell niches (Reinhard, Brösicke, Theocharidis, & Faissner, 2016), many of which either contribute to neural stem cell homeostasis or serve as reservoir for angiogenic growth factors like FGF and VEGF (Kundu et al., 2016; Lemjabbar-Alaoui, McKinney, Yang, Tran, & Phillips, 2015). Although the integrity of the blood-brain barrier (BBB) in perhaps the early stage of gliomas is relatively maintained, large infiltration of immune cells such as macrophages, dendritic cells, neutrophils and lymphoid cells, are often found in higher grade gliomas and make up a substantial amount of tumor mass (Figure 1.1.3). In fact, macrophages (both differentiated from tissue-resident microglia and bone marrow-derived macrophages) can take up to 30% of the tumor mass (Graeber, Scheithauer, & Kreutzberg, 2002; Hambardzumyan, Gutmann, & Kettenmann, 2016). The role of tumor-associated macrophages (TAM) is rather controversial, as they produce pro-inflammatory cytokines but lack the molecules for full T-cell activation (S Farzana Hussain et al., 2006). However, TAM’s involvement in vessel integrity modulation and tumor angiogenesis may have implications for modulating angiogenesis and therapeutic drug delivery (Anton & Glod, 2014; S. F. Hussain et al., 2006; C. Lu-Emerson et al., 2013).
In addition to immunoresponsive cells, other cells that pertain to brain parenchyma are also reactive to both tumor microenvironment and the anti-tumor treatments. For example, it has been shown that astrocytes shed microRNAs that may suppress PTEN expression while boosting PI3K signaling in cells that are in proximity, both of which are common molecular signatures for tumor cells that are therapy-resistant (C. Lu-Emerson et al., 2013; Piao et al., 2012; Quail et al., 2016; Sevenich et al., 2014; Zhang et al., 2015). Given that hyper-vascularity is the histological hallmark for GBM, the cells that contribute to the formation of blood vessels, such as, endothelial cells, pericytes, and astrocytes, may also be the targets of therapeutic interventions (Quail et al., 2017). The complexity and uniqueness of TME in GBM makes it an intriguing factor to consider when designing therapeutic treatments for GBM.

**Figure 1.1.3:** Composition of GBM tumor microenvironment (Modified from Upreti et al, 2013)
1.1.4 GBM TME heterogeneity

GBMs exhibit a high degree of variability of response to different treatments. Despite strong therapeutic advancement IR confers, there is a universal rate of recurrence for GBM patients with the median time to recurrence being less than 18 months. (Agnihotri et al., 2013) The mechanisms underlying treatment resistance (such as radio-resistance and AATx ineffectiveness) have always been a main focus for cancer research. In particular, the biology of GBM radioresistance has been under rigorous investigation. There is a growing body of evidence suggesting that tumor’s response to radiation are determined by both tumor cell phenotype and stromal environment (such as microvascular sensitivity), especially in the context of vascular-dependent tumor recurrence (Garcia-Barros et al., 2003; Reguera-Nuñez et al., 2014). Here, we listed some of the possible mechanisms underlying GBM radio-resistance and the failure of AATx.

1.1.4.1 Tumor cell heterogeneity

*Tumor cell genetic instability:* Microscopically, GBMs are composed of tumor cells that are in different stages of cell proliferation (Frattini et al., 2013). GBM tumor cells harbor genetic and epigenetic alterations that resulted in numerous combinations of tumor suppressor gene inactivation, oncogene activation as well as imbalanced mitotic factor production, apoptotic factor expressions and heightened DNA repair machinery (Chung et al., 2013; de Andrade et al., 2014; G. H. Jo et al., 2015). All of above events significantly increased the unpredictability of the tumor cells in response to therapeutic intervention.

*Glioma stem cells (GSCs):* The presence of particular cell population that responsible for initiating tumor mass was initially established for acute myeloid leukemia by Bonnet and Dick in 1997 (Bonnet & Dick, 1997). The first evidence of GSCs in brain tumors was reported in pediatric patients at the beginning of this century (Hemmati et al., 2003). GSCs sit at the apex of cancer cellular hierarchy and have distinctive features including their capacity for self-renewal, tissue invasion, proliferation, tumorigenesis, sustained angiogenesis, and perpetuating a tumor in orthotopic transplants (Jeon et al., 2014). Given the universal recurrence of GBM despite rigorous therapeutic intervention, it has been suggested that therapy-resisted glioma-initiating/stem-like cells may be implicated in this disease (Sundar, Hsieh, Manjila, Lathia, & Sloan, 2014). These cells could regenerate the tumor mass after treatments unless eradicated (Bao et al., 2006; Gerweck &
The intrinsic resistance of tumor-initiating cells to classical therapies has been associated with an elevated expression of drug resistance proteins (Kondo, Setoguchi, & Taga, 2004) and increased DNA damage repair ability (Bao et al., 2006).

### 1.1.4.2 Stromal cell heterogeneity

**Endothelial Cells**: ECs are the central elements of neovascularization. In contrast to the ECs in physiological vasculature, tumor-associated ECs may harbor genomic alterations in response to both tumor microenvironment and radiation therapy. While glioma stem cells and neural stem cells (NSCs) have been shown to up-regulate EC-specific markers or develop EC-like phenotypes (B. Hu et al., 2016; Quail et al., 2017; R. Wang et al., 2010), tumor-associated ECs have also been shown to have genomic alterations that resemble tumor cells (Y. Wang et al., 2009), such as EGFR up-regulation (Ricci-Vitiani et al., 2010).

**Mesenchymal stem cells**: MSCs mediate angiogenesis and contribute to radio-resistance (see section 2.2). The mechanism by which MSCs contribute to radioresistance is unclear; however, it is possible that these cells confer vascular protection thereby contributing to radioresistance. Combining the implications that the tumor tropism of MSCs that can be enhanced upon radiotherapy, their pro-angiogenic property, and role of angiogenesis in GBM recurrence, several groups have started to explore the effectiveness of inhibiting MSC migration as a way of increasing the therapeutic benefit of IR (Tseng, Vasquez-Medrano, & Brown, 2011a).

### 1.1.4.3 Alternative mechanisms

1) Vascular co-option or Vessel mimicry (VM): As early as 1999, Maniotis et al coined the phenomenal of tumor cells forming interconnected, vasculature-like structures that do not contain endothelial cells, yet had trace of red blood cells within, in melanoma as VM (Maniotis et al., 1999). Similar structure has since been reported in multiple solid tumors includes breast, ovarian and lung cancer (Passalidou et al., 2002; Shirakawa et al., 2001; Sood et al., 2001). What’s more, the cells that form VM inside a tumor expressed endothelial- and fibroblast-specific genes, which resemble the ECs from vasculogenesis during embryonic development (Bittner et al., 2000; Maniotis et al., 1999). Vascular co-option, another alternative for tumor blood supply, is described as tumors hijacking existing vasculature in proximity for blood supply and metastasis (Donnem et al., 2013). Base on an *in vivo* study done by researchers from UCSF, the inhibition of VEGF could cause an increase of
vascular cooption in GBM (Rubenstein et al., n.d.). As Holash et al demonstrated in their animal model, the booming of vascular co-option in glioma was complimentary to other phenotypes such as vessel regression, tumor hypoxia and heightened angiopoietin-2 expression (ANG2) (Holash et al., 1999a). Another clinical relevance of tumors harboring VM and vascular co-option is their implication of effective intracellular metabolic regulation, which fuels into tumor growth without triggering inflammation and hypoxia while decreasing apoptosis in tumor (Donnem et al., 2013; J. Hu et al., 2005).

Such mechanisms postulated VEGF-independent avenues for GBM tumors to obtain blood supply as well as infiltration and metastasize.

2) Thrombosis: There are also evidences provide insight into the mechanism and function of another GBM histopathologic hallmark – extensive thrombosis. Preclinical study on C6 glioma mouse model has indicated that thrombin, a major blood-clotting enzyme, can increase cell proliferation, such effect is off-set by argatranab (a thrombin inhibitor) both in vitro and in vivo presented by decreased tumor mass and neurologic deficit and ultimately improved survival time(J. S. Yu et al.). Furthermore, thrombin in the brain can not only induce edema but focal inflammation via leukocyte and microglia recruitment and activation (J. S. Yu et al.). It’s difficult to predict the clinical outcome of thrombin inhibition considered that it can potentially preclude anti-tumor effect elicited by individual’s own immune system (Fadul & Zacharski, 2005). The expression of TF, a primary thrombogenic factor, has been associated with malignancy grade in GBM (Carneiro-Lobo et al., 2014; Rong et al., 2005) as well as other cancer types (J. T. Jo, Schiff, & Perry, 2014; Rak, Milsom, May, Klement, & Yu, 2006). Tissue factor is a 47 kD protein that interact with circulating clotting factor VIIa to mediate thrombin activation, platelet aggregation and fibrin deposition (Lewis et al., 1995; Williams & Mackman, 2012). Irradiation induced TF upregulation has been documented in both tumor cells (Goldin-Lang et al., 2007) and endothelial cells (B. M. Chan, Elices, Murphy, & Hemler, 1992; Szotowski et al., 2007). The mechanisms which TF employed to facilitate oncogenesis have since been studied by various groups such as: Gessler et al used 9 different glioma cell lines to demonstrated that TF modulates cell proliferation as well as hypoxia-induced cell migration and invasion through FVIIa/protease activation receptor 2 (PAR-2)/ERK axis (Gessler et al., 2010). Another group from Brazil found inversely proportional relationship between TF expression and tumor-suppressor PTEN expression. Same group also observed heightened TF and PAR-2 expressions are co-existed with VEGF and IL-8 in primary patient samples (Carneiro-Lobo et al.,
2014). Eastern Cooperative Oncology Group had conducted a phase II trial on Dalteparin, an anti-coagulant with potent ingredient of low molecular heparin, combined with radiotherapy to newly diagnosed GBM patients. However, the trial concluded as Dalteparin reduced the thromboembolic event did not translate into higher survival (Robins et al., 2008).

1.2 Mesenchymal Stem Cells (MSCs) and MSC-medicated Angiogenesis in GBM

1.2.1 Endothelium activation

In general, activated ECs fail to maintain vascular integrity and enter a pro-thrombotic state. This can be presented by the abnormally expressed cell adhesion molecules (i.e., ICAM-1, VCAM-1, E-selectin, etc), the loss of surface anticoagulant molecules, and the expression of platelet activating factor (e.g., nitrate oxide and tissue factor) (Hunt & Jurd, 1998). Certain factors, for instance NFkB, IL-6, IL-1b, etc that are up regulated either by IR or TME have been shown to activate ECs (Liu, Pelekanakis, & Woolkalis, 2004; Mahboubi et al., 2001; Shebuski & Kilgore, 2002).

TME activated endothelium: Apart from providing blood supply and route of cell migration, endothelium in the TME are a robust and dynamic network that can be “switched on” from a dormant stage to become activated. This is partly the result of disrupted balance between pro-angiogenic factors and anti-angiogenic factors. Subsequent vessel outgrowth, inflammation, vessel leakage, etc are often found in the event of an angiogenic switch. At the cellular level, the features of activated endothelium include but are not limited to the increased expression of leucocyte adhesion molecules (e.g., E-selectin, ICAM-1 and VCAM-1) and the up-regulation of Class II human leucocyte antigen molecules (both can facilitate leucocyte recruitment and immune response).

IR-activated endothelium: When tumors are radiated, the ECs of blood vessels within the tumor and surrounding ECs also receive radiation. IR can provoke EC proliferation when given in lower doses (<10Gy) (Sofia Vala et al., 2010), increase tube formation capacity at 0.5 to 2Gy, and induce EC apoptosis when given in higher doses (>10Gy) (Langley, Bump, Quartuccio, Medeiros, & Braunhut, 1997; Paris, 2001). In addition to the dosage of IR, the response of ECs to radiation can also be dependent on the frequency of radiation, the cell culture environment (e.g., TME, serum-deprivation or MSC conditioned media), and the specific cell cycle ECs are at when receiving IR. In general,
radiation induces reactive oxygen species or its intermediates (Mikkelsen & Wardman, 2003) in ECs which can lead to the activation of NFκB (Langley et al., 1997; Quarmby, Kumar, & Kumar, 1999), and double-stranded DNA damage (DSB). Radiation also triggers molecular cascades that up-regulate the expression of pro-angiogenic factors (i.e., VEGF, TGF, HIF etc) in various cell types (Arrillaga-Romany et al., 2014; Bertolini et al., 2011; Tseng, Vasquez-Medrano, & Brown, 2011b). To add another layer of complexity of EC responses to irradiation, a series of adhesion molecules, such as E-selectin, P-selectin, ICAM-1, PECAM-1/CD31, VCAM-1 have been reported to be up-regulated in ECs after radiation (Hallahan, Kuchibhotla, & Wyble, 1996; Lewis et al., 1995; Quarmby et al., 1999; Shebuski & Kilgore, 2002). Many of these factors have been shown to have important roles in inflammatory responses and the maintenance of vascular integrity. (Herbert, Savi, Laplace, & Lale, 1992)

Radiation may also increase exocytosis and further synthesis of von Willebrand factor, which like P-selectin, can link inflammatory reaction to thrombotic event by facilitating the coagulation process via binding to tissue factor and its substrates (i.e., factor VIII)(Shebuski & Kilgore, 2002) as well as platelet and leukocyte attraction (Jahroudi, Ardekani, & Greenberger, 1996; Ruggeri, 1999). High dosages of IR can also induce double-stranded DNA breaks (DSBs) and/or single-stranded DNA breaks (SSBs). Depending on their ability to repair such DNA damages, irradiated cells will either undergo apoptosis, cell cycle arrest, and senescence, or have reduced proliferation. In summary, depending on the IR-dosage and timing of administration, IR can generate variable outcomes in ECs both at molecular aspect (i.e., intracellular expressions, cell proliferation, cell death) and functional aspects (i.e., pro-angiogenesis, etc) (Kondo et al., 2004).

1.2.2 Bone marrow-derived MSCs

Proposed by International Society of Cellular Therapy, the minimal histologic criteria of human BM-MSC are cells that do not stain for CD45, CD34, CD14 and human leukocyte antigen- antigen D related (HLA-DR), and have positive immunohistochemical staining for CD73 (which converts AMP to adenosine), CD105 (a member glycoprotein) and CD90 (GPI-linked glycoprotein (Dominici et al., 2006; F.-J. Lv, Tuan, Cheung, & Leung, 2014). The expression of additional cell markers is often observed depending on each tumor’s environmental stimuli, MSCs can also be identified by characteristic cell surface markers that indicate their plastic adherence and/or stemness (Dominici et al., 2006; E. A. Jones et al., 2002).
As a subpopulation of BMDCs, MSCs have been reported to migrate to glioma tumors or conditioned media of glioma (Doucette et al., 2011). Over the past two decades, the contribution of MSCs to tumor progression has become the subject of main investigation. Bone marrow-derived MSCs are multi-potent mononuclear cells that are known to give rise to osteoblasts, adipocytes, chondrocytes, osteocytes and endothelial cells (Figure 1.2.2) as well as their important implications for neovascularization and tumor progression (F.-J. Lv et al., 2014). MSCs can be detected in nearly all vascularized tissues such as bone marrow (Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966), placenta (Crisan et al., 2008; Fukuchi et al., 2004), umbilical cord (Akimoto et al., 2013; F. Lv, Lu, Cheung, Leung, & Zhou, 2012), endometrium (Schwab, Hutchinson, & Gargett, 2008), dental pulp (Huang, Gronthos, & Shi, 2009) and adipose tissue (Yang et al., 2014), which complement their inherent ability to differentiate into connective tissues (Leung et al., 2014; F.-J. Lv et al., 2014). However, the bone marrow remains the most widely documented and accepted origin of MSCs to date (F.-J. Lv et al., 2014). Although approximately 0.01% - 0.001% of the bone marrow cells are composed of MSCs, these cells are arguably one of the most important bone marrow cell subpopulations due to their pluripotency, remarkable proliferation capacity and (for the context of this thesis) their significant involvement in angiogenesis and radioresistance (Friedenstein et al., 1966; Janeczek Portalska et al., 2012; Penfornis & Pochampally, 2011).

Due to the variations in tissue origin and its consequential heterogeneous tendency on differentiation and proliferation, a universal definitive cell marker of MSCs has yet to be identified.
1.2.3 MSC-mediated angiogenesis

1.2.3.1 MSCs in normal physiological conditions
MSCs can differentiate into endothelial progenitor cells and later on mature ECs. Moreover, MSCs are also be found to act as perivascular supporting mural cells (pericytes) by contributing to the functionality and stability of blood vessels in multiple normal solid organs such as spleen, liver, kidney, pancreas and brain (Dominici et al., 2006; Hass & Otte, 2012; Lamagna & Bergers, 2006). MSCs are also known to contribute to migrate to site of tissue injury and facilitate wound healing by secreting pro-angiogenic factors or recruiting endothelial progenitors, as well as M2-macrophages polarization, extracellular matrix remodeling, inflammatory cytokine suppression. (Motegi & Ishikawa, 2016)
1.2.3.2 MSCs in tumor microenvironment

Two observations implicate MSCs in tumor angiogenesis:

1) MSCs have close perivascular distribution within tumor tissues. (Crisan et al., 2008; Nakamizo et al., 2005) They are known to modulate angiogenesis as well as release cytokines upon pathological stimuli such as hypoxia and radiation-induced inflammation. (J. K. Y. Chan & Lam, 2013a)

2) MSC secretomes contain pro-angiogenic factors. Some of the cytokines are known mitogenic or angiogenic regulators, such as bFGF, VEGF, PDGF, IL-6, IL-1beta (Appaix et al., 2014; Bexell et al., 2009; Du, Lu, et al., 2008; Lamagna & Bergers, 2006). Other MSC-secreted cytokines (i.e., MMP-2 and MMP-9) increase the affinity of VEGF to VEGF receptor-2. Many of the cytokines have also been implicated in tumor progression (J. K. Y. Chan & Lam, 2013b; Zimmerlin, Park, Zambidis, Donnenberg, & Donnenberg, 2013). For example, Rose et al. showed that the recruited bone marrow-derived CD45+ myeloid cells could modulate the tumor vasculature in GBM. The same group also demonstrated that the HIF-1α-augmented SDF1α signals can attract a subpopulation of bone marrow-derived cells as well as endothelial progenitor cells, which can initiate tumor angiogenesis through increasing MMP-9 activity and VEGF mobility (Du, Petritsch, et al., 2008).

In addition to the above two observations, Kong et al have shown that tumors generated by xenografting MSC-primed glioma stem cells had increased microvascular density (a reflection of angiogenesis) compared to the tumors generated from control glioma stem cells (Kong et al., 2013). However, in a separate study, Ho et al demonstrated that co-injection of MSCs and glioma cells in vivo generated tumors with smaller volumes and less vascular density compared to tumors were generated from glioma cells alone (Ho et al., 2013). These contradictory results suggested that MSCs could have bi-modal effects on tumor angiogenesis.

1.2.4 IR recruitment of MSCs and its implications in radio-resistance and radio-protection

In our laboratory, we have been able to show in vivo that bone-marrow-derived cells (BMDCs) exclusively localize to the site of radiation for both normal mouse brains and mouse brains that
harbor GBM tumor. (Figure 1.2.4) The recruitment of BMDCs appears to be dose-dependent, with
the greatest recruitment response at 15Gy radiation (Burrell, Hill, & Zadeh, 2012). The recruitment
of BMDCs to tumor-site post-radiation is thought to facilitate repopulation of the tumor mass (i.e.
tumor recurrence) (Kozin et al., 2010), as well as further tumor invasion (Busillo & Benovic, 2007).
In addition to this, it has been shown that intratumor blood vessels post-radiation at least in part are
derived from BMDCs, suggesting that BMDCs can also contribute to stromal cell structures (Ahn &
Brown, 2008; Kioi et al., 2010).

The radioresistant potential of MSCs has been previously elucidated as increased doses of IR in vivo
reduced the proliferation of BM-MSCs while preserving their ability to differentiate (Singh et al.,
2012). Moreover, it has also been shown that MSCs may in fact harbor a stronger radioresistant
potential when compared to lung and breast cancer cells (M.-F. Chen et al., 2006). It is thought that
MSCs could effectively utilize DNA repair mechanisms and increase antioxidant production to cope
with IR-induced ROS (M.-F. Chen et al., 2006) by up-regulating genes that are involved in DNA
damage responses and DNA repair, after radiation treatment (M.-F. Chen et al., 2006; Garcia-Barros
et al., 2003; Naidu, Mason, Pica, Fung, & Peña, 2010; Pawlik & Keyomarsi, 2004; Valerie et al.,
2007; Wachsberger, Burd, & Dicker, 2003).

The radioprotective role of MSCs has also been documented previously. Conditioned media from
murine BM-MSCs has been found to protect hepatic cells from IR-induced apoptosis via the
activation of Akt and ERK signaling pathways. In addition to this, it has also been suggested that
MSCs may provide radioprotection by modulating the inflammatory response and fibrosis formation
at the site of irradiation (Chargari, Clemenson, Martins, Perfettini, & Deutsch, 2013; Y.-X. Chen et
al., 2015; Chung et al., 2013; Linard et al., 2013; Saha et al., 2011). IR is also known to effectively
provoke or enhance local inflammation in tumor microenvironment (Chargari et al., 2013; Chung et
al., 2013). BM-MSCs also promote expression of anti-inflammatory cytokines in cells that are
exposed to IR (Y.-X. Chen et al., 2015). Further evidence of MSC protection against IR was
demonstrated in IR-induced epithelial injury in the gastrointestinal tract. MSCs were believed to
mitigate the harmful effects of IR by reducing inflammation and fibrosis at the sites of irradiation,
specifically by elevating the expression of IL-6, IL-10, and other anti-inflammatory cytokines
(Linard et al., 2013; Saha et al., 2011).
Figure 1.2.4: The Migration of Bone Marrow Derived Cells. (A) *in-vivo* real-time 2PLM images of normal mouse brain 7 days post IR, illustrates distinct BMDC recruitment (*Green: BMDC, blue: CD31 positive cells*). *in-vivo* real-time 2PLM images of the migration of BMDCs to (B) normal mouse brain (C) U87 Xenograft mouse brain at day 21 (*Green: BMDCs, Red: U87, Blue (CD31 positive cells)*). (D) Quantification of BMDCs on 2PLM images shows a statistically significant increase in BMDC recruitment in response to both RT and U87 tumor growth, and the most increase occurs when combined these two conditions. (Burrell et al., 2012)
1.2.5 Tumor microenvironment recruitment of MSCs

The recruitment of MSCs to tumors has been reported in breast cancer, lung cancer, melanoma, gliomas and other solid cancers. (Hall et al., 2007; Kanehira et al., 2007; Studeny et al., 2004) Different mechanisms have been postulated for the recruitment of MSCs to the tumor microenvironment. Similarities between tumor-generated inflammatory profiles and the ones generated by tissue injury makes the native inflammatory response an intriguing mechanism for MSC recruitment (Dvorak, 1986). The tumor microenvironment is a “natural” source of inflammatory cytokines, chemokine and chemoattractants given the high degree of cellular injury and cell turnover. In addition to secreting immune responsive factors, tumor cells also express high levels of membrane-bound chemoattractants such as cell adhesion molecules (e.g. VCAM1, ICAM1 and TRL1) and receptor tyrosine kinases for VEGF (Burrell, Singh, Jalali, Hill, & Zadeh, 2014; Zimmerlin et al., 2013), HGF (Kanehira et al., 2007; Soleymaniejadian, Pramanik, & Samadian, 2012), PDGF-bb (Hata et al., 2010) and IGF-1, all of which are thought to contribute to the recruitment of MSCs (Spaeth, Klopp, Dembinski, Andreeff, & Marini, 2008).

Hypoxia, a condition that has been associated with the microenvironment of most tumors including GBM, may also contribute to MSC recruitment to tumor cells (Blouw et al., 2003; Du, Lu, et al., 2008; Ozawa et al., 2005). GBM tumor cells express hypoxia-induced transcriptional factor 1α (HIF-1α) which triggers the production of VEGF and, TNF-α, as well as the activation of the transcription factor nuclear factor κB (NFκB), all of which have implications in MSC recruitment (Aggarwal & Pittenger, 2005; Winner, Koong, Rendon, Zundel, & Mitchell, 2007). GBM-secreted HIF-1α can also recruit BM-MSCs through increased stromal cell-derived factor-1 (SDF1) (Du, Lu, et al., 2008; Tseng et al., 2011a).

In terms of specific factors that involved in MSC trafficking, several growth factors and cytokines, such as TNF-α, (Egea et al., 2011; Fogarty, Kessler, & Wechsler-Reya, 2005), interleukin 6 (IL-6) (Anton & Glod, 2014), C-X-C motif chemokine receptor 1 (CXCR1) (Schmidt et al., 2005), C-X-C motif chemokine ligand 12 (CXCL12) (Komatani, Sugita, Arakawa, Ohshima, & Shigemori, 2009), CCL2/MCP1, platelet-derived growth factor (PDGF) (Hata et al., 2010), and stromal cell-derived factor-1α a (SDF-1α), epidermal growth factor (EGF) but not vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), have been implicated in MSC migration to glioma tumors (Doucette et al., 2011). Moreover, extracellular signal-regulated kinase 1/2 (ERK1/2) has
been implicated in MSC recruitment, as inhibition of mitogen-activated protein kinase (MAPK) signaling obliterates the recruitment of MSCs (Egea et al., 2011; S. M. Kim et al., 2011).

1.2.6 The function of MSCs in the tumor microenvironment

The role of MSCs in GBM initiation and progression remains controversial. As it appears that both intrinsic/biologic factors such as tissue origin and paracrine secretion in response to the given tumor microenvironment, and extrinsic/technical factors such as the time of administration, quantity of cultured condition media when doing in vitro studies, and the number of MSCs in a given microenvironment, are all paramount to decide how MSCs affect TME (Behnan et al., 2014; Ho et al., 2013; Karnoub et al., 2007; Ströjby et al., 2014; Yang et al., 2014). For example, Kucerova et al have demonstrated in xenograft mouse models that in vivo co-injection of adipose tissue-derived MSCs (AT-MSCs) with GBM cells did not promote tumor growth, unlike co-injection of AT-MSCs with melanoma cells which promoted tumor growth (Kucerova, Matuskova, Hlubinova, Altanerova, & Altaner, 2010). Moreover, Akimoto et al found that conditioned media from AT-MSC can promote glioma development, possibly via neovascularization (Akimoto et al., 2013), while others have shown that that umbilical cord blood-derived MSCs may in fact inhibit glioma development in vitro (Yang et al., 2014). Glioma cells that were co-cultured with BM-MSCs were shown to have decreased proliferation potential (Ho et al., 2013), while BM-MSCs may promote melanoma and lung proliferation via enhanced neovascularization (Suzuki et al., 2011). It appears that MSCs promote tumorigenesis indirectly via augmented angiogenesis, instead of a direct proliferative effect on tumor cells.

As mentioned in 1.2.5, TME is a reservoir of inflammatory cytokines and chemokines. In fact, robust infiltration of microglia and microphages (both classic inflammatory responders in tumors), have been reported in gliomas (Zernecke et al., 2001). Oxidative stress and inflammatory factors, both of which are abundant in the TME may initiate a senescence state or irreversible growth arrest state in MSCs. Chronic exposure to such an environment may potentially oppose the innate immuno-modulatory effect of MSCs thereby facilitating tumor cell migration and proliferation in a pathological state (Davalos, Coppe, Campisi, & Desprez, 2010; Velarde, Demaria, & Campisi, 2013). In addition to these, it has been suggested that MSCs may provide hematopoietic support associated with the secretion of bio-active factors (Caplan & Dennis, 2006) and the release of trophic
factors in response to pathologic stimuli (Doucette et al., 2011; Ehtesham et al., 2004; Schmidt et al., 2005). In addition, MSC-derived exosomes contain large number of catalytic enzymes which allows for a robust response of MSCs to injury and disease states, thus, contributing to the maintenance of homeostasis in the given tissue microenvironment (R C Lai et al., 1007; Ruenn Chai Lai, Yeo, & Lim, 2015).

Several groups have reported that MSCs can be primed for chemotaxis upon exposure to inflammatory cytokines, and thereby develop chemotactic factor receptors, such as C-C chemokine receptor type 2 (CCR2) (Altman et al., 2011; Belema-Bedada, Uchida, Martire, Kostin, & Braun, 2008). MSCs also participate in immunomodulation upon exposure to paracrine factors such as hepatocyte growth factor-1 (HGF-1), transforming growth factor beta 1 (TGF-β1), prostaglandin E 2 (PGE2), nitric oxide (NO), Insulin-like growth factor (IGF) and indoleamine 2,3-dioxygenase (IDO), many of which can suppresses T-cell activations(Haniffa et al., 2007a) and inhibit monocyte differentiation into dendritic cells by induction of cell cycle arrest (Haniffa et al., 2007b). Furthermore, the immunosuppressive effects of MSCs can be enhanced in the presence of inflammatory cytokines such as tumor necrosis factor- alpha (TNF-α), and interferon-gamma (INF-γ) (Krampera et al., 2006). It has even been suggested that such environment is necessary for MSC-mediated immune suppression to occur (S. Jones, Horwood, Cope, & Dazzi, 2007).

Last but not least, data from our laboratory has shown that the recruited BDMCs co-localizing with the tumor-associated vasculature and give rise to perivascular or inflammatory cells rather than differentiating into ECs (Burrell et al., 2014). (Figure 1.2.6) This is in contrast to previous reports which suggested that glioma infiltrated BMDCs may differentiate into endothelial progenitor cells and subsequently become incorporated into the GBM neovascularization (Achyut et al., 2016).

In summary, MSCs are a subpopulation of BMDCs that are present within the TME having a myriad of roles in tumorigenesis including angiogenesis. MSCs are relatively resistant to conventional GBM therapies. Further understanding of the biological function of MSCs and the molecular control of their behavior in human TME is urgently needed in order to develop effective treatment strategies to target this cell type.
Figure 1.2.6: Characterization of Recruited BMDCs in GBMs. (A) immunofluorescence (IF) analysis and (B) in-vivo real-time 2PLM images show no evidence of co-localization of perivascular BMDCs and ECs in U87 xenograft mouse model. IF staining of (C) U87 xenograft mice (Green: cancer cells, blue: perspective cell markers, red: BMDCs) and (D) transgenic RASB8 mice show that the recruited BMDCs differentiate into IBA\(^+\), MAC\(^3\)+ and CX11b\(^+\) cells (Green: perspective cell markers, blue: nuclei, red: BMDCs). (Burrell et al., 2014)
1.3 Central hypothesis and specific aims

It is clear that MSCs are present within the GBM TME. MSCs support vasculature integrity with secretomes that contain pro-angiogenic factors. We therefore postulated that MSCs could protect ECs from IR by increasing EC survival. We therefore also hypothesize that differential gene expressions from conditions involving MSC co-culturing and IR may be predictive of response to anti-angiogenic therapy.

1.4 Hypothesis: MSCs confer GBM radio-resistance via promoting angiogenesis.

1.4.1 Aim 1: Establish the differential expression profile of ECs when “activated” by MSCs and ionizing radiation.

1.4.2 Aim 2: Ascertain the clinical relevance of the gene signature of activated ECs in predicting survival and response to anti-angiogenic therapy

1.4.3 Aim 3: Evaluate the protective role of MSCs on EC proliferation and in response to ionizing radiation
2 Materials and Methods

2.1 Cell lines

*Human Mesenchymal stem cells (hMSC):* Poietics™ hMSC (PT-2501) were purchased from Lonza (Basel, Switzerland). They were isolated from bone marrow that was withdrawn from the posterior iliac crest of healthy adult volunteers. The company has verified the chondrogenic, adipogenic and osteogenic potential of this product with guarantee of positive expression of CD29, CD44, CD105, CD166, CD90 and CD73 as well as negative expression of CD14, CD34, CD19 and HLA-DR.

*Human Umbilical-derived Vascular Endothelial Cells (HUVECs):* HUVECs were purchased from Lonza. The cells were maintained in endothelial growth medium (EGM™-2) from Lonza (Basel, Switzerland) referred as EGM hereafter, which was made of endothelial basal medium (EBM, CC-3156) and growth factors (Lonza, CC-4176; EGM-2 SingleQuot Kit Suppl. & Growth Factors).

*U87MG:* GBM cell lines were obtained previously from ATCC (Rockville, MD, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) from Wisent (Bioproducts, St. Brune, Quebec, Canada) supplemented with 10% fetal bovine serum (FBS) from Wisent and passaged as previously described (Burrell et al., 2014).

2.2 Experimental design

*Mono-cell culture (MC):* ECs, U87s, and MSCs were individually maintained in cell culture dishes with their respective growth medium.

*Double cell co-culture (CC):* HUVECs were seeded in 6-well tissue culture plates while human MSCs were seeded to transwell insert in their prospected growth media, such as DMEM H21 (for U87) EGM-2 (for HUVECs) and MSCGM (for MSCs) when the cells on both transwell insert and 6-well plates reached 85% confluency. Different cells lines were plated on the top of the insert and the transwell chamber and combined after adjusted confluency. (Figure 2.2)
2.3 Optimization of cell concentrations

To avoid cell inhibition of cells once they reach high confluence, we tried to find the optional cell concentration that allow the seeded MSCs or HUVECs to attach within 6 hours and reach 80% confluency within 24 hours. MSCs and HUVECs were suspended in MSCGM and EGM-2, respectively before seeded onto TC dishes at 5 concentrations (0.1, 0.3, 0.5, 1, and $2 \times 10^6$ cells per 6 cm$^2$ TC dish). The TC dishes were monitored at different time points (1, 6, 12, 18, 24, 36, 48 hours) for their morphologic changes and confluency. $0.5 \times 10^6$ MSCs and $2 \times 10^6$ HUVECs were established as the optimal concentrations in experiments throughout this thesis.

The morphology of MSCs under different IR dosages and growth media (i.e., MSCGM and EBM) were measured as a reflection of MSC radio-resistance. $0.5 \times 10^6$ MSCs were seeded in 6 cm$^2$ TC dishes and were irradiated by 0Gy, 2Gy and 15Gy before switching to EBM solution for 24 and 48 hours. Neither the morphology nor the confluency of MSCs was differentiated by IR dosages or media conditions at 24 hours. (See Figure 2.3)
Figure 2.3: Light microscopic image of MSCs in different condition media. The morphologies of MSCs remain constant under IR and EBM conditions.
2.4 Condition media collection

0.6 x 10^6 MSCs were seeded in 10-cm TC dish with MSCGM, (0.5 x 10^6 for U87, seeded in DMEM supplemented with 10%FBS). 24 hour later, or upon reaching ~85% confluence, the cells were gently washed twice with lukewarm PBS (Ca^{2+} and Mg^{2+} free). The media was then switched to 11 ml of endothelial basal medium (EBM). 24 hours later, the conditioned media was collected and centrifuged. The supernatant was collected as MSCCM (or U87CM, if conditioned U87s) and used immediately or preserved in -80°C. Supernatant was warmed to 37°C prior to use.

2.5 Irradiation treatment:

The cell lines (hMSC, HUVECs etc, excluding GSCs) were preferably seeded for at least 24 hours prior to radiation treatment to ensure attachment. Radiation treatments were delivered at room temperature, using a ^{137}Cs irradiator (Shepherd Mark-I, model 68) at a dose rate of 2.5Gy/min. Four dosages of ionizing radiation were employed in this study: 0, 2, 15, 20Gy. Cells were incubated in 37°C, 5% CO_2 both before and after irradiation. Two sets of time points will be use to harvest the cells: 1) Cells will either be collected at 0, 0.5, 1, 2, 4, 8 hours post IR treatment to assess immediate cellular response such as DNA repair; 2) or at 12, 24, 48 hours post IR treatment to determine the cell apoptosis and proliferation rate.

2.6 Angiogenic assay: real time PCR-based assay

*Design of angiogenic assay:* We designed an angiogenic array consisting of 30 key angiogenic genes (Table 1). The list of genes was generated upon comparing the genes in the pre-existing commercially available angiogenic assays with selected genes recognized to be critical angiogenic factors in the literature. PCR primers sequences (forward and reverse; see Table 1 Column 2&3) were obtained from commercially available Origene™ Technologies (Rockville) for each gene. Primers were sent to Integrated DNA Technologies (Coralville) for manufacturing.

*RNA extraction:* Total RNA was extracted using TRIzol reagent from Invitrogen. (Carlsbad, CA, USA). Suspended cells (such as GSCs) were pelleted and washed with ice-cold water while adherent cells (HUVECs, U87MG etc) were washed with ice-cold water directly. 500ul of TRIzol were added to each well of 6-well plate, the cell were then scraped and collected in ependof tubes. After 3 mins
incubation in room temperature, 1/5\textsuperscript{th} volume (100ul) of chloroform was added to each tube. The cells in the tubes were mixed well and spin at room temperature at 21130 RCF/g for 10 mins. The top aqueous layers were collected and transferred to a new tube and equal volumes of isopropanol were added to each tube. After thorough mix, the RNA tubes were then been centrifuged at 14000rpm for 30 mins at 4°C. The supernatant of the tube were then discarded. 750ul of ice-cold 80\% ethanol were added to each tube and centrifuged again at 14000rpm for 10 mins at 4°C. The ethanol was then carefully removed from each tube. The remaining RNA pellets were re-suspended in RNase-free water and quantified using Nanodrop 2000c Spectrophotometer (QIAGEN, Canada). They were stored in -80°C if not used right away.

Reverse-transcription reaction: QIAGEN quantiTech Reverse Transcription Kit was used to make cDNA. Template RNA Mix was made using 1 µg of template RNA, 7x gDNA Wipeout Buffer and RNase-free water. After 2 mins of incubation at 42°C, 1 µl each of reverse transcriptase and primer mix and 4 µl of RT buffer were added to the template RNA Mix and incubated at 42°C for 30 mins before 95°C for 3 mins. Each cDNA sample was diluted 20x and pipetted into 96 wells (biological triplicates for 32 genes) of a 384-well plate with SYBR green mix and ROX reference dye as per kit instructions.

Quantitative PCR: QuantiTech Reverse Transcription Kit (Invitrogen, Canada) was used to convert RNA to cDNA. 1ug of template RNA were used to yield 20ul of cDNA base on QIAGEN kit instruction. Each cDNA sample was diluted with 380 ul of DNase/RNase-free dH2O. 3ul of diluted cDNA was mixed with 5ul of master mix, latter contains 0.25ul of forward/reverse primers (10uM) and 0.75ul of dH2O and 4ul of SYBR green mix (2x), and added into 384-wells. The plates were then put into ViiA7 RT-PCR machine (Applied Biosystems, USA) and operated under QuantStudio Real Time software (Thermo Fisher Scientific, for quantitative PCR reaction. 30 primers of angiogenesis genes (see Table 1) were tested with beta actin and RPL30 as the control.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tr>
<td>hTGFβ1</td>
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<tr>
<td>h-Bactin</td>
<td>GATGCAGAAGGAGACATCAG</td>
<td>GATCTTACTGGCTCAGGAG</td>
</tr>
</tbody>
</table>

h: human, e.g. CD31: human CD31 gene
2.7 Bioinformatics analysis

Two clinical trial databases were used in this study. Glioblastoma (GBM) TCGA data was downloaded from TCGA Data Portal previously. The TCGA dataset contains 20,000 gene sequencing data for 150 GBM IDHwt patients. Same database can be obtained via NCI Genomic Data Commons (GDC) (https://gdc.nci.nih.gov/). The patients in TCGA dataset did not undergo any treatment. The AVAglio (Bevacizumab in Glioblastoma) patient dataset was downloaded from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84010). The AVAglio dataset contains 786 gene sequencing data on 349 GBM IDHwt patients. There are 171 of the patients were assigned to treatment group (Bevacizumab) and 178 of the patients were assigned to the placebo arm. Hierarchical clustering (HC) was conducted on both AVAglio database and TCGA database. The heat map was created using Partek Genomics Suite software (Partek, St. Louis USA). To investigate the clinical relevance of those cluster groups, we conducted Kaplan-Meier survival analysis on cluster groups using Partek Genomics Suite. We calculated p-values using a log-rank test. Spectral clustering was performed in Bioconductor R software (http://www.bioconductor.org/) and derived cluster membership accordingly. The gene expressions profiles of patients were recorded from naïve tumors for the AVAglio dataset (i.e., before they were exposed to any treatment or placebo). For AVAglio database, the 5 gene signatures were analyzed using spectral clustering (SC) algorithm in Similarity Network Fusion (SNF) package to obtain the gene clustering groups, and HC algorithm was applied to 1) check the consistency of cluster groups and robustness of the clustering methods we chose; 2) visualize the up and down regulation gene characteristics.

2.8 Trypan blue exclusion assay:

Equal numbers of cells were plated in triplicate or quadruplicate in six-well dishes in EGM-2 until optimal confluence, and then followed by 0, 2 or 15Gy of IR treatment. Cells were washed with 1x PBS at 2 hours post IR and switched to EBM or MSCCM of equal volume. At 24 or 48 hours post IR, the viability of cells was subsequently assessed by Trypan Blue exclusion assay (Sigma). At 24 and 48h post IR, cells were trypsinized and re-suspended in 500ul of PBS with a dilution factor of 2. 10ul of Trypan Blue solution was added into each tube and assessed using Vi-Cell™ XR Cell
viability analyzer. (Beckman Coulter Inc; Ontario, Canada) Viable cells with diameter of 5~50 microns were counted.

2.9 Western blot analysis

Whole cell lysate collection: To assess the level of DNA damage repair and apoptotic proteins, cells will grow on 6 cm or 10 cm plates. First submerge cells in 2mg/ml EBC lysis buffer (2M Tris pH 8, 5M NaCl, 100% NP-40 in water) that was supplemented with proteinase inhibitor and phosphatase inhibitor on ice. Samples will then be scraped off and collected in eppendorf tubes. Sonicating the eppendorf tubes in 4°C water for two 15-seconds interval separated with 15-seconds waiting period then sent to centrifuge at 1200 rpm for 10 mins, 4°C. The supernatant will be collected and quantified.

Bradford protein assay: Protein concentrations are quantified using Bradford assay. Add 5 ul of standards to each well of 96-well plate (BSA in EBC from with 8 increments of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0mg/ml). Add 5 ul of protein lysate (or 1ul of lysate plus 4 ul of EBC buffer so its 1:5 dilution) in each well of 96 –well plate. Add 250ul of Bradford buffer on top of each 5ul. Gently mix them by pipetting and measure the protein concentration with SpectraMax® 190 Microplate Reader form Molecular Devices (Sunnyvale, CA, USA). Sample buffer were added to the protein lysates before stored in -80°C.

Western Blot: Total of 20-30ug of cell lysate per sample will be separated on 8%, 10% and 12% SDS- polyacrylamide (SDS-PAGE) cell electrophoresis and transferred onto 0.2um or 0.4 um nitrocellulose membranes from BioRAD (Germany). After blocking the membrane with PBS (or TBS) containing 5% nonfat dry milk (BioShop, Burlington, Canada) and 0.1% Tween 20 from Sigma-Aldrich (St. Louis, MO, USA) for 1 hour, the membranes will be incubated with primary antibodies overnight at 4 degrees or 1 hour at room temperature. The following antibodies will be tested: 1:20,000 β-actin, p-Akt, p-ERK1/2, p-p53 (s15), and p21 (Sigma-Aldrich). Densitometry analysis will be performed using Image Studio™ Lite provided by LI-COR Biosciences (Lincoln, NE, USA).
Figure 2.9: Schematic representation of cell culture procedure for cell viability assay and western blot.

2.10 Tube formation assay

50ul of growth factor-reduced Matrigel matrix (BD biosciences; Mississauga, Ontario, Canada) was add to 96-well and allowed to polymerized for 30-6- minutes at 37°C incubator. After gel formation, cells of interested were re-suspended in base media (EBM for HUVEC, DMEM for U87 and DMEMH21:F12 for GSC267), and MSCCM made by conditioning MSC in respective base media. The cells were finally seeded at a density of 3 x 10^4/50ul to the matrigel well. Incubate the angiogenesis assay plate for 3 hours at 37°C, 5% CO2 atmosphere. The media were then carefully removed from the plates, and the plates were washed for HBSS by adding 200ul of HBSS to each well, and aspirated. And Cells were labeled by adding 50ul/well of 8ug/ml Corning Calcein AM in HBSS and incubated again for 30mins. Calcein AM solution were then removed from each well and washed with HBSS and washed each well with HBSS. White field and fluoresces images were
photographed with a phase contrast microscope (Olympus CK2; Olympus, Tokyo, Japan) coupled to a digital camera (Olympus DP21). Angiogenesis Analyzer from ImageJ software (http://imagej.nih.gov/ij/; provided by the National Institutes of Health in the public domain, Bethesda, MD, USA) was used to analyze the images.

2.11 Statistical analysis

All experiments were performed with 3-6 biologic replicates. Where appropriate, direct comparisons were conducted using an unpaired two-tailed Student’s t-test (for cell viability) or log-Rank algorithm (for gene clustering). * denotes significance defined as p<0.05. For the five treatment regimens we tested in this project; the results of angiogenic array were analyzed using cut-offs of either relative quantification (RQ>=2 or RQ=< 0.5) or Z-score (Z> 1.955 or Z<-1.955; Z=RQi-Mean(RQ))/StDev(RQ)) to decide the EC gene signatures.
3 Results

3.1 Aim 1 – Bone marrow-derived mesenchymal stem cells and IR result in differentially expressed gene sets in endothelial cells

The genomic instability of cancer cells causes a large number of mutations that provides evolutionarily favorable variants to thrive in the host and resist therapies more effectively. Therefore, targeting genetically stable “normal” cells within the TME that support the growth of the solid tumor might be a more effective approach to tackle GBM therapeutic resistance. Two such cell types are MSCs and ECs. The former is a stromal cell subpopulation that was commonly recruited to the site of tumor, while the latter contributes to the growth of neo-vasculature that is tightly involved in GBM tumorigenesis.

Radiation or IR is part of the 1st line treatment of GBM and demonstrates a bi-modal effect on ECs depending on the dosages and the specific microenvironment in which the ECs reside. Radiation is also a trigger for MSC recruitment, via generation of local inflammatory responses. Here we examined how MSCs, radiation or the combination of both, can influence the gene expression profiles of angiogenic factors in ECs. We postulated that the angiogenic gene signature might be used to predict patient survival as a surrogate of how aggressive a tumor is in response to therapy, and furthermore predict clinic responses to anti-angiogenic therapy.

3.1.1 The establishment of the HUVEC gene expression profiles in response to IR

We first aimed to establish the differential angiogenic gene expression profile in ECs in response to IR. We used an in-house angiogenic array, consisting of 30 angiogenic factors that are established and recognized to be involved in blood vessel formation (Table 1). We treated HUVECs with ionizing radiation at varying doses, 0, 2 and 15Gy. HUVECs were subsequently lysed 24 hours post IR followed by RNA extraction. The RNA samples were then reverse transcribed into cDNA and used as template for Real Time PCR with the primers listed in Table 1. HUVECs treated with 0Gy of radiation were used as baseline control. The relative quantification (RQ) values were normalized or reported as percentages compared to ECs with null IR treatment and null MSC co-culture.

We showed that IR augmented gene expression of angiogenic factors in ECs. With 2Gy of IR, three of the 30-angiogenic genes: CXCL5 (Figure 3.1.1-1), CXCL10 (Figure 3.1.1-2), matrix
metallopeptidase 9 (MMP9) (Figure 3.1.1-3) were unregulated with at least 2-fold in ECs. Similarly only one angiogenic factor was down regulated with less than 0.5 fold: hepatocyte growth factor (HGF) (Figure 3.1.1-4). See section 2.11 for cut-offs.

Figure 3.1.1-1: Relative gene expression of hCXCL5 in HUVECs 24 hours post IR. CXCL5 gene expression is IR-dose dependent and would get up regulated upon MSC co-culture.
Figure 3.1.1-2: Relative gene expression of hCXCL10 in HUVECs 24 hours post IR. 2Gy of IR was able to up-regulate CXCL10 regardless of MSC co-culture.
Figure 3.1.1-3: Relative gene expression of hMMP9 in HUVECs 24 hours post IR. 2Gy of IR was able to up-regulate MMP9 regardless of MSC co-culture, though with low statistical significance.
At 15Gy of IR, CXCL5 (Figure 3.1.1-1), fibroblast growth factor 2 (FGF2) (Figure 3.1.1-5), neuropilin-2 (NRP2) (Figure 3.1.1-6), platele-derived growth factor-A (PDGFA) (Figure 3.1.1-7), plasminogen activator inhibitor 1 precursor (SERPINE1) (Figure 3.1.1-8) and tissue factor (TF) (Figure 3.1.1-9) were upregulated in HUVECs while vascular cell adhesion molecule 1 (VCAM1) (Figure 3.1.1-10) was down regulated.

**Figure 3.1.1-4: Relative gene expression of hHGF in HUVECs 24 hours post IR.** No clean pattern between IR dosage and HGF gene expression. MSC alone was able to up-regulate HGF though such trend was not seen when combined MSC and 2Gy of IR. There were no clear trends implied by conditions that involve 15Gy of IR.
Figure 3.1.1-5: Relative gene expression of hFGF2 in HUVECs 24 hours post IR. 15Gy of IR was most efficient in up regulating FGF2 gene expression as compared to low dose of IR (i.e., 2Gy) as MSC co-culture.
Figure 3.1.1-6: Relative gene expression of hNRP2 in HUVECs 24 hours post IR. No clean pattern between MSC co-culture and HGF gene expression. The gene expression of NRP2 appeared to be IR dose dependent.
Figure 3.1.1-7: Relative gene expression of hPDGFA in HUVECs 24 hours post IR. No clean pattern between MSC co-culture and PDGFA gene expression. The gene expression of PDGFA appeared to be IR dose dependent.
Figure 3.1.1-8: Relative gene expression of hSERPINE1 in HUVECs 24 hours post IR. No clean pattern between MSC co-culture and SERPINE gene expression. The gene expression of SERPINE1 appeared to be IR dependent, however did not differentiated between 2Gy and 15Gy of IR.
Figure 3.1.1-9: Relative gene expression of hTF in HUVECs 24 hours post IR. No clean pattern between MSC co-culture and TF gene expression. The gene expression of TF appeared to be IR dependent, however did not differentiated between 2Gy and 15Gy of IR.
Figure 3.1.1-10: Relative gene expression of hVCAM1 in HUVECs 24 hours post IR. MSC co-culture alone was able to up regulate VCAM1 gene expression, even under 2Gy of IR. 15Gy of IR largely decrease the VCAM1 gene expression.
3.1.2 MSCs result in distinct gene expression profile on EC

In order to determine the effect of MSCs on ECs, we employed the same experimental set up to assess the angiogenic gene expression of ECs in response to MSC stimulation. HUVECs were co-cultured with MSCs in endothelial base media (EBM) for 24 hours (see Figure 2.2 for transwell co-culture system). Angiogenic array analysis indicated that MSCs up-regulated the gene expression of angiopoietin 1 (ANG1) (Figure 3.1.2-1), HGF (Figure 3.1.1-4), TF (Figure 3.1.1-9), VCAM1 (Figure 3.1.1-10) and vascular endothelial growth factor-c (VEGF-c) (Figure 3.1.2-2) in HUVECs. There was no gene expression down regulation in ECs when co-cultured with MSCs.
Figure 3.1.2-1: Relative gene expression of hANG1 in HUVECs 24 hours post IR. MSC co-culture alone was able to up regulate ANG1 gene expression, but no clear trend was identified under IR.
Figure 3.1.2-2: Relative gene expression of hVEGFC in HUVECs 24 hours post IR. MSC co-culture alone was able to up regulate VEGFC gene expression, however in less extend when combined with 2Gy, and none with combined with 15Gy. IR alone did not appear to affect VEGFC gene expression.
3.1.3 MSC-mediated pro-angiogenic profile in ECs upon IR

We next examined the expression profile of HUVECs when treated with IR with the presence of MSCs. When combined 2Gy of IR and MSCs co-culture, we saw the gene expression of ICAM1 (Figure 3.1.3-1), SERPIN1, TF, CXCL5 and CXCL10 (the latter two cytokines were shown to be up regulated by 2Gy alone previously) went up, while that of VCAM-1 went down. The protein expression of ICAM in HUVEC (Figure 3.1.3-2) in response to IR and/or MSC corroborated with RNA expression. In addition to the gene set that was altered by 15Gy of IR alone, the combination of MSCs co-culture and 15Gy also up-regulated the expression of HGF and FGF1 (Figure 3.1.1-4& 3.1.3-3) but not FGF2 (Figure 3.1.1-5) in HUVECs, although with little statistic significance (reflected by huge error bars) Surprisingly, despite distinctive and persistent dose-dependent up-regulation of TF at the RNA level in the presence of MSC, protein expression of TF remained the same across all conditions. (Figure 3.1.3-4)
Figure 3.1.3-1: Relative gene expression of hICAM1 in HUVECs 24 hours post IR. MSC co-culture alone was able to up regulate ICAM1 gene expression, however in less extend when combined with 2Gy, and none with combined with 15Gy. IR alone did not appear to affect ICAM gene expression.
Figure 3.1.3-2: A representative western blot result for the protein expression of ICAM in HUVECs 24 hours post IR. MSC co-culture alone was able to up regulate ICAM protein expression, however in less extend when combined with 2Gy, and none with combined with 15Gy. IR alone did not appear to affect ICAM protein expression.
Figure 3.1.3-1: Relative gene expression of hFGF1 in HUVECs 24 hours post IR. The expression of FGF1 is neither dose dependent nor MSC co-culture dependent
3.1.4 Discussion for aim 1:

To qualitatively understand the significance of ECs activation in tumor progression, we first tried to benchmark the gene expression profile of ECs in response to MSC and IR. We used ionizing radiation and mesenchymal stem cells as stimulators to generate 6 disrupted/activated states (one of which, the one with null IR and MSC treatment, was used as the baseline) in EC in vitro. We demonstrate that MSCs result in a distinct gene expression profile when used alone as a stimulator of EC, while IR elicited a differential responses based on radiation dose. Combination of MSC with IR alters the differential gene expression profile in EC. In vitro, 2Gy of IR increased CXCL5 and CXCL10, both can act as chemokines and modulators of inflammatory response in GBM tumors (Reynolds et al., 2016). There are previous studies to show that they can be triggered by TNFα (P. F. Yu et al., 2016) and IL-1β (Balyasnikova et al., 2014; Bertazza & Mocellin, 2010; Guha, Klamar, Reinhart, & Ayyavoo, 2015). CXCL5 is also called epithelial-derived neutrophil-activating peptide, hinting at its ability to attract neutrophils that mediate angiogenesis and potentially immune responses (Keglowich, Tamm, Zhong, Miglino, & Borger, 2014). While overexpression of CXCL10 and its receptor, CXCR3, have been correlated with heightened tumor progression in GBM (Sharma, Siraj, Sharma, & Singh, n.d.), CXCL10 was also reported to down-regulate the angiogenic process (Reynolds et al., 2016). 2Gy of IR also increased MMP9, a gene that codes for signal peptides that can break up extracellular matrix. There is a concurrent decreased HGF expression with 2Gy IR, a
growth factor that is recognized for contributing to vascular stability (Q.-H. Chen, Liu, Qiu, & Yang, 2015; Nagase & Woessner, 1999).

In addition to the inflammatory factors that were seen in HUVECs treated with 2Gy of IR, higher dose of IR (15Gy) also stimulated growth factors in HUVECs such as FGF2, a cellular stress marker (Gama Sosa, De Gasperi, Hof, & Elder, 2016); PDGFA, an cell surface marker that mediates angiogenesis and pro-inflammatory reaction (Verstraete et al., 2015); Neuropilin 2, co-receptor for VEGFR-2/3 that contributes to endothelial cell survival and migration (Favier et al., 2006); as well as TF and SERPINE1, which are key mediators for thrombotic cascades (Campello et al., 2011; O’Reilly, Casper, Otto, Sexton, & Swerlick, 2003; Rak et al., 2006; Szotowski et al., 2007). Intracellular gradient of TF has a biphasic effect on endothelial cells viability: moderate TF expression in EC promote cell proliferation via integrin-mediated ERK-MAPK pathway (Collier & Ettelaie, 2010); overexpression of TF will cause EC apoptosis via stabilization of p53 inside nucleus, causing up-regulation of bax and p21 that lead to apoptosis, such signaling transduction can be prevented by suppressing p38α (ElKeeb, Collier, Maraveyas, & Ettelaie, 2015). However, neither MSC nor IR appeared to up-regulate the protein expression of TF in HUVECs as to its gene expression. (Figure 3.1.3-3) This could due to the high TF protein expression in our control group, which masked the relatively smaller increase of protein expression in ECs upon IR. Lastly, the decrease of VCAM1 in HUVECs caused by 15Gy of IR has functional implication in vascular permeability. Lower IR dose turn to cause inflammatory response and instability while higher IR dose provoked chemokines as well as factors that pertain to vascular instability and thrombotic reaction in HUVECs.

MSC co-culture with ECs on the other hand caused a gene expression profile that mostly resembles vascular maturation, such as up-regulation of ANGI (the ligand for Tie-2, Figure 3.1.2-2), HGF (Figure 3.1.1-4), VEGF-c (Figure 3.1.2-2) and VCAM1 (Figure 3.1.1-10). Tissue factor was also upregulated (Figure 3.1.1-9). The connection between MSCs and HGF secretion for endothelial cells permeability has been reported in the literature (Q.-H. Chen et al., 2015). MSC co-culture also up-regulated VEGF-c in ECs. VEGF-c belongs to the large VEGF superfamily, and primarily binds to VEGFR-3 and promotes endothelial cell survival with additional major roles in lymphatic vessel growth (Joukov et al., 1996). The heightened TF expression added another dimension to MSC’s seemingly angiogenic, vascular maturing effect. However protein expressions did not vary between different media conditions and IR treatments (Figure 3.1.3-3). Since the amount of TF detected was
very high across all conditions, there is a possibility that IR or MSC increased TF was masked by its baseline TF expression, i.e., they were saturated.

The presence of MSCs had little effect on the expression of IR-induced inflammatory and thrombotic factors in HUVECs directly. However MSCs increased the expression of adhesion molecules and growth factors, for example, HGF and VCAM1, both of which can potentially antagonize the vascular instability secondary to inflammatory and the thrombotic responses of ECs caused by IR.

This set of data suggested a plausible and exciting potential: these expression profiles potentiated by IR signified an inflammatory, thrombotic and unstable state which can contribute to more aggressive transformation or progression of tumor growth therefore a worse outcome. MSCs in turn counter the disruption caused by IR via stabilizing endothelial cells. Stabilized endothelial cells translated to vascular homeostasis therefore can potentially fuel tumor growth as well as better perfusion of anti-tumor drugs.

3.2 Aim 2 – clinical inference of ECs gene expression profiles

AATx, particularly the ones targeting vascular endothelial growth factor, have shown efficacy in preclinical and early phase human clinical studies in patients with glioblastoma. However, this response is variable across different subtypes, and therefore prognostic markers that can predict response to AATx are needed. Having established differential angiogenic profiles for the ‘activated’ ECs we next explored whether these gene sets predict and distinguish specific subsets of GBM clinically, with respect to existing molecular subclasses or outcomes. To accomplish this aim, we correlated the gene profiles that were established from different combination of IR and MSCs to the clinical outcome of GBA patient databases. We analyzed two distinct GBM patient databases, the TCGA GBM database and the AVAglio database. The AVAglio database has a smaller gene sequence repository (786 genes) as compared to the TCGA database (20,000 genes). See section 3.8 for more detail regards to the databases. For analysis on AVAglio patient outcomes we focused on the common denominator genes within the gene sets from previous chapters.
3.2.1 Correlation of differential angiogenic gene signature profiles to survival in TCGA GBM patients

We examined five angiogenic profiles that were introduced in Chapter 5. Gene set A: angiogenic factors up-regulated in response to 2Gy IR (CXCL5, CXCL10, MMP9 and down regulation of HGF), Gene set B: angiogenic factors up-regulated in response to 15Gy (CXCL5, FGF2, NRP2, PDGFA, SERPINE1, TF and down regulation of VCAM1), Gene set C: angiogenic factors up-regulated in response to MSC alone (ANG1, HGF, TF, VCAM1, VEGF-c), Gene set D: angiogenic factors up-regulated in response to MSC plus 2Gy of IR (CXCL5, CXCL10, ICAM1, SERPINE1, TF, VCAM1) and Gene set E: angiogenic factors up-regulated in response to MSC plus 15Gy of IR (CXCL5, FGF1, HGF, NRP2, PDGFA, SERPINE1, TF and down regulation of VCAM1) (Table 2).

Table 2: List of gene sets that were significantly altered in HUVECs upon IR and/or MSC

<table>
<thead>
<tr>
<th>Gene Set A</th>
<th>Gene Set B</th>
<th>Gene Set C</th>
<th>Gene Set D</th>
<th>Gene Set E</th>
</tr>
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<tbody>
<tr>
<td>2Gy</td>
<td>15Gy</td>
<td>MSC 0Gy</td>
<td>MSC 2Gy</td>
<td>MSC 15Gy</td>
</tr>
<tr>
<td>CXCL10</td>
<td>CXCL5</td>
<td>ANG1</td>
<td>CXCL10</td>
<td>CXCL5</td>
</tr>
<tr>
<td>CXCL5</td>
<td>FGF2</td>
<td>HGF</td>
<td>CXCL5</td>
<td>FGF1</td>
</tr>
<tr>
<td>MMP9</td>
<td>NRP2</td>
<td>TF</td>
<td>ICAM1</td>
<td>HGF</td>
</tr>
<tr>
<td>PDGFA</td>
<td>VCAM1</td>
<td>SERPINE1</td>
<td>NRP2</td>
<td></td>
</tr>
<tr>
<td>SERPINE1</td>
<td>VEGFC</td>
<td>TF</td>
<td>PDGFA</td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>VCAM1</td>
<td>SERPINE1</td>
<td>TF</td>
<td></td>
</tr>
<tr>
<td>HGF</td>
<td>VCAM1</td>
<td>VCAM1</td>
<td>TF</td>
<td></td>
</tr>
</tbody>
</table>

HC analysis of these five gene sets on the TCGA GBM database showed significant patient survival difference, we found that clustering of gene set A, separated patients into two groups (Figure 3.2.1-1a). Patients that were clustered as Group 2 possess gene expression that resembles that of ECs when receiving 2Gy of IR, whereas patients in Group 2 have the opposite of gene expression profile.
Group 2 demonstrated a worse patient survival compared to Group 1 when plotted to the patient overall survival of the TCGA database with approximately 3.6 months of survival difference. (Figure 3.2.1-1b; P-value= 0.02) Moreover, about 76% of the GBM patients in Group 2 correlated with mesenchymal subtype, while patients from Group 1 were equally distributed in all 4 subtypes of GBM. (Table 3)
Figure 3.2.1-1a: Hierarchical clustering of GBM patient database from TCGA on gene set A. The hierarchical clustering algorithm generated two patient groups based on their gene expression within gene set A.
Figure 3.2.1-1b: The Kaplan-Meier overall survival curve of patient clustering groups generated by hierarchical clustering on TCGA database using gene set A. Group 2 (in red) depicts the gene cluster that resembles the gene set A characteristics, whereas Group 1 (in green) resembles the opposite of said gene signature. The overall survival was shown in blue. Group 2 has a significant worse survival than Group 2 (p-value < 0.05). Use median survival as a reflection, there is nearly 3.6 mo difference between Group 1 and Group 2.
The same hierarchical clustering analysis on the TCGA database for gene set B generated two clustering groups with group 2 representing the up-regulation of most of the genes in the list. (Figure 3.2.1-2a) Similar to gene set A, the TCGA patients that were clustered into Group 2 by using gene set B had worse survival disadvantage comparing to Group 1. (Figure 3.1.2-2b; P-value= 0.085) The patients within Group 2 were primarily (80%) diagnosed as mesenchymal subtype whereas an even distribution in four GBM subtypes was observed in Group 1 patients. (Table 4)
Figure 3.2.1-2a: Hierarchical clustering of GBM patient database from TCGA on gene set B. The hierarchical clustering algorithm generated two patient groups base on their gene expression within gene set B.
Figure 3.2.1-2b: The Kaplan-Meier overall survival curve of patient clustering groups generated by hierarchical clustering on TCGA database using gene set B. Group 2 (in red) depicts the gene cluster that resembles the gene set B characteristics, whereas Group 1 (in green) resembles the opposite of said gene signature. The overall survival was shown in blue. Group 2 has a marginally worse survival than Group 2.
We next examined if by combining the angiogenic profile of gene set A & B would generate similar patient outcome. This combined angiogenic profiles (Figure 3.2.1-3a) divided the two patient groups with the biggest survival difference of approximately 3.7 months. (Figure 3.2.1-3b; p-value= 0.009)

When we looked at the distribution in GBM subtypes, not surprisingly, majority (81%) of the Group 2 patients have been diagnosed as mesenchymal subtype. (Table 5)

Table 4: GBM subtypes of gene set B-generated TCGA patient clustering groups

<table>
<thead>
<tr>
<th>Number of occurrences</th>
<th>Proneural</th>
<th>Neural</th>
<th>MES</th>
<th>Classical</th>
<th>Total</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

<table>
<thead>
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<th>MES</th>
<th>Classical</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
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<td>34%</td>
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<td>100%</td>
<td>77%</td>
</tr>
<tr>
<td>Group2</td>
<td>3%</td>
<td>11%</td>
<td>80%</td>
<td>6%</td>
<td>100%</td>
<td>23%</td>
</tr>
</tbody>
</table>
Figure 3.2.1-3a: Hierarchical clustering of GBM patient database from TCGA on gene set A&B. The hierarchical clustering algorithm generated two patient groups base on their gene expression within gene set A&B.
Figure 3.2.1-3b: The Kaplan-Meier overall survival curve of patient clustering groups generated by hierarchical clustering on TCGA database using gene set A&B. Group 2 (in red) depicts the gene cluster that resembles the gene set A& B characteristics, whereas Group 1 (in green) resembles the opposite of said gene signature. The overall survival was shown in blue. Group 2 has a significantly worse median overall survival than Group 2 by nearly 3.7 months (p
Interestingly, when clustering gene profiles of gene set C, D, E, or the combination of them, the resulted gene expression profiles did not differentiate in terms of the patient survival of their perspective gene clustering groups (Figure 3.2.1-4-7).

<table>
<thead>
<tr>
<th>Number of occurrences</th>
<th>Proneural</th>
<th>Neural</th>
<th>MES</th>
<th>Classical</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
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<td>19</td>
<td>21</td>
<td>28</td>
<td>93</td>
</tr>
<tr>
<td>Group2</td>
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<td>46</td>
<td>3</td>
<td>57</td>
</tr>
<tr>
<td>Percentage</td>
<td>Proneural</td>
<td>Neural</td>
<td>MES</td>
<td>Classical</td>
<td>Total</td>
</tr>
<tr>
<td>Group1</td>
<td>27%</td>
<td>20%</td>
<td>23%</td>
<td>30%</td>
<td>100%</td>
</tr>
<tr>
<td>Group2</td>
<td>0%</td>
<td>14%</td>
<td>81%</td>
<td>5%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 5: GBM subtypes of gene set A&B-generated TCGA patient clustering groups
Figure 3.2.1-4a: Hierarchical clustering of GBM patient database from TCGA on gene set C. The hierarchical clustering algorithm generated three patient groups based on their gene expression within gene set C.
Figure 3.2.1-4b: The Kaplan-Meier overall survival curve of patient clustering groups generated by hierarchical clustering on TCGA database using gene set C. None of the group differentiated the patient survival.
Figure 3.2.1-5a: Hierarchical clustering of GBM patient database from TCGA on gene set D. The hierarchical clustering algorithm generated two patient groups base on their gene expression within gene set D.
Figure 3.2.1-5b: The Kaplan-Meier overall survival curve of patient clustering groups generated by hierarchical clustering on TCGA database using gene set D. None of the groups differentiated the patient survival.
Figure 3.2.1-6a: Hierarchical clustering of GBM patient database from TCGA on gene set E. The hierarchical clustering algorithm generated two patient groups based on their gene expression within gene set E.
Figure 3.2.1-6b: The Kaplan-Meier overall survival curve of patient clustering groups generated by hierarchical clustering on TCGA database using gene set E. None of the group differentiated the patient survival.
3.2.2 Correlation of differential angiogenic gene signature profiles to survival in AVAglio GBM patients

We next sought to determine whether the angiogenic gene profiles (Gene set A~E) have specific implication in patient response to AATx. We applied SC algorithms with gene set A~E to the AVAglio patient database (see section 3.8 for details regard to the database). Interestingly, out of the five groups, gene set A (specifically HGF and CXCL10 generated three different gene clusters (P-value= 0.013) in the treatment arm, coined group 1, 2 and 3 (Figure 3.2.2-1A). Post-algorithm analysis (Kaplan-Meier estimator) of the three SC-generated groups in terms of patient overall survival revealed distinct outcomes. (Figure 3.2.2-1B) The three groups corresponded to significantly different patient OS with Group 2 having the worst median OS by 5 months compared to Group 1 and 3 (Figure 3.2.2-1B). What’s more, when using HC algorithm as previously described on the same gene list for the treatment arm, the result corroborated the SC algorithm, as there was 100% concordance, suggesting the robustness of our algorithms. (Figure 3.2.2-1C) The HC algorithm also allowed visualization of gene characteristics exhibited as down-regulations of both CXCL10 and HGF for Group 1 (in green), down-regulation of CXCL10 and up-regulation of HGF for Group 2 (in red), and up-regulation of CXCL10 and down-regulation of HGF for Group 3 (in blue) exhibited (Figure 3.2.2-1C) However, when applied the same analysis to the placebo arm of the AVAglio database using the same gene set, we found that a.-the SC algorithm did not generate gene clusters with differentiated patient survival (Figure 3.2.2-2A&B); b.-The SC algorithm-generated result have a low level of concordance with the HC algorithm generated result (Figure 3.2.2-2C). We also applied SC algorithm for the Kaplan-Meier progression free survival curves in both the treatment arm and placebo arm; a similar trend was found whereas the HGF & CXCL10 signature only generated groups with differentiated survival at treatment arm (Figure 3.2.2-3A, P-value = 0.0007) but not placebo arm (Figure 3.2.2-3B, P-value = 0.141).
Figure 3.2.2-1: Generation of prognostic groups and signature analysis based on expression levels of gene set A on AROglio dataset treatment arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups, there is a 5-month median survival difference between group 2 and group 1&3; (C) Hierarchical clustering generated using gene set A shown in heat map.
Figure 3.2.2-2: Generation of prognostic groups and signature analysis based on expression levels of gene set A on AVAglio dataset **placebo arm**. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Hierarchical clustering generated using gene set A shown in heat map.
Figure 3.2.2-3: Prognostic signature analysis base on expression levels of gene set A on AVAglio dataset. (A) Kaplan-Meier progression free survival curves in treatment arm based on SNF-generated clustering groups; (B) Kaplan-Meier progression free survival curves in placebo arm based on SNF-generated clustering groups

The same bioinformatics analysis using Gene set B–E were applied on AVAglio database, however none of them generated positive result as gene set A, see appendix for details (Figure 3.2.2-4~11).
Figure 3.2.2-4: Generation of prognostic groups and signature analysis base on expression levels of gene set B on AVAglio dataset treatment arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set B shown in heat map.
Figure 3.2.2-5: Generation of prognostic groups and signature analysis base on expression levels of gene set B on AVAglio dataset placebo arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set B shown in heat map.
Figure 3.2.2-6: Generation of prognostic groups and signature analysis base on expression levels of gene set C on AVAglio dataset treatment arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set C shown in heat map.
Figure 3.2.2-7: Generation of prognostic groups and signature analysis based on expression levels of gene set C on AVAglio dataset placebo arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression-free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set C shown in heat map.
Figure 3.2.2-8: Generation of prognostic groups and signature analysis base on expression levels of gene set D on AVAglio dataset treatment arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set D shown in heat map.
Figure 3.2.2-9: Generation of prognostic groups and signature analysis base on expression levels of gene set D on AVAglio dataset placebo arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set D shown in heat map.
Figure 3.2.2-10: Generation of prognostic groups and signature analysis base on expression levels of gene set E on AVAglio dataset treatment arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set E shown in heat map.
Figure 3.2.2-11: Generation of prognostic groups and signature analysis based on expression levels of gene set E on AVAglio dataset placebo arm. (A) Spectral clustering in SNF package estimated clustering groups; (B) Kaplan-Meier overall survival curves based on SNF-generated clustering groups; (C) Kaplan-Meier progression-free survival curves based on SNF-generated clustering groups; (D) Hierarchical clustering generated using gene set E shown in heat map.
3.2.3 HGF and CXCL10 individually did not differentiate clinical outcomes in AVAglio patients

In order to account for the possibility that one of the two genes might be the main indicator for patient survival, we applied SC algorithm on treatment arm base on the up-regulation and down-regulation of HGF (Figure 3.2.3A, P-value= 0.15) and CXCL10 (Figure 3.2.3B, P-value= 0.44) individually. Neither of the genes alone was capable of differentiating patient survival as shown in Figure 3.2.3.

Figure 3.2.3: Prognostic signature analysis base on individual gene expression levels in AVAglio dataset treatment arm. (A) Kaplan-Meier overall survival curves based on HGF expression; (B) Kaplan-Meier overall survival curves based on CXCL10 expression.
3.2.4 The **CXCL10, HGF** combined genotype preferentially predicts patient’s response to anti-angiogenesis therapy

We next want to determine whether the aforementioned genetic profile (**HGF & CXCL10**) can predict patient OS in GBM patients who did not receive AATx to ensure it was a specific signature relevant to the predictive power for the Bevacizumab efficacy. We performed SC and HC algorithms on the GBM database form TCGA using HGF and CXCL10. The bio-informatics analysis generated two gene clusters: Group 1 showed low levels of both **CXCL10** and **HGF** whereas Group 2 showed high levels of **CXCL10** and **HGF**. (Figure 3.2.4A) However, these two groups did not appear to differentiate patient OS and PFS. (Figure 3.2.4B)

![Figure 3.2.4: Generation of prognostic groups and signature analysis base on expression levels of gene set A on TCGA GBM database.](image)

**(A)** Kaplan-Meier overall survival curves based on SNF Groups

**(B)** Hierarchical clustering generated using gene set A shown in heat map.
3.2.5 Quantitative PCR analysis revealed high expression of HGF and low expression of CXCL10 in cancer cells and MSCs relative to HUVECs

Since GBM tumors, are heterogeneous entities composed of tumor, stromal and immune cells, we wanted to know if the HGF and CXCL10 expression pattern is EC-specific. We tested the RNA levels of HGF and CXCL10 genes in U87s, MSCs and HUVECs. We also investigated the effect of MSCs or IR (0, 2, 15Gy) treatments on the 3 cell lines. The data showed that for the same cell type, the expressions of HGF and CXCL10 were independent of MSCs and IR dosages. For example, HUVECs that received 0, 2, 15Gy of IR and/or MSCCM produced marginal differences in the HGF and CXCL10 expressions as compared to HUVECs that received null treatment. (Figure 3.2.5) When compare in between different cell types, relative to HUVECs, MSCs on average have 3000 times higher amount of HGF whereas U87s have on average 13000 times higher amount of HGF expression than that of HUVECs (Figure 3.2.5A). Therefore, IR and MSCCM have marginal effects on the level of CXCL10 in HUVECs. Furthermore, the baseline expressions of CXCL10 in HUVECs are higher than that of MSCs and U87s and remain such trend upon of IR and MSC treatment (Figure 3.2.5B).
Figure 3.2.5: Quantitative PCR analysis of gene expression in HUVECs, MSCs and U87s under different conditions. (A) The gene expression of HGF; (B) The gene expression of CXCL10.
3.2.6 Discussion for aim 2:

Apart from the molecular subtypes and genetic alterations that pertain to tumor cells (such as, IDH mutation and 1p19q co-deletion) (Sandmann et al., 2015; Thomas et al., 2013), TME determinants can make large contribution in both GBM progression and therapeutic efficacy. It is recognized that ECs of the tumor blood vessel are not necessarily quiescent. The above experiments are an in vitro experimental model to establish the gene expression profiles in ECs when they are “activated” by IR and MSC. We used these gene sets for clustering the GBM database from TCGA for patient survival. We found that the group 2 of the gene clusters generated using gene sets A&B (i.e, gene signatures generated by irradiating ECs) were correlated with worse patient survival (Figure 3.2.1-1&2). A worse patient survival of nearly 24 months was observed in group 2 relative to that of group 1. (Figure 3.2.1-3, p-value= 0.001) The same group (shown as Group 2 in Table 3) also has as high as 81% of the patients that were diagnosed as mesenchymal subtype; the latter was associated with the worst prognosis among all 4 subtypes of GBM; whereas group 1 did not differentiate between 4 subtypes of GBM. (Table 5) Later when applied the same hierarchical clustering analysis to gene sets that acquired from MSC co-culture or irradiation combined with MSC co-culture, no significant difference in patient survival was found. (Figure 3.2.1-4~7, p-value > 0.05 or of little biological significance) Such observation suggested prognostic potential for the “activated” EC gene expression profile in GBM.

To further explore and refine the prognostic potential of the “activated” EC gene expression profile with respect to AATx, we then performed both SC algorithm and HC algorithm on AVAglio GBM database based on the gene set A~E. We analyzed the clinical responses of each group for both the treatment arm and the placebo arm of AVAglio patients and found that only the gene set A, more specifically, the combination of CXCL10 and HGF alterations separated the treatment group of AVAglio patients into 3 groups (Group 1–3) with differentiated clinical outcomes (Figure 3.2.2-1A&B). Group 2 with down-regulation of CXCL10 and up-regulation of HGF distinguished itself from the other two groups by correlating to both the worst patient OS (Figure 3.2.2-1A&B; p-value= 0.0139) and the worst PFS (Figure 3.2.2-3A, p-value= 0.0007) in AVAglio treatment arm but not in the placebo arm (Figure 3.2.2-2, Figure 3.2.2-3B). The connection between HGF secretion and endothelial cell permeability has been reported in the literature where they showed decreased para-vascular and trans-cellular permeability resulting from a MSC-medicated HGF-rich microenvironment (Q.-H. Chen et al., 2015). We also looked at the individual gene expressions of
HGF and CXCL10 in the context of patient survival and found that they do not differentiate clinical outcome when used alone. (Figure 3.2.3) Our data suggested that the subpopulation of GBM patients with up regulation of HGF and down regulation of CXCL10 is correlated to the worst clinical response with AATx. Therefore, the high HGF and low CXCL10 gene expression profiles in GBM patients could be a convincing prognostic indicator for negative Bevacizumab response (i.e., worse clinical outcome). Our results thereby draw interesting connections between the HGF and CXCL10 genes and AATx-resistance, latter often partnered with heightened tumor invasion and metastasis in GBM (Ellis & Reardon, 2009).

Since the AVAglio database was established from patient samples that were taken non-discriminatorily from the tumor tissue instead of isolated ECs from the tumor, we tested the HGF and CXCL10 levels in MSCs and U87s under different stimuli (i.e., IR and MSCs). The drastically higher expression of HGF in U87 cells and MSCs (to a lesser extent, however physically closer to ECs) relative to endothelial cells corroborated with the postulation that the tumor cells and MSCs could be the major sources of HGF levels for ECs. (Figure 3.2.5) Furthermore, such genetic expression patterns could be a whole TME feature instead of that for a single cell type.
3.3 Aim 3 – bone marrow-derived MSCs increase EC viability upon IR

Propelled by the proangiogenic and mitotic gene expression profiles that are mediated by MSCs in endothelial cells and to further explore the mechanisms by which MSCs assists in mitigating IR-induced apoptosis, we attempted to dissect different stages of cellular response for IR injury in endothelial cells. Co-culture systems established earlier in the literature and cell viability assay were applied to differentiate between insufficient DNA damage repairs from lost reproductive integrity for cells that receive IR.

Ionizing radiation (IR) is part of the standard care for post-surgical treatment of GBM. The cell-killing effect of IR is largely rested on its ability to cause double stranded DNA breaks (DSB) in rapidly dividing cells (Naidu et al., 2010; Valerie et al., 2007). Yet MSCs can up-regulate genes that involved in DNA damage response and DNA repair for ECs and cancer cells (Hass & Otte, 2012; R C Lai et al., 1007; Prockop, 2009). Collectively, the IR-conditioned cell cycle dynamics, apoptosis, differentiation capacity can change the course of which MSC cultivating tumor microenvironment in a temporal manner.

3.3.1 MSCCM promotes EC viability in vitro upon ionizing radiation

We employed trypan blue exclusion assays to study whether MSCCM promotes EC viability in the context of ionizing radiation. At 24 hours, we did not observe any significant differences in viable cell number counts between EBM and MSCCM upon ionizing radiation, nor did MSCCM provide any proliferative advantage under null IR treatment. (Figure 3.3.1A) At 48 hours, 2Gy of IR marginally decreased HUVEC viable cell number in EBM condition by 15% while 15Gy of IR decreased HUVEC proliferation in EBM condition by 29%. In contrast, MSCCM was able to increase the cell proliferation of HUVECs by 2 folds at 48 hours, and approximately 80% and 50% increases when HUVECs were irradiated with 2Gy and 15Gy respectively. (Figure 3.3.1B)
Figure 3.3.1: Trypan blue elusion assay analysis for HUVECs. (A) The viability of HUVECs at 24 hours post IR under EBM and MSCCM; (B) The viability of HUVEC at 48 hours post IR under EBM and MSCCM.

* p-value < 0.05
3.3.2 MSCCM promotes EC survival via phosphorylation of Akt but not ERK1/2 upon IR

In the absence of IR (i.e., 0Gy), both EBM and MSC-conditioned EBM (MSCCM) caused increases in Akt phosphorylation, but MSCCM triggered higher levels of Akt phosphorylation in HUVECs as early as 2 hours after media switch, and this continuously increased to larger extents than EBM increased p-Akt for at least 4 more hours. IR in general decreased p-Akt levels in HUVECs compared to null IR treatment, whether they were cultured under EBM and MSCCM conditions (Figure 3.3.2-1). At the same time frame as the assessment of Akt phosphorylation, the phosphorylation of ERK1/2 remained constant either in response to IR alone or IR in combination of MSCCM (Figure 3.3.2-2).

![Figure 3.3.2-1: A representative western blot for Akt phosphorylation in HUVECs at different time points, IR dosages, and media conditions.](image-url)
MSCCM does not alter double stranded DNA damage repair

At 1-hour post IR, phosphorylation of p53 at serine 15 was detected in HUVEC. The level of p53 phosphorylation is directly proportional to IR dosage (Figure 3.3.3-3A). The same trend was seen in both EBM and MSCCM (Figure 3.3.3-1A). 2Gy of IR caused increase of p53 phosphorylation in HUVECs immediately after receiving IR and decreased after 16 hours. 15Gy of IR triggered higher levels of p53 phosphorylation and decreased at a slower rate at 16 hours in HUVEC without MSC co-culture as opposed to MSC co-cultured HUVECs (Figure 3.3.3-1B).

The up-regulation of p21 is IR dose dependent and independent of the MSC co-culture system at 24 hours post IR as shown in Figure 3.3.3-2A. The up-regulation of p21 in HUVEC's was detected as early as 4 hours post IR (Figure 3.3.3-2B). 15Gy of IR induced greater p21 protein expression than 2Gy of IR and carried on to 24 hours post IR, as seen in Figure 3.3.3-2C.

Figure 3.3.2-2: A representative western blot for Erk phosphorylation level in HUVECs at different time points, IR dosages, and media conditions.
Figure 3.3.3-1: Representative western blot of P53 phosphorylation in HUVECs. (A) p-P53 expression in HUVECs at 1 hour post IR under different media conditions; (B) p-P53 expression in HVUECs at different time points and IR dosages and MSC co-culture.
3.3.4 MSCCM might alter HVUECs’ response to IR-provoked DNA damage

Both 2Gy and 15Gy was able to induce ATM phosphorylation in HUVECs immediately. Lower IR dose (i.e., 2Gy)-induced p-ATM signal faded out after 8 hours under MSCCM as contrast to EBM, which the signal persisted. (Figure 3.3.4-1) IR also increased PARP cleavage in HUVECs, a later event that appeared more prominently at 8 hours post IR in all media conditions. (Figure 3.3.4-2) Higher c-PARP level was detected in HUVECs under MSCCM at 8 hours as compare to that of HUVECs under EBM, regardless of the dosage of IR that induced it. (Figure 3.3.4-2)
Figure 3.3.4-1: A representative western blot of ATM phosphorylation in HUVECs. The expression of p-ATM in HUVECs at 24 hours post 0Gy, 2Gy and 15Gy under different media conditions.

<table>
<thead>
<tr>
<th>Hours post 2Gy</th>
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<th>EBM</th>
<th>MSCCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ATM(1981)</td>
<td>-1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hours post 15Gy</th>
<th>EGM</th>
<th>EBM</th>
<th>MSCCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ATM(1981)</td>
<td>-1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 3.3.4-2: A representative western blot of cleaved PARP in HUVECs. The expression of c-PARP in HUVECs at different time points post 2Gy and 15Gy under different media conditions.

<table>
<thead>
<tr>
<th>Hrs post IR</th>
<th>Media condition</th>
<th>IR dose</th>
<th>EGM</th>
<th>EBM</th>
<th>MSCCM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGM</td>
<td>0Gy</td>
<td>-1</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>EBM</td>
<td>2Gy</td>
<td>0</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>MSCCM</td>
<td>15Gy</td>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

| c-PARP/GAPDH | 1.0 | 0.6 | 0.6 | 0.5 | 2.0 | 3.1 | 0.5 | 0.8 | 0.9 | 2.8 | 4.1 |

| c-PARP       |     |     |     |     |     |     |     |     |     |     |     |
| GAPDH        |     |     |     |     |     |     |     |     |     |     |     |
3.3.5 Discussion for aim 3:

In the first body of work we establish that IR and MSC can induce distinct differential gene expression profiles in EC and subsequently the correlation between IR-activated gene expression profile and worse clinical outcome (as shown on Section 3.1 & 3.2). Here we establish that MSCs can provide a protective role for EC, by increasing EC viability and decreasing cell death. We showed that MSCs increase HUVEC viability by nearly two folds when cells are treated with condition media of MSCs. Furthermore, there is significant, 80% increase in cell viability when EC are treated with MSC condition media following treatment with 2Gy IR and a 50% increase in viability when treated with 15Gy of IR. (Figure 3.3.1B) To further decipher the pathways that are upregulated to increase cell viability we looked at a number of downstream regulators known to modulate cell proliferation and growth. We found that p-Akt (Figure 3.3.2-1) but not p-EKR1/2 (Figure 3.3.2-2) expression was significantly elevated under MSCCM, suggesting that MSC are more likely aiding in HUVEC viability by promoting cell survival than cell proliferation. This partly agreed with another study where the author showed MSC protect against endothelial cells apoptosis through phosphorylation of both Akt and ERK1/2 (Y.-X. Chen et al., 2015).

We also looked at phosphorylation of p53 at serine 15 as an indication of both double-stranded DNA damage and the activation of ATM signaling cascade, which is one of the major DNA damage repair pathway. The level of p-p53 appeared to be IR dose-dependent and independent of media condition at 1 hour post IR (Figure 3.3.3-1). One of the DNA damage repair pathways is represented by activation of p-ATM at serine 1981, which can selectively phosphorylate p53 at serine 15, in turn up-regulates p21^waf1 and cause cell cycle arrest at G1/S, so that the cells can undergo homologous recombination (HR) to repair DSB (Pawlik & Keyomarsi, 2004). p21 was elevated in HUVECs dose-dependently as early as 4 hours post IR, suggesting activation of DNA DSB repair. There was no difference in p21 up regulation in HUVECs between MSCCM and EBM conditions. (Figure 3.3.3-2) The increased p53/p21 level in HUVECs could explain the general trend of IR-cytotoxic as suggested in the literature. (Nübel, Damrot, Roos, Kaina, & Fritz, 2006)

With the understanding that the cytotoxic effect of IR is largely via DNA damage and subsequently apoptosis if irreversible, we also looked at if MSC enhanced the DNA damage repair mechanism of HUVECs upon IR, which translated into cell survival. ATM is one of the most proximal signal transducers after DNA damage. We showed that similar levels of ATM activation (ATM
phosphorylation) were generated in HVUECs immediately after 2Gy or 15Gy, yet p-ATM generated by 2Gy of IR reverted back to baseline level in MSCCM compared to control. (Figure 3.3.4-1) Since p-ATM gets recruited to the nucleus by both double stranded and single stranded DNA break (DSB and SSB), we next measured PARP1 level to reflect on SSB events. We found that although MSCCM cause more e-PARP than EBM, such signal was not detected until 8 hours post IR, suggesting other sources of single stranded DNA. (Figure 3.3.4-1) A possible explanation for the delay of PARP1 cleavage could be that MSCCM stimulate more HR. In the process of which, it breaks up more normal double stranded DNA into single strand to serve as the template. PARP1 couldn’t differentiate between DNA SSB and single strand template that generated during the cellular response of repairing DSB events which resulted in “false positive” of SSB events in HVUECs.

Taken together our data supported that the MSCs that were recruited to the TME maybe providing a protective role for EC in GBMs, by promoting EC viability and decreasing DNA DSB. These results will need further validation in animal studies as well as human tumor samples.
4 General Discussions

4.1 Overall conclusion:

One of the notorious features of GBM is its heterogeneity in terms of cell types, cell cycle, proliferation, and mutation status (Quail et al., 2017). Nearly 1/3 of cell population in the bulk of tumor is comprised of stromal cells. However, the impact of the genetic profiles of tumor-associated stromal cells in cancer progression is largely overlooked. In addition, the traditional subtypes of GBM (i.e., classic, proneural, neural and mesenchymal) have limited predictive power for patient outcome. Better classifiers are needed for GBM prognosis. Abnormal vascularity is a histopathological hallmark of glioblastoma multiforme (GBM). AATx has shown efficacy in preclinical and early phase human clinical studies in patients with glioblastoma. Though, this response is variable across different subtypes, and therefore prognostic markers that can predict response to AA therapy are needed. In this thesis, we have established five angiogenic gene expression profiles (Gene set A–E; refer to Table 3) by stimulating ECs with different combinations of IR and MSC. In summary, radiation generated profiles (gene set A & B) have prothrombotic/pro-inflammatory potential whereas gene expression profiles in the context of MSC (gene set C–E) were relatively pro-angiogenic and hierarchical and spectral clustering of gene set A-E in the AVAglio database and post clustering analysis revealed that high HGF and low CXCL10 levels in patients with GBM had the worst response to AATx. Using qPCR in various cell types we showed that GBM cells have the highest expression of HGF and the lowest expression of CXCL10 relative to endothelial cells. Similar trends were detected with MSCs relative to ECs. Together, our findings highlighted an important angiogenic genetic profile that is predictive of response to AATx in patients with glioblastoma. We’ve also showed a plausible mechanism for MSC-medicated radioresistance in ECs by showing higher cell viability and Akt phosphorylation of ECs when cultured in MSCCM as compared to base media. However, the data did not draw connection between Akt phosphorylation and P53/p21 expression as indicated in literature as Akt-conferred cell survival mechanism for endothelial cells.

Documenting variation in radiation susceptibility of ECs and tumor cells among different culturing conditions can give indication to how the intricate tumor microenvironment and heterogeneity affect ECs’ response to radiation therapy. Latter is crucial for giving accurate prediction for the outcome of
classical treatment at a cellular biology level and boost the efficacy and specificity of anti-cancer regime by fine-tuning the therapeutic targets and concomitant therapy kinetics.

4.2 Limitations:

4.2.1 Potential mismatch between gene expression and biological function

The in-house angiogenesis array is a very robust assay as it can capture the mRNA levels of the cells, which reflect the transcription of DNA into protein. However gene expressions are known to be insufficient in reflecting the functional efficiency in the host. For example, we’ve confirmed that the MSC- dependent *ICAM* expression (Figure 3.1.3-1) correlates on the protein level (Figure 3.1.3-2), however, the MSC- and IR- dependent *TF* expressions (Figure 3.1.1-9) were not substantiated at the protein levels (Figure 3.1.3-3). Such inconsistency can be at least in part due to the limited amino acid storage that was available to synthesize the encoded protein in the cells given the overwhelming growth signals pertain to TME. Also, post-translational modification may be needed for certain proteins to be functional (i.e., Akt, ERK, etc). Given that all the gene expressions profiles that were recorded for the patients in both TCGA and AVAglio cohorts were from naïve tumors (i.e., before they were exposed to any treatments or placebo), identification of the set of proteins that were abnormally expressed in GBM prior to treatments can help elucidating the mechanisms underlining therapy resistance.

4.2.2 Restricted mechanism inference from an *in vitro* experimental setting

We’ve shown, in Aim 3, a correlation between MSCCM and EC survival and the potential signaling pathways that contribute to such correlation. The *in vitro* experimental set up in this project allowed us to study the targeted pathway in an isolated manner both spatially and temporally. However the actual TME is much more complicated system than *in vitro* environment due to the involvement of a broader range of cell types, the cross communications with other cell types over time as well as events that are secondary to cell interaction, therapeutic intervention and tumorigenesis (i.e., hypoxia, thrombosis, immune response, etc). Therefore, further experiments in animal models as well as human samples are in need to confirm the suggested mechanism definitively.
4.2.3 The use of non-brain blood vessel cells

HUVEC, our chosen endothelial cell line is well characterized both in the literature and in the laboratory setting. The vasculature within the brain has certain unique characteristics. For instance, brain blood vessels form BBB, which is a tightly regulated network that prevents free entry of neurotoxins (Deeken & Loscher, 2007). To achieve this, the endothelial cells that made up the BBB are connected by tight junctions continuously, and covered with pericytes and astrocytic foot processes. Such intricate fenestrated system of BBB is further fortified by two ATP-binding cassette (ABC) efflux transporters: P-glycoprotein (Hegmann, Bauer, & Kerbel, 1992) and breast cancer resistance protein (Agarwal, Hartz, Elmquist, & Bauer, 2011). Although our thesis focused on investigating the mechanisms underlining radioresistance of GBM, the influence of the efflux transporters and vesicular integrity in brain blood vessels can have importance inference on the resistance of chemotherapy; latter is also an important component of the GBM standard care. Therefore, it would be helpful to validate our results with specific brain tumor derived endothelial cells in order to take into account the uniqueness of the CNS vasculature.
5 Future Directions:

5.1 Protein expression confirmation of established gene expression profiles in ECs

There are overlaps between the 5 gene signature profiles (gene sets A~E) that were generated in ECs upon different combinations of radiation and MSC treatments. What’s more, the heat map produced from hierarchical clustering shows that certain genes have more contribution to the gene clustering than others, latter being the ones are uniformly up-regulated or down-regulated in both groups. Such pattern shows that certain genes can be more indicative than others in stratifying patients’ clinical outcome, thereby further refinement and identification of the key elements of the gene signature is needed.

Our bioinformatics analysis of the five gene signatures, particularly the high HGF and low CXCL10 expression, in predicting worse clinical response to Bevacizumab was based on gene sequencing data from the AVAglio patients. It is worthwhile to also confirm those gene signatures at the protein levels on the pre-existing patient tumor samples from our tumor bank using immunohistochemistry (IHC) to 1) test the predictive power of the gene signatures in GBM patients; 2) identify the key players within the gene sets.

5.2 Prospective observational clinical study

Once we have confirmed the key gene signatures from experimentation from local tumor samples, we will seek to translate our findings into clinical application. The prognostic power of CXCL10 and HGF that was shown in Aim 2 has important clinical inference. To further explore the potential of this gene signature as one of the selective criteria for precision medicine in GBM patients, we plan investigate its utility in clinical trials. Since Bevacizumab has been approved in recurrent GBM (2\textsuperscript{nd} line of treatment), there is proof-of-principle for its safety, and therefore we can conduct a phase III clinical trial to test whether it can benefit newly diagnosed GBM patients, i.e., used in adjuvant with1\textsuperscript{st} line of treatment. As demonstrated in the schematic illustration of the clinical procedure (Figure 5.2), all patients with newly diagnosed GBM will undergo surgical resection followed by adjuvant chemoradiation, in standard fashion. After surgery, patient tumors will undergo genotyping, and patients who have HGF upregulation and CXCL10 don-regulation will also receive adjuvant
Bevacizumab in addition to standard treatment. Patients who have HGF upregulation and CXCL10 down regulation will only receive standard adjuvant treatment. The overall survival and progression-free survival of two groups will be measured and compared to decide whether Bevacizumab confers survival benefit as part of 1st line treatment for GBM patients.

Figure 5.2: A simplified schematic representation of the prospective phase III clinical trial procedure.
5.3 Further mechanistic analysis of MSC rendered radioresistance in ECs

The heightened cell viability of ECs was observed in MSCCM as compared to EBM at 48 hours (but not 24 hours) as shown in Figure 3.3.1. This improved viability can be attributed to MSC-increased DNA damage repair (caused by radiation), MSC-increased EC proliferation or MSC-mediated cell cycle arrest. We have shown that increased p-Akt levels but not p-ERK1/2 in ECs under MSCCM may be responsible for this. Drug inhibition of Akt or siRNA can be used to further confirm the Akt-dependency of ECs in radioresistance. We also looked at p53 phosphorylation, p21 and ATM phosphorylation and PARP cleavage of ECs in between different condition media and IR dosages (More experiments are needed to ensure the reproducibility of the results). Furthermore, additional DNA damage markers, cell proliferation markers, etc can be tested to map out the signaling pathways that render the high cell viability of ECs described earlier.

5.4 Functional analysis of MSC-mediated ECs

Both p21 and p53 phosphorylation in ECs are IR dose dependent and MSC independent (See section 3.3 results). The implication of DNA damage (reflected by p53, pATM, cPARP, etc), cell cycle arrest (p21) and cell viability conferred by MSCCM posed interesting question about the functional efficacy of the viable ECs in TME (Figure 3.3.1). An experimental model that emulates the reorganization stage of angiogenesis, more specifically the formation of tubal structure, the tight junctions that prevent leakage of the tubal structure, the appropriate extracellular matrix support and the intricate balance between pro- and anti-angiogenic factors of brain vascular system is needed to decipher the impact of MSCs in irradiated ECs in a pragmatic prospective. Since the first angiogenesis model that was published in 1980 (Folkman & Haudenschild, 1980), a plethora of in vitro and in vivo assays have been developed to exam the angiogenic potential of perspective targets. They can be roughly classified into 2 categories: 1) two-dimensional model such as fibronectin- and gelatin-based matrix, matrigel and amnionic membrane (Folkman & Haudenschild, 1980; Kubota, Kleinman, Martin, & Lawley, 1988; Kulke et al.; Kuzuya & Kinsella, 1994; Madri & Williams, 1983; Pelletier, Regnard, Fellmann, & Charbord, 2000); 2) three-dimensional model such as clotted chick plasma, collagen based matrix, and zebrafish (Chávez, Aedo, Fierro, Allende, & Egaña, 2016; Korff & Augustin, 1999; Nicosia, Tchao, & Leighton, 1982). These assays are chosen by
researchers base on their biological, technical, economical concerns. Our lab has established a robust tube formation assay derived from the BD bioscience tumor formation assay protocols. We’ve optimized the number of HUVECs required to be seeded to observe the effect of IR and MSCCM in tube formation capacity. Our preliminary results suggest that neither MSCCM nor 2Gy of IR at 24hours incubation seems to affect the tube formation capacity of HUVECs as shown in Figure 5.4.

**Figure 5.4: Immuno-fluorescence analysis of tube formation assay on HUVEC optimization.**

Two IR dosages (0Gy and 2Gy) were tested under EBM and MSCCM conditions.
5.5 The Investigation of MSCs radio-protection in GBM cells

Another postulation for radiotherapy resistance in GBMs is that RT triggers molecular cascades that up-regulate the expression of pro-angiogenic factors (i.e., VEGF, TGF, HIF etc) in various cell types (Arrillaga-Romany et al., 2014; Bertolini et al., 2011; Tseng et al., 2011b). This thesis focused on the role of MSCs in GBM angiogenesis, more specifically, investigating whether MSCs confer radioresistance in ECs, which can potentially be translated into increased angiogenesis and eventually tumor progression or recurrence. However, some studies have also shown that MSCs are implicated in tumorigenesis independent of angiogenesis. For instance, there are studies which show that MSCs can promote tumor metastasis by improving the motility, invasive potential of cancer cells (Behnan et al., 2014; Karnoub et al., 2007). Other research groups have shown that MSCs induce glioma apoptosis and differentiation (Yang et al., 2014) as well as potentiating peripheral immunotherapy against tumor cells (Ströjby et al., 2014). Perivascular association of MSC or BMDC in various cancer types including glioma has been well documented (Behnan et al., 2014; Fons et al., 2015; Hardee & Zagzag, 2012). Overall, the complex mechanisms underlining IR and the vasculature targeting agents (i.e. Bevacizumab) are likely a result of interactions between the tumor stroma and vasculature and the tumor cells. We postulate that the interactions between MSCs, tumor cells and tumor-associated endothelial cells collectively contribute to the heterogeneous outcomes of GBM therapies. Therefore it is worthwhile to investigate the effect of MSCs in GBM radioresistance in the context of both tumor cells and stromal cells.

5.5.1 Experimental procedure overview

To pinpoint which type of interaction (direct versus humoral) is attributed for MSC to exert radioprotection for EC and glioma cells, we can employ the cell co-culture system from Section 2.6 and re-engineer it into a triple cell culture system. Two conditions of double cell co-culture systems will be used, one will allow physical contact between two cell types, and another will prevent it. As shown in Figure 5.51, Cell type A will either be planted on the bottom of the well or the transwell insert with prospective growth media on day 1. After reaching optimal confluency, on Day 2, we can either plate cell type B on the bottom of the transwell insert or switch to the condition media of Cell B. After 24 hours of conditioning, we can either assemble the transwell insert that has two cell types on each side to a new well that is pre-seeded with cell type C or switch to the condition media of
Cell C. Three different IR dosages will be applied, and cell type A will be collected and assess for cell viability, gene expression, protein expression, clonogenic capacity as well as tube formation capacity at different time point post IR.
Figure 5.5.1: A schematic representation of the experimental procedure for assessing the effect of MSC and IR on GBM cell lines.
We have conducted preliminary experiments based on the protocol above, using U87 and GSC267 as cell type A. Below are some of the preliminary data we’ve collected pursuing this postulation.

5.5.2 The MSC-mediated apoptosis pathway in GBM cell lines

The levels of caspase-3/7 in U87 (a commercially available, immortalized human GBM line) were measured using flow cytometry as a reflection of apoptotic events (to be differentiated from necrosis). Based on the optimization shown in Figure 5.5.2-1, 15Gy at 48 hours were set to be the optimal IR dosage and time point for maximal cell death.

![U87p Caspase 3/7-dependent apoptotic rate](chart.png)

**Figure 5.5.2-1:** The optimization of Caspase 3/7–dependent apoptotic events in U87 measured by Flow cytometry. Three time points (12h, 24h, and 48h) and 4 IR doses (0Gy, 5Gy, 10Gy and 15Gy) were measured.
Based on our results measuring cleaved caspase-3/7 in U87, MSCCM was able to marginally reduce the apoptosis of cancer cells that resulted from 15Gy of IR as compared to U87 growth media (U87GM). (Figure 5.5.2-2)

![Flow cytometry result of U87 apoptotic rate at 48 hours.](image)

**Figure 5.5.2-2: The Flow cytometry result of U87 apoptotic rate at 48 hours.** Two IR doses were used for 3 biological replicates.
5.5.3 MSCs promote protein expression of DNA damage repair markers

The phosphorylation of p53 in U87 was observed immediately after IR, and persisted up to 16 hours post IR independent of the presence of MSCs as shown in Figure 5.5.3-1. However, starting from 24 hours post IR, MSCCM appeared to generate higher level of p53 phosphorylation in U87 cells than the control media. MSCCM was also found to up-regulate Erk1/2 phosphorylation 24 hours earlier in U87 post IR. (Figure 5.5.3-2)

In responses to IR-induced DNA damage (Fig. 5.5.3-3) activation of checkpoint kinase 2 (Chk2), the direct upstream of p53 that is responsible for phosphorylation at serine 15, is up regulated in all media conditions at 24 hours post IR, however, more prominent in MSC CM and MSCIRCM. This suggests that DSB are generally sensed and elicited an ATM-dependent kinase cascade possibly involving DNA repair and cell cycle arrest. By 48 hrs, the effect of condition media seem to fade away, as the p-Chk2 level started to reverse back to the baseline level (Figure 5.5.3-4).
Figure 5.5.3: Western blot analysis of DNA damage repair markers. (A) A representative blot of p-p53, p-Akt, -p-p53, p-ERK1/2 expressions in U87 cells at different time points post 0Gy and 15Gy of IR with or without MSCCM or MSC co-culture; (B) The protein expression of p-Chk2 in U87 at 24 hours post IR; (C) The protein expression of p-Chk2 in U87 at 48 hours post IR.
5.5.4 MSCs increase the viability of U87 upon radiation

Combing the increase of p-p53 level and decreased metabolic rate at 24hrs and reverse of both incidents at 48hrs, we next tried to differentiate whether such fluctuation of metabolic rate is caused by a general increase of cell numbers that have low metabolism or limited cell numbers with hyperactive metabolism and how this may affect cell proliferation. Using a trypan blue exclusion assay, we measured the viable cells in different condition media with or without radiation. As demonstrated in Figure. 5.5.4, MSSCM alone did not result in an increase in viable U87 cells when compared to control at any time point of incubation. However, MSCCM when combined with 15Gy increased the number of viable U87 cells at 72hours incubation when compared to controls without an identifiable pattern of change in U87 metabolic rate.

Figure 5.5.4: The temporal viability and metabolic rate of U87 upon 2Gy and 15Gy of IR under different media conditions. (A) U87 viability measured by Trypan blue elusion assay; (B) The metabolic rate of U87 measured by Alamar blue assay.
5.5.5 MSCs up-regulated angiogenic factors in GBM cell lines

We also looked at whether MSCs can up-regulate angiogenic factors in GBM cell lines in attempt to identify the possible reconfigurations that tumor cells undergo prior to VM or vessel co-option as mentioned in section 1.1.4.3. We tested all 30 genes (listed in Table 1) in both U87 and GSC 267 cells (a glioma stem cell line that was derived from GBM patients in MD Anderson Cancer Centre). Here we want to highlight two genes with important implication: MSC co-culture significantly up-regulated the gene expression of CD31 in U87 (Figure 5.5.5A), VEGFR-2 in GSC267 (Figure 5.5.5B).

Figure 5.5.5: Gene expression of angiogenic factor in two GBM cell lines with or without MSC co-culture. (A) The gene expression of CD31 in U87 measured by quantitative PCR; (B) The gene expression of VEGFR-2 in GSC267 measured by quantitative PCR.

To conceptualize the preliminary data shown in section 5.5, we suggest that IR-recruited BM-MSCs protect against IR-induced cell death, which enhances survival of a sub-population of tumor cells with a smaller proliferation rate. BM-MSC may confer radioresistance possibly by protecting against apoptosis and facilitating DNA damage repair in surviving tumor cells which eventually reconstitute
tumor mass post treatment. Tumor microenvironment is a dynamic compartment that is also responsive to therapeutic interventions.

However, the number of samples within each condition of experiments in section 5.5 are low, therefore this needs to be supplemented with further experimentation to achieve statistical significances and further studies, both in vivo and in vitro, are in demand to elucidate the role of MSCs in tumor angiogenesis.
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