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Investigating The Role Of Exosomal Communication In The Glioma Microenvironment

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INVESTIGATING THE ROLE OF EXOSOMAL COMMUNICATION

IN THE GLIOMA MICROENVIRONMENT

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A

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Dedication

I dedicate my dissertation to my parents, for their unwavering support. To my father, thank you for instilling the wonder and love of science in me at an early age. Your guidance through life is one of the reasons for my success, and I strive every day to make you proud. To my mother, thank you for imparting a strong work ethic in me during childhood. I would not be where I am today without your support, and I endeavor every day to live by your standards. I love you both very much and I am lucky to have such caring, encouraging, and devoted parents.

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INVESTIGATING THE ROLE OF EXOSOMAL COMMUNICATION WITHIN THE GLIOMA MICROENVIRONMENT

Javier Miguel Figueroa II, M.S.Supervisory Professor: Frederick F. Lang, M.D.

Evidence indicates that human cancers are maintained by a population of cells with stem-like properties called cancer stem cells (CSCs). However, the influence of the surrounding stromal cells on the behavior of the CSCs remains poorly understood. We have recently shown that the micro-environment of human gliomas, the most aggressive human brain tumors, contains both glioma stem cells (GSCs) and cells that resemble human bone marrow-derived mesenchymal stem cells (BM-MSCs), called Glioma Associated-MSCs (GA-MSCs). We have also shown that GA-MSCs generate a cytokine-mediated increase in the growth and self-renewal (clonogenicity) of GSCs. However, other paracrine interactions between GA-MSCs and GSCs have not been fully explored. Recent studies have suggested that nano-sized vesicles, termed exosomes, may contribute to intercellular communication within the tumor niche. Therefore, I hypothesized that GA-MSC-derived exosomesincrease the growth and evolution of gliomas. Here I show for the first time that exosomes can be isolated from patient-derived GA-MSCs and that these exosomes contain oncogenic microRNAs. Importantly, *in vitro* delivery of exosomes isolated from GA-MSCs significantly increased both the proliferation and clonogenicity of GSCs. Furthermore, GSC xenografts,treated with GA-MSC-derived exosomes, inthe brains of nude mice resulted in a greater tumor burden and significantly decreased animal survival. Lastly, delivery of microRNA identified as both highly expressed and highly enriched in GA-MSC-derived exosomes, altered gene expression in recipient GSCs resulting in the glioma-enhancing effects described. I conclude that GA-MSC-derived exosomes represent an alternative intercellular communication mechanism for the transfer of specific microRNAs, which enhance the aggressive nature of Gliomas.

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Abbreviations

BM-MSC: Bone Marrow-Mesenchymal Stem Cell

CAF: Cancer Associated Fibroblast

CD: Cluster of Differentiation

CM: Conditioned Medium

CSC: Cancer Stem Cell

EF-CM: Exosomes-Free Conditioned Medium

EM: Electron Microscopy

ESCRT: Endosomal Sorting Complex Required for Transport

GA-MSC: Glioma Associated-Mesenchymal Stem Cell

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GBM: Glioblastoma Multiforme

GSC: Glioma Stem Cell

ISCT: International Society for Cellular Therapy

LV: Lentiviral

mRNA: Messenger-Ribonucleic Acid

miRNA: Micro-Ribonucleic Acid

MSC: Mesenchymal Stem Cell

MVB: Multivesicular Body

NCOR1: Nuclear Receptor Corepressor-1

NSC: Neural Stem Cell

RISC: Ribonucleic Acid-Induced Silencing Complex

TA-MSC: Tumor Associated-Mesenchymal Stem Cell

TMZ: Temozolomide

Chapter 1

Introduction

Introduction

Breakthroughs in biological science are typically the result of cutting-edge research exploring novel mechanisms of physiologic and pathologic processes. For intercellular communication, the forefront of present research has recently shifted from protein signal transduction to exosomal paracrine mechanisms. For cancer, the vanguard of current research has moved from the study of tumor propagating cells to the investigation of the complex interactions within the tumor micro-environment. These front lines of discovery converge in an area evaluating the exosomal paracrine intercellular signaling within the microenvironment of tumors. Considering the variety of possible exosomal contents, the crosstalk between tumor propagating cells and the surrounding tumor niche via the transfer of exosomes is likely paramount to that of protein receptor activation. Therefore, my research investigates the role of exosomal communication within the micro-environment of tumors. Specifically, I focused my studies on the interaction between glioma stroma-derived exosomes and glioma propagating cells, and the resulting effects of this communication pathway. This subject has never been studied in gliomas, and significant findings will establish a novel mechanism for communication within the tumor micro-environment, as well as a potential target for adjunct therapy. Given the complexity of cancer, the success of future curative therapies relies on a full understanding of tumor biology that includes evaluation of the intercellular exosomal communication system within the tumor microenvironment.

1.1 Exosome Background

Intercellular communication has previously been characterized as the transfer of ions, proteins, hormones, and lipids.[1-4] However, evidence for an alternative form of communication between neighboring cells, involving the delivery of proteomic and genetic elements via exosomes, is building. Exosomes are quickly being recognized as an important part of cell biology in both physiologic and pathologic conditions.[5-9] Therefore, in cancer research, it is essential that we evaluate this mechanism of cell-to-cell interaction in order to move towards a more complete understanding of the microenvironment in which a tumor forms and progresses.

The term exosome is used to specify a subset of small microvesicles, and is not to be confused with the unrelated intracellular RNA degrading exosome-complex.[10-11] Exosomes belong to a class of extracellular vesicles, and are the smallest in the group. An exosome is a 40-100 nm diameter particle composed of the same lipid bi-layer membrane from the originating cell.[12] Thus the size of exosomes limits their analysis by light microscopy, which has a lower limit of approximately 200nm, and instead requires the utilization of electron microscopy.[13] Conversely, light microscopy can be used to observe larger extracellular vesicles, specifically microvesicles (200-1000nm in diameter) and apoptotic bodies (1-5µm in diameter).[13] Exosomes are also found to have a density range of 1.10-1.19 g/mL, which differs from that of heavier microvesicles (1.17-1.25 g/mL) and apoptotic bodies (1.24-1.28 g/mL).[13] The formation of exosomes takes place intracellularly within the endosomal/lysosomal system, after which they are excreted from the cell via exocytosis. This process also distinguishes exosomes from larger microvesicles and apoptotic bodies, which form by external membrane budding or blebbing, and fragmentation of the plasma membrane, respectively.[14]

Exosomes were first described via electron microscopy (EM) in 1981 by Trams, et al, as a smaller subset of a group of larger microvesicles derived from the C-6 rat glioma cell line.[15] Here, the authors were investigating the ecto-5'-nucleotidase activity of a group of large microvesicles (500-1000nm in diameter) that budded from the plasma membrane of glioma cells. Their EM analysis however,

demonstrated the presence of a smaller subset of microvesicles (40-100nm in diameter) that had no ecto-5'-nucleotidase activity and did not seem to form from plasma membrane blebbing. Trams and colleagues were the first to propose the term *exosomes* for the nano-sized vesicles.[15] Subsequently in 1983, Pan, et al, described a physiologic role for exosomes in the elimination of the transferrin receptor during reticulocyte maturation.[16] Here, investigators found that mature sheep reticulocytes no longer required transferrin receptors to maintain intracellular iron homeostasis. Unutilized receptors were then recycled to endosomal compartments within the cell, and then excreted into the extracellular space in exosomes.[16] These two seminal papers revealed exosomes to be a novel part of cell biology, and since that time the literature on exosomes has increased exponentially.

An important part of studying exosomes is understanding how they are formed. This knowledge could lead to more accurate detailing of their composition, and help determine their function. Exosomes form within the endosomal system. This system is responsible for intracellular protein trafficking between various organelles and the cell membrane. The formation of exosomes initially involves the recycling of plasma membrane receptors and proteins, via clathrin-associated endocytosis, resulting in the extracellular domain of membrane proteins now within the intra-endosomal compartment. This process signifies the creation of the early endosomes in the cytosol (**Figure 1**).[17] Early endosomes then follow one of two pathways, depending on their content and function. The first pathway involves fusion with a lysosome, resulting in degradation and recycling of intra-endosomal contents.[17] The second pathway involves lysosomal escape which is thought to be signaled by specific endosomal membrane components. Lysosomal escape results in the eventual maturation of early endosomes into late endosomes.[17] The membrane of late endosomes then undergoes the process of reverse endosomal budding, which is essentially endocytosis of the endosomal membrane. This process forms smaller vesicles within the endosome, which now have the extracellular domain of recycled plasma membrane proteins in the extra-vesicular compartment. The formation of these intra-endosomal vesicles, which are future exosomes, signifies the creation of the multi-vesicular body (MVB).[17] MVBs then traffic their

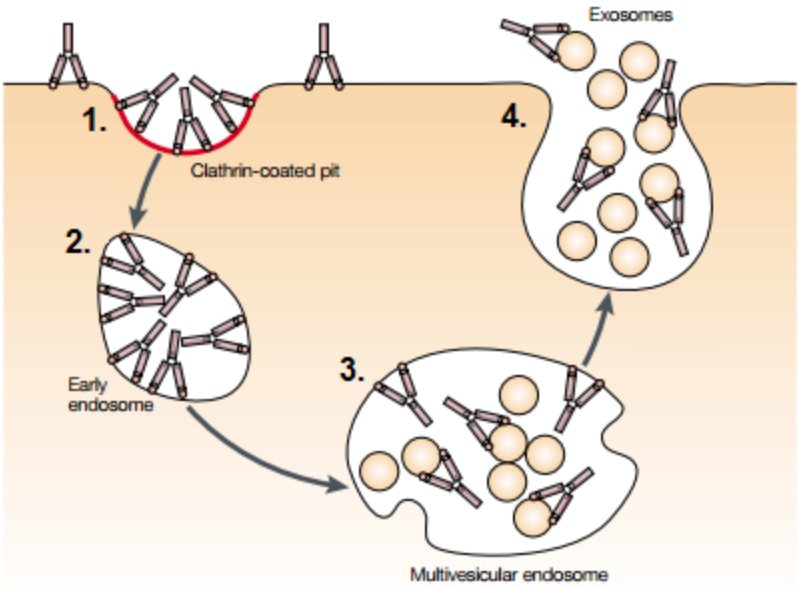


Figure 1. Exosomes are formed by an intracellular process. (1) Clathrin-coated pit endocytosis, recycling plasma membrane components. (2) Formation of an early endosomes that escape lysosomal degradation, becoming a late endosome. (3) Reverse-inward budding resulting in exosomes within intra-endosomal compartment, and forming a multi-vesicular body (MVB). (4) Fusion of the MVB membrane with the plasma membrane, releasing exosomes into the extracellular space.[17]

cargo of exosomes through the cytoplasm to the plasma membrane.[17] Fusion of MVBs with the cell membrane occurs in an energy-dependent, calcium-mediated, process.[18-19] This process is similar to the mechanism used by neurons to release pre-packaged neurotransmitter vesicles from the axon terminal into the synaptic space. After the MVB fuses with the plasma membrane, the exosomes are released with similar, but not identical, membrane receptors and proteins characteristic of the originating cell. Once in the extracellular milieu, exosomes are able to bind to target cells and complete the intercellular interaction by transferring their internal cargo.[17]

One key aspect of exosome formation that is poorly understood is the concentrating and packaging of specific cellular elements in the exosomal membrane and inside exosomes. The composition of the exosomal membrane is not identical to that of the cell membrane from which it was derived. Although the two membranes are similar to each other, exosomal membranes lack many of the common cluster of differentiation (CD) and fragment crystallizable (Fc) surface proteins, as well as various integrins, that are present on the plasma membrane.[20] Thus, the components of the membrane are altered during protein recycling and exosome formation, with certain lipids and proteins being removed or concentrated.[20] For instance, compared to the cell of origin, exosomes have an increased concentration of ceramide, an important lipid membrane constituent capable of cellular signaling.[21] This indicates that the composition of the exosome membrane is distinctive and purposeful, and is not indiscriminately compiled. Similarly, the intra-exosomal content is comparable, but not identical, to that of the parental cell.[22] The packaging of cellular elements into exosomes has been linked to the ESCRT (endosomal sorting complex required for transport) machinery, which is part of the endosomal system and responsible for trafficking of proteins and remodeling of cellular membranes during exosome formation.[23] Variations in this process can lead to either enrichment or depletion of exosomal content depending on the needs of the cell.[22] For instance, specific nucleotide motifs on RNA molecules can be recognized by ribonucleoproteins (RNPs), which lead to the cell preferentially packaging messenger-RNA (mRNA) or microRNA (miRNA) into exosomes.[24-26] The unique differences between the composition of exosomes and the cell of origin

indicate that exosome formation is not a random process, and that both the membrane constituents and intra-exosomal content are assembled for a distinct purpose in cellular biology.

Exosomes are known to contain a wide variety of cellular elements that are in the exosomal membrane (**Figure 2**).[27] Likewise, the function of these membrane constituents is broad. Evidence shows that exosome membrane can contain integrins, which can mediate binding in the extracellular space and may play a role in internalization by a recipient cell.[28-29] Additionally, studies show that the membranes of exosomes can contain major histocompatibility complexes (MHC) which can participate in antigen presentation and immune responses to infection and cancer.[30-31] Many other membrane proteins have been described in exosomes, some of which have no known or accepted function at this time. Specifically, proteins in the tetraspanin family, which are thought to be membrane scaffold proteins, have been ubiquitously described in exosomes. However, the role of these proteins in exosomes is still under investigation. Included in the tetraspanin family are various cluster of differentiation proteins, including CD9, CD37, CD63, and CD81, which are all used as surface markers for exosomes.[32-33] CD63 is one of the most utilized tetraspanin surface markers in exosomes from a variety of cell types, and has been linked to antigen presentation via association with MHC molecules.[34] This assortment of proteins represents only a fraction of the exosomal membrane constituents, and new functional components are still being discovered.

Similar to the exosomal membrane components, the intra-exosomal compartment is home to a wide variety of cellular elements (**Figure 2**).[27] However unlike the membrane components, the roles of nearly all cellular elements within exosomes are well known. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known to play a major role in intracellular vesicular trafficking, and may also indicate a metabolic role for exosomes.[35-36] The intra-exosomal compartment can also contain functional genetic elements, not present in exosomal membranes. The transfer of mRNA via exosomes has been described in a variety of disease processes, including cancer. Once inside the recipient cell,

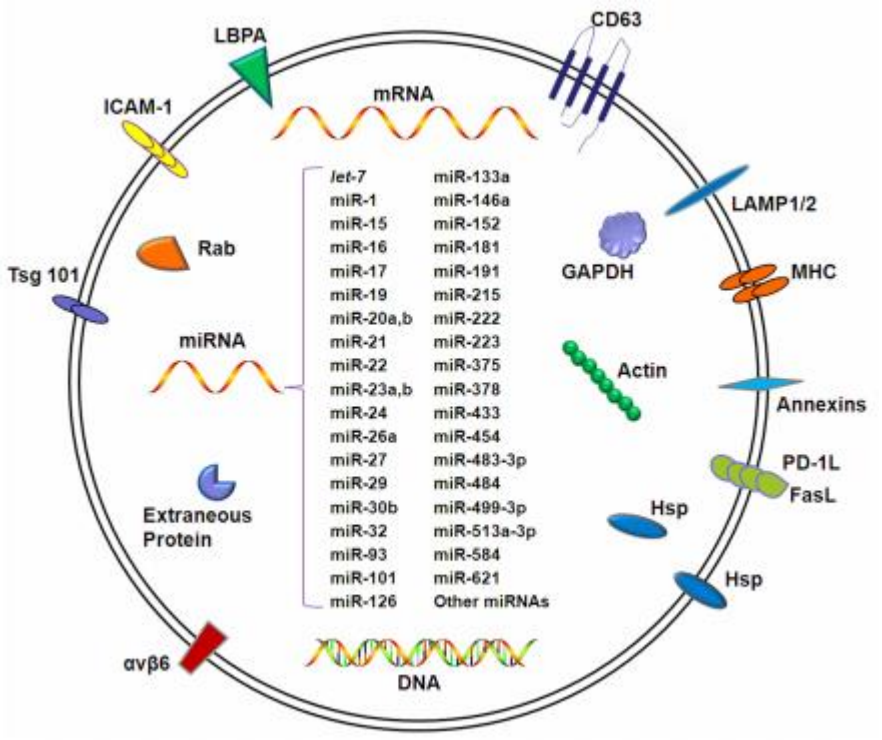


Figure 2. Exosome contain cellular elements. The exosomal membrane contains a variety of proteins, including the family of tetraspanins used as exosomal markers. The intra-exosomal compartment contains various cellular elements, including soluble proteins (GAPDH) and RNA.[27]

exosome-delivered mRNA can be translated by recipient cell machinery. Evidence shows that oncogenic mRNA within exosomes can enhance gene expression in non-cancer cells, thereby modifying cellular biology.[37-38] Additionally, miRNA have been extensively described in exosomes from numerous cell types. Once in the cytoplasm of the recipient cell, miRNA can regulate gene expression by binding to complementary regions in the 3-prime untranslated region (3'UTR) of mRNA, and inhibiting translation. Studies show that the exosome-mediated transfer of oncogenic miRNAs can alter the biology of non-cancer cells, while the transfer of tumor-suppressor miRNAs can inhibit tumor growth.[39-42] Importantly, exosome profiling has drastically expanded the list of known contents, which adds to the complexity of this intercellular communication system.

Interaction of exosomes with recipient cells has been shown to occur in two distinct forms, both of which are receptor mediated: direct fusion or endocytic internalization (**Figure 3**).[43] The exosomal membrane constituents include proteins that can serve to target exosomes to specific recipient cells. For example, exosomes from platelets readily bind to endothelial cells and macrophages, but not to neutrophils.[44] This highlights the complexity of exosome function, and suggests that they are not universally taken up by all cell types. Thus, targeting of exosomes to a specific destination must be encoded into the exosomal membrane for recognition by a specific recipient cell type. Upon receptor interaction with the desired cell, exosomes can either fuse with plasma membrane or be internalized by endocytosis.[45-46] Either pathway will eventually result in transfer of receptors to the cell plasma membrane, as well as the transmission of exosome contents, including RNA, into the cytosol.[43] In the case of immediate fusion with the plasma membrane, the contents of the exosomes can readily interact with their cytosolic targets, for instance miRNA can begin to bind and inhibit mRNA gene transcripts.[27] However, the fate of exosomes which undergo endocytosis is variable, with some exposed to lysosomal degradation or recycling to the extracellular space.[17] Only exosomes which escape these degradative processes and release their content into the recipient cell cytoplasm, will have successfully navigated the entire exosomal intercellular communication pathway, to induce a targeted effect.

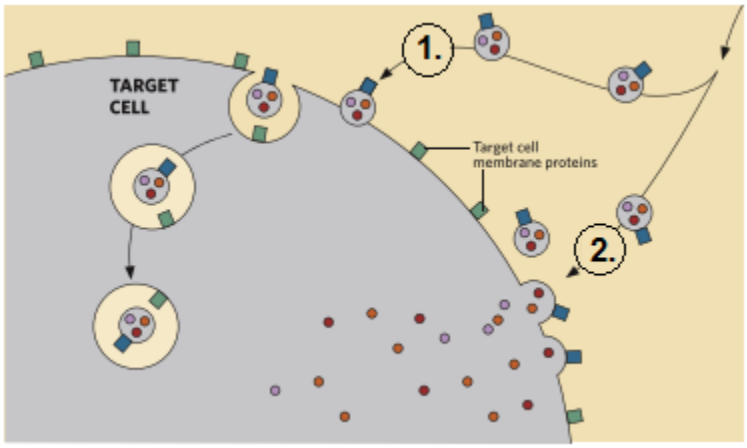


Figure 3. Exosomes can be taken up by target cells. (1) Exosome can bind receptors on the plasma membrane of the recipient cell, and initiate endocytosis for internalization into the cytoplasm. (2) Exosomes can fusion with the plasma membrane of the recipient cell, releasing their contents into the cytoplasm.[43]

1.2 Gliomas and Exosomes

Exosomes were first described in the setting of cancer, therefore it is fitting that the majority of research since that time has been devoted to exploring their role in malignancy.[15] To date, exosomes have been described in a wide variety of cancer types and in numerous aspect of tumor biology, from promoting metastases and malignant progression, to utilizations as diagnostic/prognostic markers, and as novel delivery vehicles for therapeutics.[47-53] Furthermore, exosomes have been implicated in modulation of the immune response to tumors, subjugation of the microenvironment, and resistance to chemotherapy.[54-59] Exosomes derived from lung, breast and prostate cancers are the most widely studied.[60-62] However, with respect to gliomas, the evidence for exosome interactions within malignancies of the brain is less extensive.[63-65] Nevertheless, the unique anatomical nature and isolated environment of the brain only add to the potential for significant exosomal function in localized tumors.

At present time, there are a limited number of published studies pertaining to human gliomas and exosomes. These few reports can be grouped into three broad categories; reviews, basic science, and translational. The reviews sought to correlate the knowledge of exosomes from research areas outside the central nervous system, to that of gliomas.[63-65] The review published by van der Vos, et al., focused on the changes mediated by transfer of exosomal RNA in the glioma microenvironment, as well as the utilization of exosomes as a diagnostic biomarker.[63] A subsequent review by, D'asti and colleagues, addressed the biogenesis and characterization of exosomes in glioma, as well as the oncogenic potential of the exosomal cargo.[64] The latest review, by Gonda, et al., concentrated on the modulation of the tumor microenvironment by exosomes in glioma, as well as the therapeutic applications for exosomes against gliomas.[65] These comprehensive reviews cover much of the currently published research on gliomas and exosomes, and connect the findings with those from other areas of exosome research.

Basic science studies investigating gliomas and exosomes, range from proteomic and genetic characterization of GBM exosomes to evaluating the

exosomal influence on tumor neo-angiogenesis.[66-73] Bastida and colleague published the first study on gliomas and exosomes in 1984. Here researchers demonstrated the presence of tissue factor (TF) in exosomes derived from the U87 human glioblastoma (GBM) line. These TF-laden GBM-derived exosomes had pro-thrombotic capabilities, which were linked to the activation of the coagulation cascade and promotion of platelet aggregation.[66] Svensson, et al., then demonstrated that TF in glioma-derived exosomes could interact with endothelial cells in a paracrine manner. These TF-laden glioma-derived exosomes could activate pro-thrombotic properties in recipient endothelial cells under hypoxic conditions.[67] These studies highlighted the role of glioma-derived exosomes in tumor thrombosis. However, the majority of basic science exosome research in gliomas focuses on their content. In 2008, Al-Nedawi, et al., identified the mutant EGFRvIII in exosomes derived from glioma cells. This EGFR variant is oncogenic due to constitutive activity, and exosomes mediated the transfer to non-EGFRvIII expressing glioma cells.[68] This study was the first to demonstrate exosome-mediated transfer of tumor-promoting proteins, and laid the foundation for exosome characterization. The first profiling of glioma-derived exosomes was performed by Graner and colleagues.[69] Here researchers described the presence of mutant EGFRvIII, as well as immunosuppressive TGF-β, within exosomes released into the systemic circulation by gliomas.[69] Research then shifted to the genetic profiling of glioma-derived exosomes. Guescini, et al., showed that exosomes secreted by GBM cells harbored mitochondrial DNA (mtDNA), but not nuclear DNA (nDNA). These exosomes mediated the transfer of mtDNA to neighboring cells, which could modulate mitochondrial activity.[70] This exosomal transfer of genetic elements represented a new mechanism of intercellular communication in gliomas. Subsequently, Bolukbasi and colleagues reported the presence of specific nucleotide (nt) sequences in the 3'UTR of mRNA in glioma cells that resulted in targeted enrichment into exosomes for transport out of the cell. This 25nt "zip-code" sequence also contained a complementary binding site for miR-1289, which was found to play a role in the enrichment process into glioma-derived exosomes.[71] Furthermore, Li, et al., identified the enrichment of non-coding RNA, particularly

miRNA and vault-RNA (vtRNA), in glioma-derived exosomes. Interestingly, they also described the miRNA profile of these exosomes as distinct compared to the glioma cells from which they originate.[72] These studies uncovered the significant amount of genetic material present in glioma-derived exosomes, however this characterization did not yet correlate with function. In 2013, Kucharzewska and colleagues, described hypoxic enhancement of mitogenic and angiogenic properties in glioma-derived and tumor stroma-derived exosomes. When compared to normoxic conditions, glioma-derived exosomes contained increased levels of matrix metalloproteinases (MMPs), IL-8 and PDGFs, when isolated under hypoxic conditions. Similarly, exosomes derived from hypoxic endothelial cells harbored increased levels of growth factors and cytokines which stimulated PI3K/AKT signaling in pericytes.[73] Together, these basic science papers demonstrate the broad range of exosome function in gliomas, and highlight the need for further investigation of exosomes in other areas of glioma biology.

Translational studies explored patient oriented applications from exosomal RNA and immunologic therapeutic strategies to the diagnostic and prognostic utilities of exosomes.[74-81] Recently, Katakowski and colleagues found that GBM cells had decreased levels of miR-146b when compared to normal human astrocytes, which also held true for the 9L rat glioma cell line. Delivery of BM-MSC-derived exosomes, packaged with miR-146b, to rat glioma xenografts resulted in decreased tumor burden, due to miRNA-mediated EGFR down-regulation.[74] Additionally, Munoz, et al., showed that GBM cells had low levels on miR-9, which targets the drug efflux transporter, P-glycoprotein. Increasing the levels of this specific miRNA in GBM cells, via delivery of miR-9 laden MSC-derived exosomes, resulted in the down-regulation of P-glycoprotein, and increased the chemosensitivity of GBM cells to temozolomide (TMZ).[75] Furthermore, Bronisz and colleagues demonstrated that glioma cells had very low expression of miR-1, and that over-expression of this miRNA resulted in diminished tumorigenicity. Interestingly, GBM cells which were modified to over-express miR-1, released exosomes that were enriched in miR-1. These miR-1 laden GBM cell-derived exosomes decreased tube formation of human brain microvascular endothelial cells (HBMVECs) *in vitro*, as well as increased the

invasion of GBM cells through the extracellular matrix *in vivo*.[76] The findings from these studies support the investigation and utilization of miRNA-packaged exosomes as a therapeutic strategy. In the area of tumor immunology, Bu, et al., demonstrated that glioma-derived exosomes contained tumor antigens that were recognized by recipient antigen-presenting dendritic cells. After exposure to glioma-derived exosomes, dendritic cells were able to activate T-lymphocytes that had robust cytotoxic activity against autologous glioma cells.[77] This study highlighted the potential for the use of tumor-derived exosomes as a vaccine for immunization against gliomas. With regard to treatment of gliomas with ionizing radiation, Arscott and colleagues showed the increase of connective tissue growth factor (CTGF) mRNA and insulin-like growth factor binding protein 2 (IGFBP2) in glioma-derived exosomes after glioma irradiation. Furthermore, exosomes derived from irradiated gliomas promoted the activation of neurotrophic tyrosine kinase receptor type 1 (TrkA) and proto-oncogene tyrosine-protein kinase Src, in recipient non-irradiated glioma cells. Delivery of growth factors and kinase activation resulted in an increase in glioma cell migratory and invasive properties.[78] This study indicates that pre-surgical radiation of gliomas may promote further invasion of glioma cells that have escaped radiation-induced cell death. Thus, this mechanism may play a role in tumor recurrence at the surgical margins. With respect to the diagnostic properties of exosomes, Noerholm and colleagues found that glioma-derived exosomes, isolated from the serum of glioma patients, contained numerous RNA elements including a significant amount of small non-coding RNA species < 500nt in length. This exosome RNA profile was distinct from that of exosomes derived from normal subjects, specifically in the down-regulation of 121 genes, many of which encoded for ribosomal proteins.[79] This study outlined the potential for profiling of intra-exosomal content as a diagnostic approach in gliomas. However, other researchers sought to utilized exosomal surface markers as an alternative diagnostic method. Skog, et al., found that glioma-derived exosomes carried numerous mRNA, miRNA and angiogenic proteins, which were functional upon transfer to recipient cells in the tumor.[80] This exosomal communication facilitated neovascularization and promoted proliferation of tumor stroma cells. Interestingly, many of these glioma-derived

exosomes were able to escape into the circulatory system due to blood-brain barrier breakdown in the tumor. These circulating glioma-derived exosomes were found to contain glioma markers, particularly mutant EGFRvIII, and could be used as an adjunct diagnostic tool.[80] Subsequently, Shao and colleagues, developed micro-nuclear magnetic resonance (μNMR) techniques to analyze exosomes from the serum of glioma patients. When compared to serum-exosomes from normal subjects, those from glioma patient had increased expression of EGFR, EGFRvIII, podoplanin (PDPN), and mutant isocitrate dehydrogenase 1 (IDH1) R132H. Analysis of this panel of markers by μNMR resulted in a glioma diagnostic test with 90% sensitivity and 85% specificity. The glioma marker panel was also shown to have prognostic capabilities by predicting patient response to temozolomide chemotherapy.[81] This important study supports the utilization of glioma-derived exosomes for both diagnostic and prognostic purposes. Together, the findings from these translational studies demonstrate the practicality and efficacy of exosome research towards improving patient outcomes.

Importantly, in every basic science and translational study discussed above, the research is focused on investigating exosomes derived from the tumor-propagating glioma cells and not exosomes released by cells in the surrounding microenvironment. To my knowledge there are no published studies evaluating the role of tumor stroma-derived exosomes in the development and evolution of glioma. Investigation into this aspect of glioma biology would add to the growing body of knowledge for glioma and exosomes, and is crucial to fully understand this complex tumor system. This will translate to more effective therapeutic strategies against gliomas in the future.

1.3 The Glioma Microenvironment

Cancer is the second leading cause of death in the United States, falling just behind heart disease, and will account for over half a million deaths in 2014. Of the wide range of malignant neoplasms, lung, colon, breast, pancreatic and prostate cancers will be responsible for nearly 55% of those fatalities.[82] Rare in comparison, brain tumors only account for 2.5% of cancer related deaths in United States citizens, 80% of which will be the result of primary malignant gliomas. However, the incidence and prevalence of gliomas is increasing. Additionally, the course of the disease process is among the most devastating, with a majority of patients succumbing to the disease within the first year, and less than 5% living beyond 5 years after diagnosis.[83-84] Furthermore there is no way to screen for, or prevent, gliomas. People can avoid smoking to decrease their risk of lung cancer. Females can be screened for breast cancer by receiving regular mammograms. Males can be screened for prostate cancer by obtaining regular prostate exams. Colon cancer can be discovered early during routine colonoscopy screenings. These preventative and early diagnostic measures are not available in glioma, and patients typically present late in the disease process. If caught early enough, a few of the other cancer types can be treated with a combinations of radiation, surgery and chemotherapy, and many patients given the designation of “in remission” or “cured”. Unfortunately this is rarely the case with glioblastoma (GBM), the most common primary malignant glioma in adults. Instead, clinicians tend to speak realistically in terms of life extension rather than cancer free outcomes. Thus, these bleak results are the driving force for new research into the full understanding of gliomas.

One of the major discoveries in the last decade was the characterization of glioma propagating cells isolated from patient tumor samples.[85-87] Investigation of these cells in tumor development is the focus of a majority of glioma research. Additionally, study of these cells aided in the development of a wide range of therapeutic strategies aimed at eradicating gliomas.[88] However, glioma propagating cells are only one of the cell types present in any given tumor, with many other cells establishing an intricate support network termed the glioma microenvironment.[89]

Cancer stem cells (CSCs) were first described by Bonnet and Dick in leukemia in 1997, and subsequently described in gliomas by Singh, et al, in 2003, and Galli, et al., in 2004.[85-87] These cells possessed stem-like properties, particularly the ability to undergo asymmetric replication (self-renewal) and multi-lineage differentiation (potency).[90] Furthermore, when compared to normal brain tissue, brain CSCs, also known as glioma stem cells (GSCs), expressed many genetic aberrations that promote cell proliferation and inhibit apoptosis. These properties were termed *oncogenic*, and subsequently linked to the presence of several tumor promoting genes, termed *oncogenes*, found in GSCs; mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase (ERK), retrovirus-associated sequences (RAS), cellular myelocytomatosis (c-MYC), and protein kinase B (Akt).[91-96]

Work performed in the Lang laboratory before I joined included the isolation of GSCs from patient surgical specimens, by adapting the protocol published by Singh and colleagues.[86] Briefly, after enzymatic digestion of the gross tumor sample, GSCs were identified by non-adherent neurosphere growth in serum-free neural stem cell medium (**Figure 4**). Isolated GSCs were also found to express the known neural stem cell marker CD133 (**Figure 5)**.[86] Lastly, GSCs were able to form tumors when implanted as xenografts into the brains of nude mice (**Figure 6)**. Importantly, GSCs harbored the same genetic aberrations known to be present in many glioblastomas (**Figure 7**).[96] The isolation of these GSC lines formed the basis for my future experiments into the glioma microenvironment and novel therapeutic strategies.[97] To date, 40 GSC lines have been isolated, characterized, and genetically profiled.

After the discovery of GSCs, trials of various targeted therapeutic agents, including viral vectors, vaccines, and stem cell therapies, were pursued in hopes of decreasing the collateral damage to non-cancerous cells caused by traditional radiation and chemotherapy.[98-99] However, the combination of surgery, radiation, plus concomitant and adjuvant temozolomide (TMZ) chemotherapy remains the standard of care for all malignant gliomas, as described by Stupp and colleagues in 2005.[100] The Stupp protocol extends survival from less than one year to nearly 15

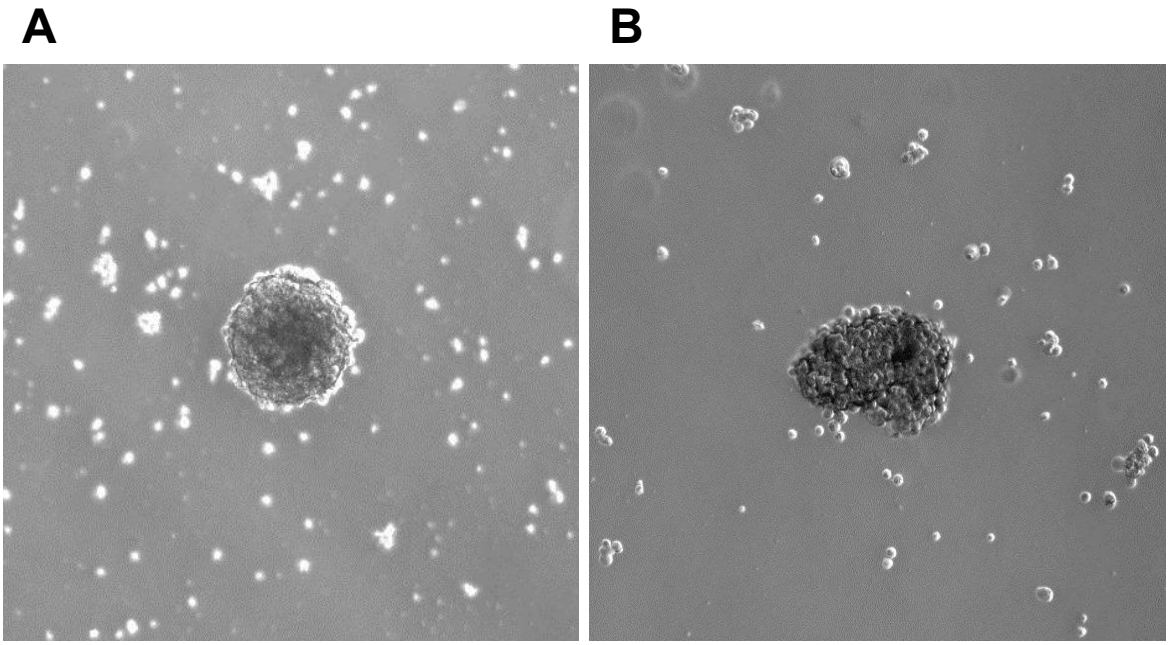


Figure 4. GSC exhibit specific morphology in cell culture. (A) Non-adherent growth of GSC 262 as a classical neurosphere in neural stem cell medium. (B) Non-adherent growth of GSC 20 as a non-classical neurocluster in neural stem cell medium.

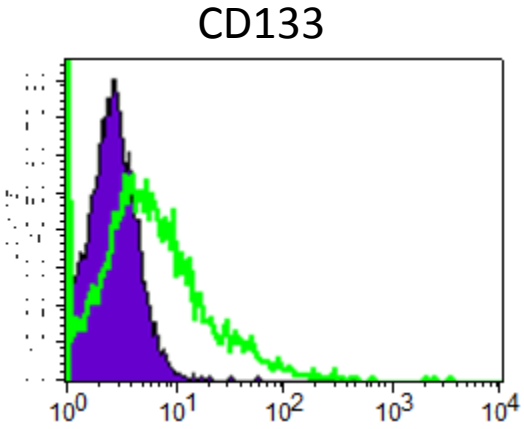


Figure 5. GSCs express a glial stem cell marker. Flow cytometry histogram for GSC 262 demonstrating the expression of the CD133 neural stem cell marker.[97]

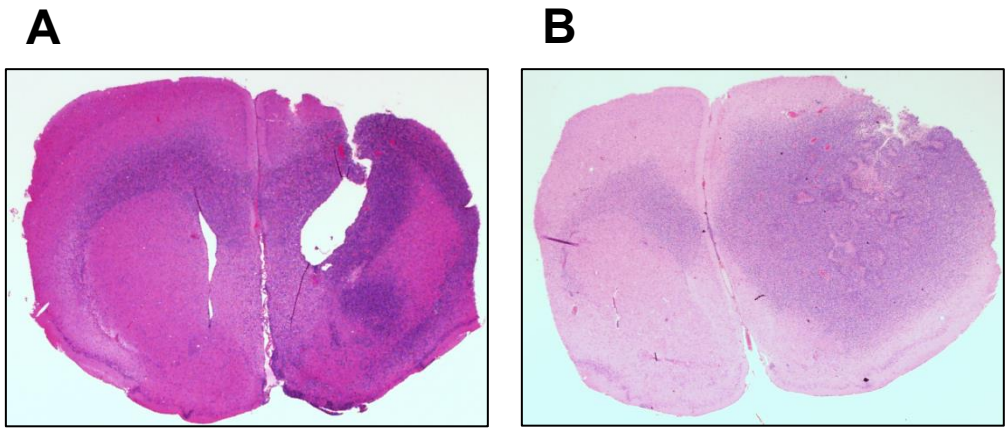


Figure 6. GSCs are tumorigenic. (A) H&E staining of histologic sections though the brain of untreated control mice euthanized 30 days after the sham injection procedure, demonstrating no tumor growth. (B) H&E staining of histologic sections through glioma xenografts from mice euthanized 30 days after implantation of GSCs, demonstrating significant tumor growth.[97]

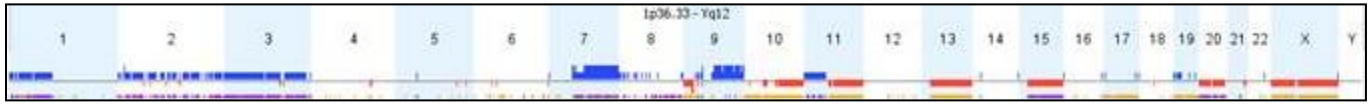


Figure 7. GSCs harbor known mutations of gliomas. Oncoscan assay for GSC 262 demonstrating a gain of function in chromosome 7, and a loss of function in chromosome 10, consistent with known amplifications and deletions described in gliomas.[97]

months, and since its inception there have been no studies in which outcomes have improved using any of the new therapeutic strategies targeted against GSCs. One reason may be the lack of a full understanding of the complex microenvironment of gliomas. This microenvironment not only involves the GSC component, but also includes the tumor stroma which is composed of many other cell types.

The idea of a nurturing tumor microenvironment is not new, and was first postulated by the English surgeon Stephen Paget in 1889.[101] In his paper, Dr. Paget referred to malignancy as a combination of “seed and soil”, in which neither on their own could grow a “plant”. Thus, the idea of a cultivating microenvironment for cancer cells to grow and develop into a tumor was born. Interestingly, Dr. Paget also added that some tumors had a predisposition for metastasis to specific organs.[101] A century later in 1989, the “seed and soil” theory was finally validated by Halachmi and colleagues, when they found that the tumor microenvironment has a positive effect on tumor growth. Specifically, they demonstrated that cancer cells passaged *in* vivo, were more tumorigenic compared to those cultured *in* vitro.[102]

The description of a microenvironment in gliomas was elucidated by Chen, et al, in 1993. In their seminal paper, researchers showed that tumor necrosis factor-alpha (TNF-α) secreted by astrocytes in the tumor stroma, increased the proliferation rate of malignant astrocytoma cell lines.[103] These studies outlined processes by which the surrounding cytoarchitecture of a tumor can significantly affect the progression of the disease. To date, endothelial cells, perivascular cells, immune cells, and organ parenchymal cells and organ stem cells have been described as part of this tumor niche. For gliomas, this includes the endothelium, pericytes, microglia, astrocytes and neural stem cells.[89, 104-105] Of these cell types, each has been implicated in creating pro-glioma conditions in which the malignancy will thrive and progress. Endothelial cells, which function to maintain the blood-brain barrier in normal brain tissue, have been shown to secrete mitogenic factors that increase the self-renewal and invasiveness of GSCs.[106-107] Pericytes which form a framework for the neuro-vasculature, have been found to stabilize tumor neovascularization as well as suppress the immune response to the malignancy.[108-109] Microglia, which are the

resident immune cells in the brain, have also been found to increase tumor invasiveness and induce immunosuppression within gliomas.[110-111] Astrocytes, the local parenchymal cells, can increase tumor cell survival as well as decrease the activation of the immune system.[112-113] Finally, neural stem cells, which are capable of differentiating into neurons, astrocytes and oligodendrocytes, are readily recruited to the hypoxic environment of the tumor site. Once incorporated into the tumor niche, neural stem cells may undergo malignant transformation.[114-115] Thus, the synergistic nature of tumor propagating GSCs and the nurturing glioma microenvironment may contribute to the inadequacy of current glioma therapy which does not target both. To effectively combat this devastating disease, requires full working knowledge not only of the genetic aberrations and tumorigenic features of GSCs, but also an understanding of the mechanisms by which the glioma microenvironment supports the malignancy. This knowledge will facilitate the development of more effective therapies aimed at both the tumor propagating cells and the tumor stroma.[116]

1.4 Glioma-Associated Mesenchymal Stem Cells

In 1963, Becker, McCulloch and Till first demonstrated that transplanted bone marrow cells in mice could undergo clonal expansion in the spleen.[117] Researchers showed that these cells were undifferentiated, but under certain culturing conditions were able to differentiate along mesodermal lines. Specifically, these progenitor cells were able to differentiate into the mesenchymal subtypes: osteocytes, chondrocytes and adipocytes. Furthermore, these cells replicated by asymmetric division creating one clonal cell identical to the parental cell, and one daughter cell programmed for differentiation. These transplanted bone marrow cells were the "stem" of multiple mesenchymal lineages, and were termed *mesenchymal stem cells* (MSCs).[117]

During the evolution of MSC research, the definition of a mesenchymal stem cell became inconsistent and non-uniform between research groups. For this reason the International Society for Cellular Therapy (ISCT) established a discrete set of requirements to denote mesenchymal stem cells.[118] In addition to MSCs being undifferentiated and multipotent, they must grow adherent in *in* vitro culture with spindle morphology. Furthermore, the MSCs must express the mesenchymal surface markers, CD73 (ecto-5'-nucleotidase), CD90 (thymocyte differentiation antigen-1), and CD105 (endoglin), and lack the hematopoietc stem cell marker CD34 (platelet/endothelial cell adhesion molecule-1), the panleukocyte marker CD45 (protein tyrosine phosphatase, receptor type, C), and the neural stem cell marker CD133 (Prominin 1).[119-121] These parameters ensured that research groups were conducting investigations utilizing a consistent and uniform definition of an MSC.

After the discovery of MSCs from human bone marrow (BM-MSCs), stem cells were found in other organ systems. Importantly, in 1989, Temple demonstrated progenitor cells in the subventricular zone (SVZ) of adult human brains, termed *neural stem cells* (NSCs).[122] Later, research groups began exploring cancer for stem cells, given that tumor growth results from a clonal expansion of cells.[123] After the discovery of cancer stem cells (CSCs) in 1997, and glioma stem cells (GSCs) in 2003, researchers began looking for non-tumor propagating stem cells within the tumor stroma of various malignancies.[85-86] One cell of focus in tumors was MSCs

due to their ability to home to sites of tissue injury to aid in wound healing.[124-126] The similarities between tumors and chronic wounds are well established, considering the damage to surrounding stromal structure that occurs as a malignancy grows.[127] Therefore it is reasonable that MSCs would home to tumors to participate in tissue repair. In 2001, Wallace and colleagues first identified MSCs within primary sites of multiple myeloma.[128] These tumor-associated MSCs (TA-MSCs) were found in the perivascular niche of the tumor and were distinct from tumor-propagating plasma cells in both morphology and genetic profile. TA-MSCs were subsequently described in breast, prostate and lung cancer.[129-131] Cancer stem cells were then shown to produce certain chemotactic factors that promoted the recruitment of MSCs to the primary tumor sites.[132] Additionally, the phenomenon of MSC recruitment was first demonstrated *in* vivo by Kidd and colleagues in 2009, using a mouse model to show the localization of systemically delivered luciferase-labeled MSCs to breast cancer xenografts.[133] Interestingly, this homing functionality of MSCs prompted investigation into their use as a delivery vector for anti-cancer agents, such as interferon-β.[134-135] Thus, MSCs are now accepted as a distinct component of the tumor stroma.

Mesenchymal stem cells were only recently demonstrated in normal brain tissue by Paul and colleagues in 2012.[136] Researchers found a subgroup of cells resembling pericytes lining the cerebral microvasculature in select areas of the brain. However, unlike pericytes, these cells expressed many of the mesenchymal markers, but lacked endothelial, microglial, hematopoietic, glial, and neural stem cell markers. Additionally, these cells had stem-like properties in their ability to differentiate along mesodermal lines.[136] These results show that MSCs are present in the microvasculature of normal brain tissue. Since MSCs are known to migrate to sites of tissue injury, it is reasonable that these organ-specific MSCs may play a role in the acute repair of local tissue.[124-126] Thus, the origin of tumor-associated MSCs (TA-MSC) could be from locally recruited MSCs in the tumor perivascular niche or from distantly recruited MSCs from the bone marrow. However, regardless of the origin, TA-MSCs have never been described in brain tumors. Although MSCs are known to migrate towards gliomas, and are used as a therapeutic delivery vehicle, they have never been isolated from patient-derived tumor specimens.[137-142]

Research conducted by the Lang laboratory before I joined, focused on the isolation and characterization of TA-MSCs in gliomas, termed *glioma-associated mesenchymal stem cells* (GA-MSCs).[97] Initially, surgical specimens were obtained within 4 hours of resection, and processed in accordance with the protocol published by Pittenger and colleagues, with a modification for whole tissues.[143] Briefly, after enzymatic digestion of the gross tumor sample, GA-MSCs were identified by plastic-adherent growth in serum-containing cell culture medium (**Figure 8**). Furthermore, GA-MSCs expressed the characteristic MSC markers of CD73, CD90 and CD105, and lacked the hematopoietc marker CD34, the panleukocyte marker CD45, and the neural stem cell marker CD133 (**Figure 9**). Lastly, GA-MSCs were able to differentiate into chondrocytes, adipocytes and osteocytes by growth medium induction (**Figure 10**). Importantly, GA-MSCs were genetically distinct from GSCs isolated from the same specimen (**Figure 11**), and did not form tumors when implanted as xenografts into the brains of nude mice (**Figure 12**). The discovery of this novel cell line in gliomas prompted further research investigating the intercellular interactions between GA-MSCs and GSCs, in order to elucidate their function in the microenvironment.[97]

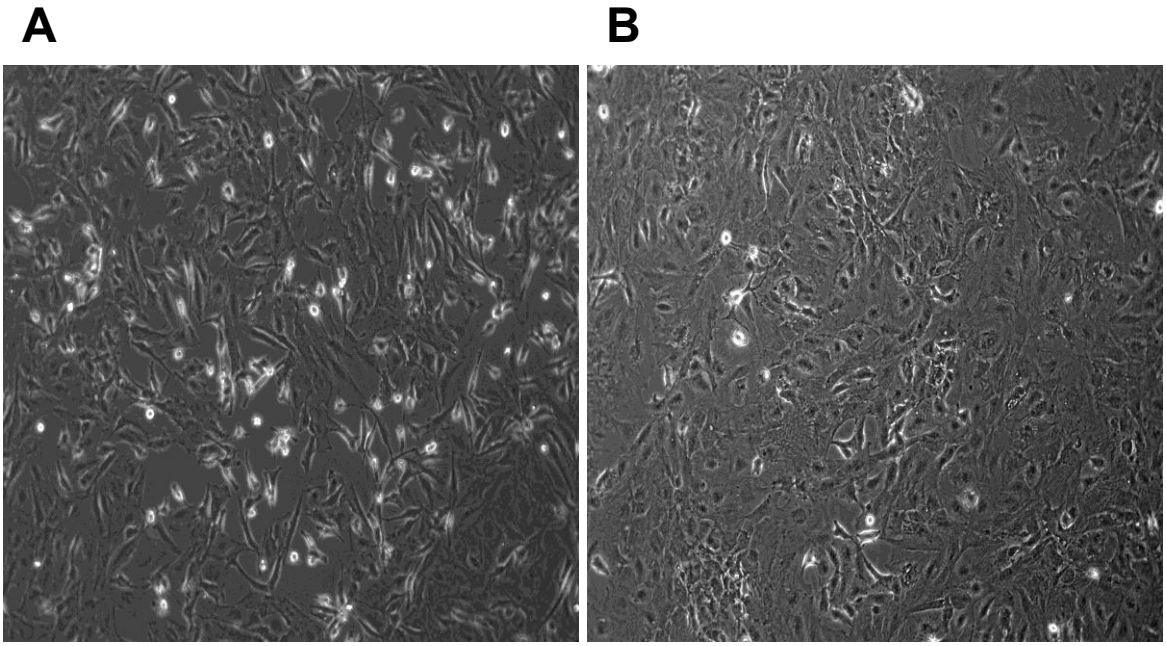


Figure 8. GA-MSCs exhibit specific morphology in cell culture. (A) GA-MSC 262 adherent growth and classical spindle morphology in MSC medium. (B) GA-MSC 20 adherent growth and classical spindle morphology in MSC medium.

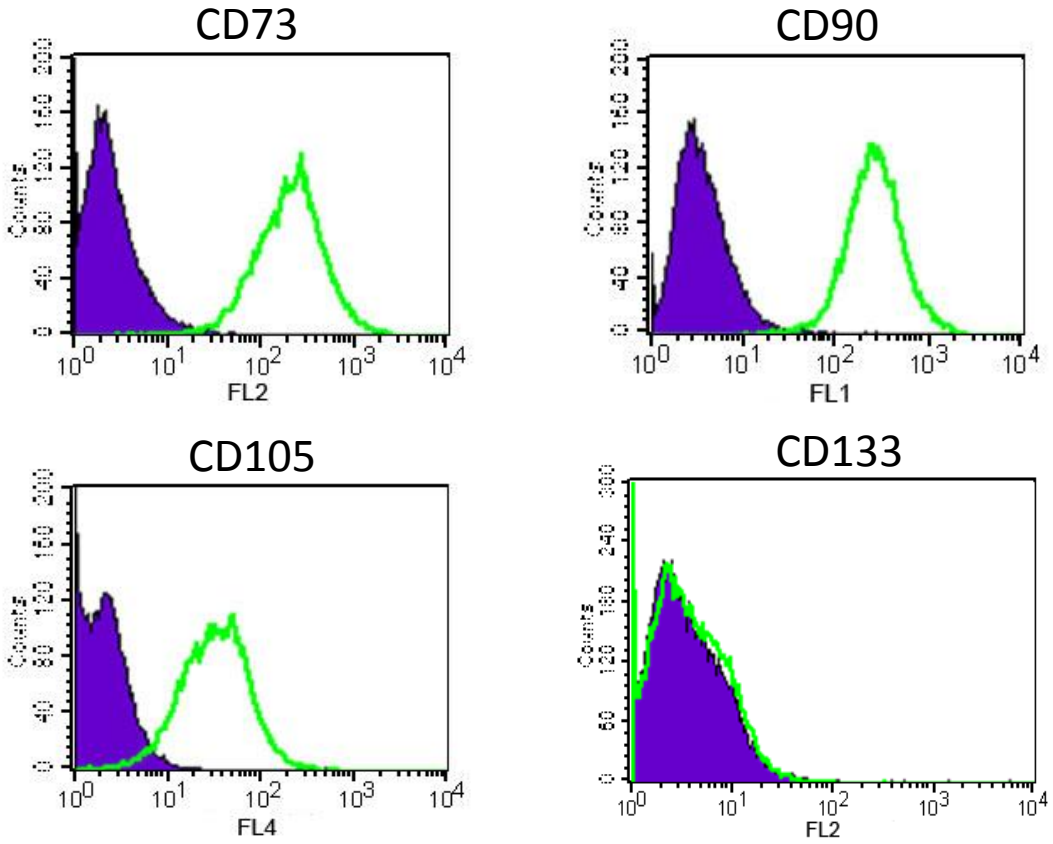
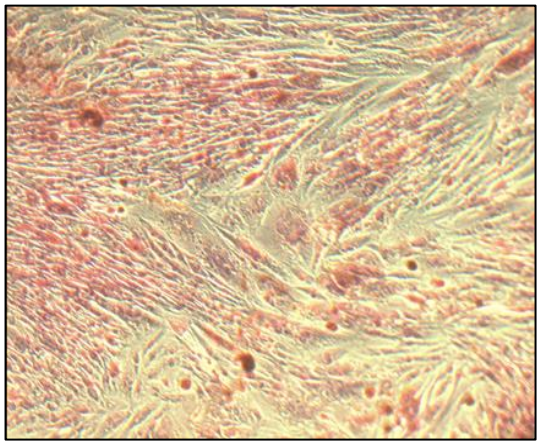
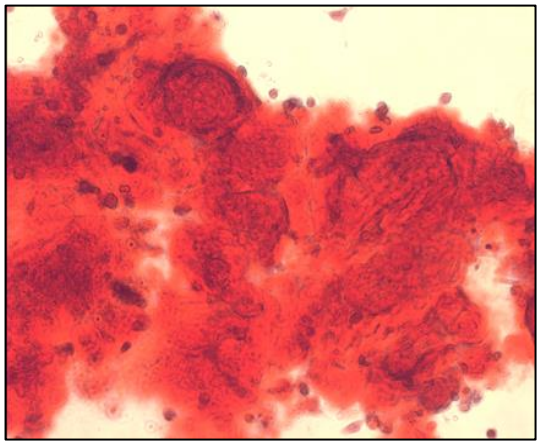


Figure 9. GA-MSCs express known MSC markers. Flow cytometry histograms for GSC 262 demonstrating the expression of the MSC markers CD73, CD90 and CD105, and lack of expression of the neural stem cell marker CD133.[97]

Adipogenic



Osteogenic



Chondrogenic

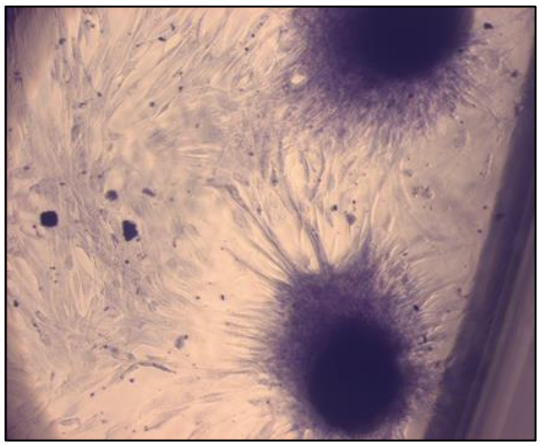


Figure 10. GA-MSCs can tri-differentiate along mesenchymal lines. Light microscopy demonstrating the characteristic adipocyte, osteocyte and chondrocyte morphology of differentiated GA-MSCs after culture in differential medium.[97]

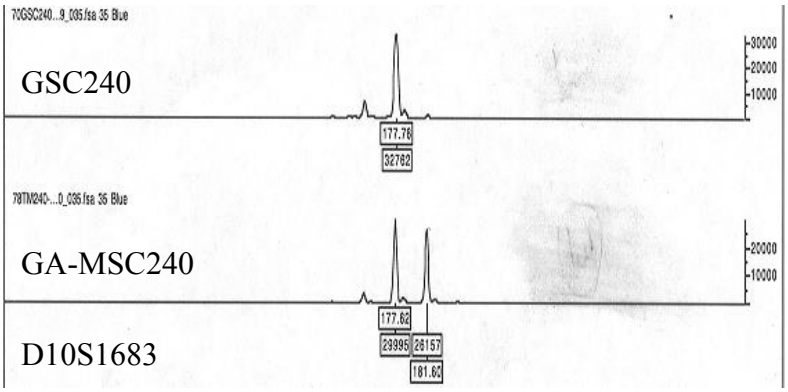


Figure 11. Matching GSCs and GA-MSCs do not harbor the same genetic mutations. Chromosomal analysis demonstrating loss of heterogeneity for chromosome 10 in GSC 240, which is preserved in the matching GA-MSC 240 isolated from the same patient tumor.[97]

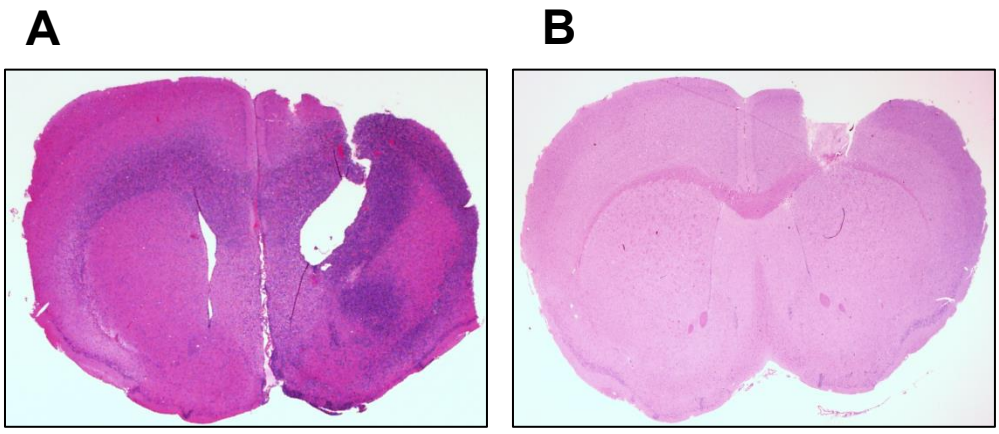


Figure 12. GA-MSCs are not tumorigenic. (A) H&E staining of histologic sections though the brain of untreated control mice euthanized 30 days after the sham injection procedure. (B) H&E staining of histologic sections through the brains of mice euthanized 30 days after implantation of GA-MSCs.[97]

1.5 The Role of Mesenchymal Stem Cells in the Tumor Niche

The fact that BM-MSCs are recruited to primary tumor sites was initially attributed to a role in maintenance and growth of the perivascular niche. However, soon after their description as part of the tumor microenvironment, research began on the interactions between BM-MSCs and CSCs. In 2007, Karnoub and colleagues first demonstrated the tumor-promoting capabilities of BM-MSCs on breast cancer stem cells.[144] Researchers found that co-injection of BM-MSCs and breast cancer stem cells in mice resulted in increased invasion and metastasis. Interestingly, this phenomenon was linked to the secretion of chemokine cytokine ligand 5 (CCL5) by the BM-MSCs. CCL5 would then act via a paracrine mechanism to stimulate the motility of neighboring breast cancer stem cells.[144] Subsequently, the tumor-promoting properties of MSCs were described in other malignancies, such as colon and prostate cancer.[145-148] Interestingly, the enhancement of tumors was not limited to the promotion of metastasis, but also included induction of immunosuppression and advancement of tumor growth.[149-150] However, each of these studies utilized BM-MSCs in their investigations, and did not examine the tumor-promoting properties of native TA-MSCs isolated from the tumor microenvironment.

Research accomplished by the Lang laboratory before I joined, investigated the interaction between GA-MSCs and GSCs.[97] Results from this study were the first demonstration of GA-MSCs promoting the growth and aggressive nature of gliomas. *In vitro* co-culture experiments showed that GA-MSCs increased the proliferation and clonogenicity of GSCs, when compared to untreated controls (**Figure 13**). Additionally, the co-implantation of GA-MSCs and GSC into the flanks of mice resulted in a decrease in median survival (**Figure 14**). Furthermore, tumor burden was significantly increased with co-implantation of GA-MSCs and GSCs into the flanks of mice (**Figure 15**). These glioma-promoting properties were linked to the secretion of interleukin-6 (IL-6) by GA-MSCs into the extracellular space and subsequent utilization by neighboring GSCs (**Figure 16**). Lastly, the IL-6 mediated tumor-promoting effects were found to be the result of downstream activation of the JAK/STAT3 pathway in GSCs. (**Figure 17**).[97]

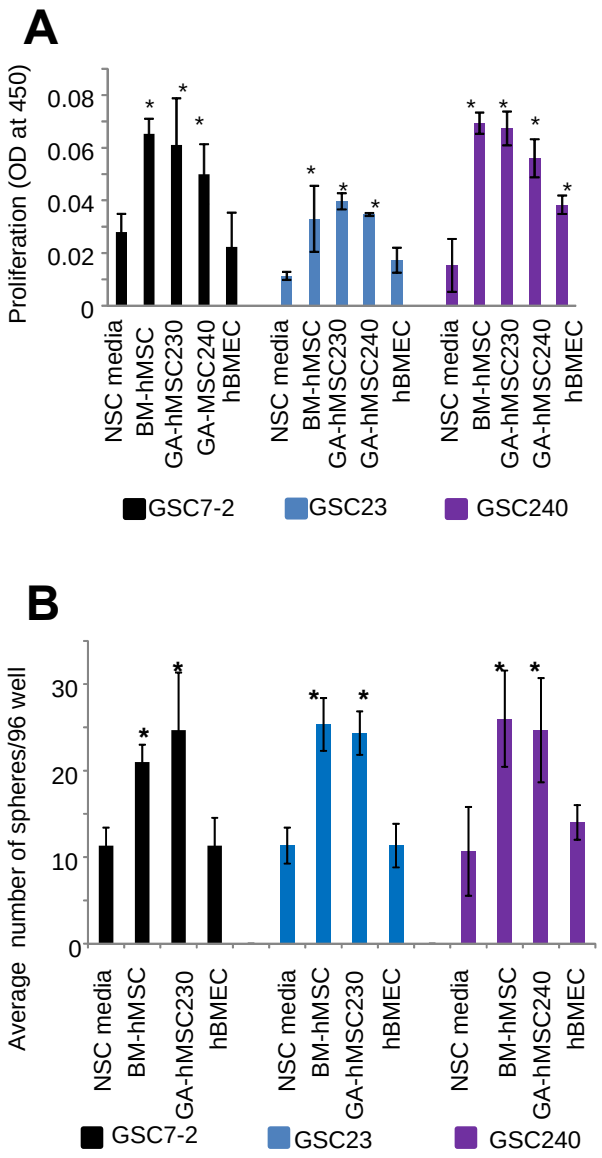


Figure 13. GA-MSCs promote the growth and self-renewal of GSCs. (A) Proliferation assay for 3 GSC lines demonstrating significant increases in viability after culturing in conditioned medium derived from BM-MSC and 2 GA-MSC lines. (B) Clonogenic assay for 3 GSC lines demonstrating a significant increase in neurosphere formation after culturing in conditioned medium derived from BM-MSC and GA-MSC.[97]

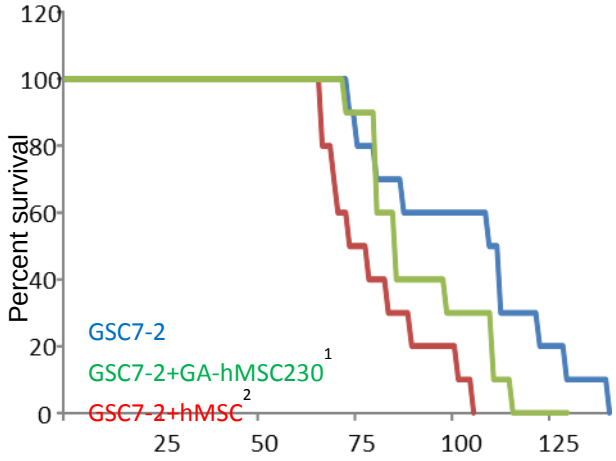


Figure 14. GA-MSCs decrease median survival in mice with GSC xenografts. Survival curve for GSC 7-2 demonstrating a significant decrease in median survival after co-injection with BM-MSC and GA-MSC 230.[97]

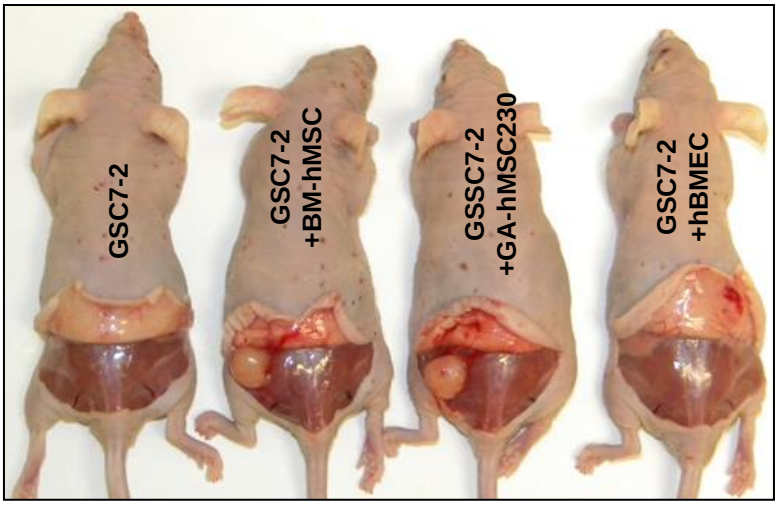


Figure 15. GA-MSCs increase tumor burden in mice with GSC xenografts. Flank tumors for mice with GSC 7-2 xenografts demonstrating an increase in tumor burden after co-injection with BM-MSC and GA-MSC 230.[97]

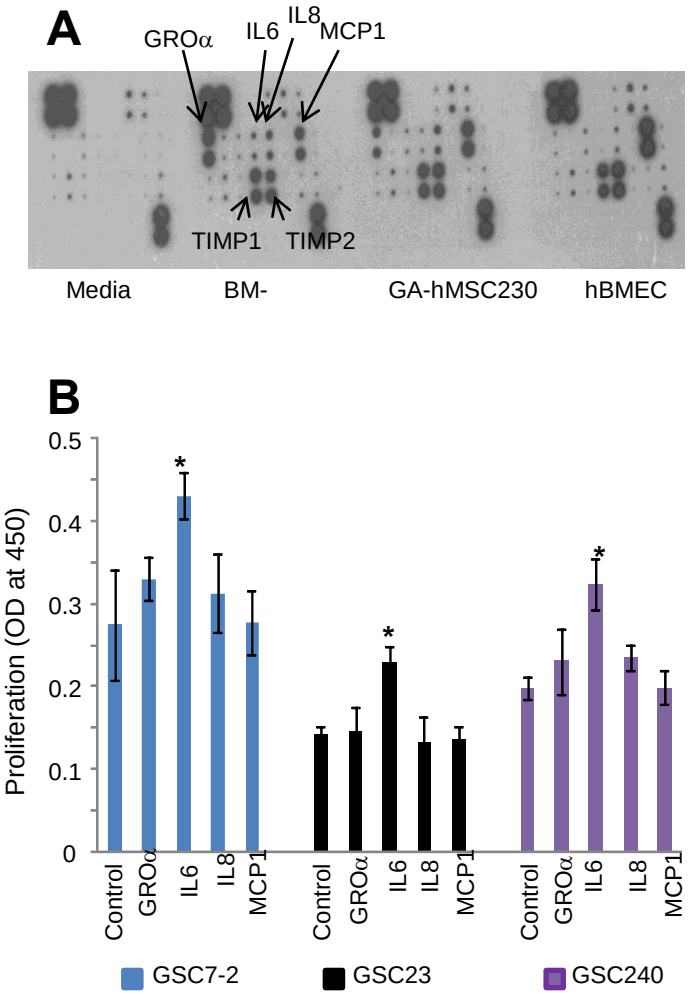


Figure 16. GA-MSCs secrete growth promoting cytokines. (A) Cytokine array immuno-blot demonstrating the secretion several cytokines by BM-MSC and GA-MSC 230. (B) Proliferation assay for GSCs demonstrating a significant increase in viability after treatment with IL-6.[97]

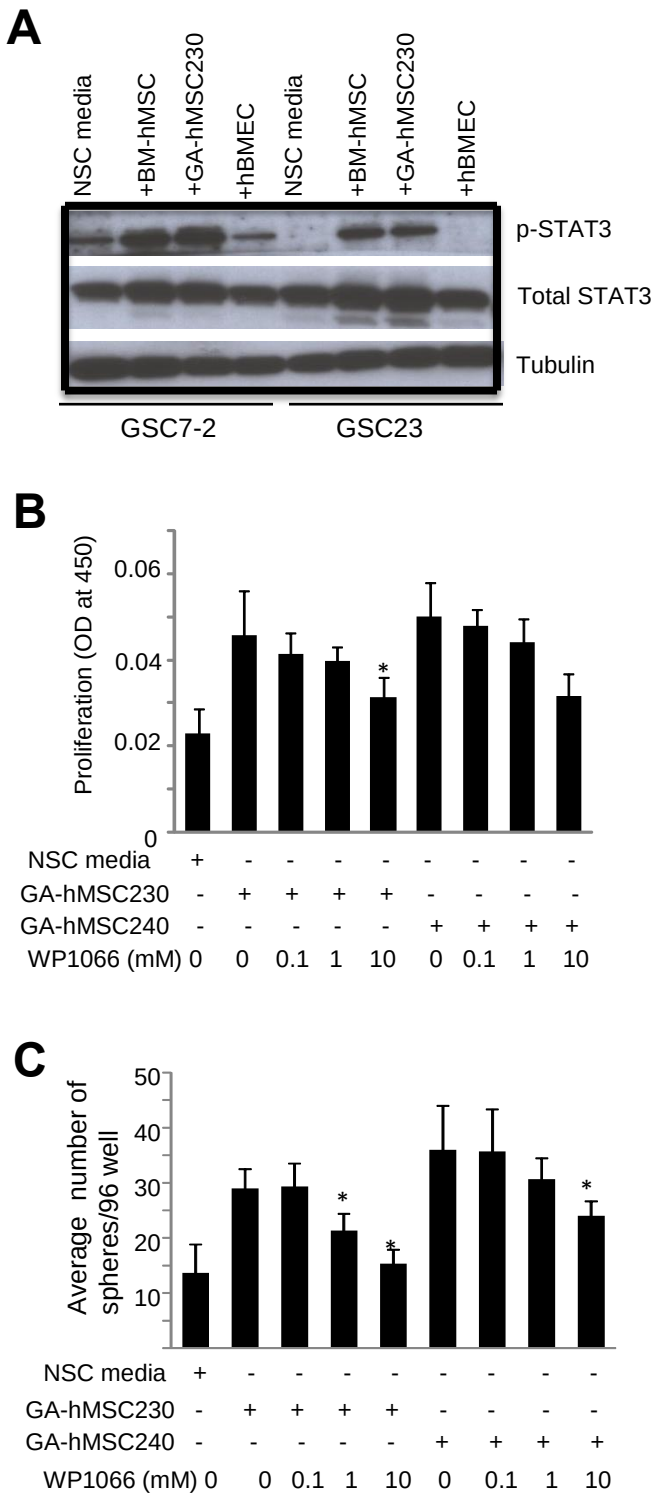


Figure 17. IL-6 secretion from GA-MSCs activates STAT3. (A) Western blot analysis for GSC demonstrating increased p-STAT3 expression after co-culture with BM-MSC and GA-MSC 230. (B) Proliferation assay for GSC demonstrating significant dose dependent decreases in viability after treatment GA-MSC and STAT3 inhibitor (WP1066). (C) Proliferation assay for GSC demonstrating dose dependent decreases in neurosphere formation after treatment with GA-MSC and STAT3 inhibitor (WP1066).[97]

The findings from studies investigating the role of MSCs in gliomas, and thus the intercellular communication between MSCs and GSCs, is limited to the description of soluble tumor-promoting proteins by MSCs. However, one key component of paracrine intercellular interactions that has been overlooked is that of exosome communication. Although the physiologic and pathologic release of exosomes has recently been investigated in a variety of disease processes, including cancer, the function of exosomes secreted from non-cancerous cells within the tumor niche has never been explored.[151] This research disparity, combined with the extensive evidence describing the various contents of exosomes, highlights a gap in knowledge as to the function of MSC-derived exosomes. Thus, further examination is necessary to evaluate this novel intercellular exosomal communication pathway between MSCs and GSCs.

Currently, there are only two reports investigating the role of tumor stroma-derived exosomes within the microenvironment. In 2013, Luga, et al., found that exosomes released from cancer associated fibroblasts (CAFs) in breast cancer, increased the invasion and motility of breast cancer cells.[152] This effects was linked to the activation of the Wnt-planar cell polarity (Wnt-PCP) signaling pathway, via Wnt11 molecules packaged in CAF-derived exosomes. Luga and colleagues concluded that this intercellular communication system led to enhanced metastatic capabilities of breast cancer.[152] This is the first study demonstrating the tumor-promoting capabilities of exosomes derived from stromal cells isolated from the tumor microenvironment. Recently, in 2014, Wang and colleagues, demonstrated that exosomes from gastric carcinoma-mesenchymal stem cells (GC-MSCs) were capable of increasing the growth and migration of human gastric carcinoma (HGC) cells.[153] These effects were found to be mediated by the delivery of miR-221 to HGC cells by GC-MSC-derived exosomes. The onco-miRNA miR-221 is linked to the down-regulation of certain cyclin dependent kinase inhibitors and anti-apoptotic factors.[153] Together these two studies demonstrate the tumor-promoting role of stroma-derived exosomes, and define a mechanism by which the exosomal contents can mediate the tumor-enhancing effects.

Another study published by Roccaro, et al., showed the tumor-promoting properties of BM-MSC-derived exosomes in multiple myeloma (MM), albeit using cells that were not part of the microenvironment in the primary tumor.[154] Here researchers found that MSCs harvested from the bone marrow (BM-MSCs) of patients with MM, had different genetic and proteomic profiles than BM-MSCs harvested from normal subjects. These differences carried over to differences in the genetic and proteomic profiles of exosomes derived from both BM-MSC lines. Roccaro and colleagues found that BM-MSC-derived exosomes from MM patients had higher levels of levels of oncogenic proteins, cytokines and adhesion molecules, when compared to BM-MSC-derived exosomes from normal subjects. These tumor promoting proteins were shown to transfer to plasma cells in both *in vitro* and *in vivo* mouse models.[154] Importantly, the BM-MSCs of MM patients were harvested from abnormal bone marrow, not primary tumor sites, and therefore not associated with the microenvironment. However, MM is thought to arise from abnormal bone marrow, and these abnormal BM-MSCs may play a role in the initial malignant transformation of plasma cells in MM.[154] Nevertheless, this study demonstrates the tumor-promoting capabilities of exosomes derived from BM-MSCs, which can be recruited to, and engrafted in, gliomas.

In 2011, Zhu, et al., investigated the tumor promoting effects of BM-MSC-derived exosomes on human gastric carcinoma and colon cancer cell lines.[155] They found that tumor stem cells treated with BM-MSC-derived exosomes had increased proliferation *in vitro*, and promoted tumorigenicity in *in vivo* mouse xenografts. Additionally, they showed that these results were due to the increased expression of vascular endothelial growth factor (VEGF) in the cancer stem cells, via exosomal activation of extracellular signal-regulated kinase 1/2 (ERK1/2).[155] Their findings suggested a novel mechanism by which intercellular communication could take place within a tumor.

Although the Roccaro and Zhu articles demonstrate the tumor promoting properties of BM-MSC-derived exosomes, these cells are not the most accurate representation of MSCs in the tumor microenvironment. Once recruited and

entrenched in the tumor niche, BM-MSCs are influenced by neighboring CSCs and undergo specific changes that alter their biology.[156-161] Although they still exhibit the classical MSC morphology, surface markers, and tri-differentiation capabilities, TA-MSCs differ from normal BM-MSCs in proliferation rate and genetic signature.[97] Therefore the efficacy of these studies could be improved by investigating the effects of TA-MSC-derived exosomes on the progression of malignancy. Thus, the focus of my thesis is the investigation of exosomal communication within the glioma microenvironment, utilizing GA-MSCs and GSCs isolated from patient tumor.

1.6 Conclusions

In this chapter I described that exosomes are 40-100nm in diameter with a lipid bilayer, are formed and packaged intracellularly within the endosomal system, and are secreted from the cell via exocytosis. I discussed how exosomes are different from other larger microvesicles, in formation, content and morphology. I described the specific protein constituents of the exosomal membrane, the cellular elements that have been found in the intra-exosomal compartment, and how exosomes interact with recipient cells via fusion or endocytosis. I explored the studies evaluating exosomes in gliomas, from the transfer of an oncogenic protein receptor, to diagnostic properties in the serum, to new therapeutic strategies. I described the constituents of the glioma microenvironment, and how it is nurturing to resident GSCs. I discussed how we isolated GA-MSCs from glioma surgical specimens, and that GA-MSC-mediate promotion of tumor growth is link to cytokine secretion. I described how MSC-derived exosomes promote malignant growth in other tumor types, but have not been studied in gliomas. Based on this background, investigating the effects of GA-MSC-derived exosomes on GSCs will provide a better model to study the role of stroma-derived exosomes in the glioma microenvironment.

1.7 Hypothesis

Given the capability of exosomes to participate in paracrine communication between cells, and also the potential of exosome content to alter the biology of recipient cells, I hypothesized that GA-MSC-derived exosomes increase the growth and evolution of gliomas via the delivery of specific miRNA to recipient GSCs. To test this hypothesis I investigated three specific aims:

1. I hypothesized that GA-MSCs produce exosomes with unique proteomic and genomic profiles when compared with parental GA-MSCs. To test this hypothesis, I propose to:

    a. Demonstrate that GA-MSCs produce exosomes.
    b. Characterize the production system of GA-MSC-derived exosomes.
    c. Characterize the content of GA-MSC-derived exosomes.

2. I hypothesized that GA-MSC-derived exosomes can be internalized by GSCs and can increase the tumorigenicity of GSCs. To test this hypothesis, I propose to:

    a. Demonstrate that GSCs can internalize GA-MSC-derived exosomes.
    b. Show that GA-MSC-derived exosomes increase the proliferation and clonogenicity of GSCs *in vitro.*
    c. Establish that GA-MSC-derived exosomes increase the tumorigenicity of GSCs *in vivo.*

3. I hypothesized that MicroRNAs delivered via GA-MSC-derived exosomes increase the tumorigenicity of GSCs. To test this hypothesis, I propose to:

    a. Identify miRNA in GA-MSC-derived exosomes that could potentially influence the growth of GSCs.
    b. Demonstrate that specific miRNA in GA-MSC-derived exosomes increase the proliferation and clonogenicity of GSCs *in vitro*.
    c. Establish that specific miRNA in GA-MSC-derived exosomes increase the tumorigenicity of GSCs *in vivo*.

Chapter II

Isolation and Characterization of GA-MSC-Derived Exosomes

Isolation and Characterization of GA-MSC-Derived Exosomes

Understanding the role of exosomes in the communication between GA-MSCs and GSCs requires evidence that GA-MSCs release exosomes, and that these GA-MSC-derived exosomes contain elements that can alter the biology of GSCs. To my knowledge there is little information about the production of exosomes by GA-MSCs and detailing of the content of GA-MSC-derived exosomes has never been performed. Therefore, I hypothesized that GA-MSCs produce exosomes with unique proteomic and genomic profiles when compared with parental GA-MSCs. To test this hypothesis, I first show that GA-MSCs produce nano-vesicles that qualified as exosomes. These results proved that this tumor stroma constituent is capable of exosome production. I then determined the extent to which external cellular stressors affected production of GA-MSC-derived exosomes. These experiments showed that exosomes are released under conditions that simulate the tumor niche. Finally, I characterized the content of GA-MSC-derived exosomes; specifically, I interrogated the protein and miRNA profiles. These experiments identified exosomal miRNAs that have the potential to alter the biology of GSCs.

2.1 Experimental Methods

To investigate the role of exosomes in the communication pathway between GA-MSC and GSCs, I utilized commercially available human BM-MSCs (Lonza, Inc.) and also GA-MSCs isolated from human glioma surgical specimens. GA-MSCs were isolated from the surgical specimens by applying the same methods used to isolate human BM-MSCs.[143] I selected four GA-MSCs lines that spanned the range of glioma grades (**Table 1**), in order to assess for any exosomal differences among degree of tumor pathology. All four GA-MSC lines met the criteria for MSCs as outlined by the International Society for Cellular Therapy (ISCT).[118] Specifically, all GA-MSC lines have spindle shape morphology and are adherent in culture. Additionally, all GA-MSC lines expressed the mesenchymal surface markers CD73, CD90 and CD105, and did not express the endothelial surface marker CD34, the hematopoietic surface marker CD45, or the neural stem cell marker CD133. Finally, all GA-MSC lines possessed the ability to tri-differentiate into adipocytes, chondrocytes and osteocytes.[118]

I also chose four GSC lines which were isolated from human glioma surgical specimens using the methods described by Singh, et al. (**Table 2**).[86] Importantly, two of these GSCs (GSC-20 and GSC-262) were isolated from the same tumor specimens from which were isolated two of the GA-MSC lines (GA-MSC-262 and GA-MSC-20). This provided us with two matching GA-MSC/GSC pairs. Importantly, the GA-MSCs are unique from the corresponding GSCs. GA-MSCs do not express the same genetic aberrations as GSCs, nor do they form tumors in mouse xenografts as do GSCs.[97] Interestingly, GA-MSCs also differ from normal human BM-MSC in both genetic and growth profiles.[97]

Isolation of GA-MSC-Derived Exosomes

In order to isolate MSC-derived exosomes, BM-MSCs and GA-MSCs were expanded to approximately 10^6 cells in MSC growth medium: Eagle's Minimum Essential Medium Alpha (Sigma-Aldrich), 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine. GA-MSCs were then washed and allowed to

Cell Line	Pathology	% CD105	% CD90	% CD73	% CD73/90/105	% CD45	% CD34	% CD133	Adipo-genesis	Chondro-genesis	Osteo-genesis	*In Vivo* Growth
GA-MSC 230	Grade II	99.9	48.0	99.9	48.0	0.0	0.0	0.0	+	+	+	NG
GA-MSC 247	Grade III	99.9	54.7	99.8	53.0	0.0	0.0	0.0	+	+	+	NG
GA-MSC 262*	Grade IV	93.5	75.6	88.4	65.9	0.8	0.0	0.0	+	+	+	NG
GA-MSC 20*	Grade IV	52.5	99.1	84.4	46.3	0.0	0.2	0.4	+	+	+	NG

Table 1. GA-MSCs exhibit MSC-like characteristics. GA-MSC lines express the CD73, CD90, CD105 mesenchymal markers, do not express the CD34, CD45 and CD133 endothelial, hematopoietic, and neural stem cell markers, can differentiate along mesenchymal lines, and are not tumorigenic (NG = no growth).

Cell Line	Pathology	% CD73/90/105	% CD133	*In Vivo* Growth
GSC 11	Grade IV	4.0	59.0	G
GSC 7-2	Grade IV	2.9	75.0	G
GSC 262*	Grade IV	6.4	28.6	G
GSC 20*	Grade IV	4.0	26.0	G

Table 2. GSCs exhibit CSC-like characteristics. GSC lines express the CD133 neural stem cell marker, do not express the CD73, CD90 and CD105 mesenchymal markers, and are tumorigenic in mice (G = growth).

incubate for 48 hours in serum-free NSC medium: Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 mix with L-Glutamine (Corning CellGro), 2% B-27 supplement (Gibco), and 1% penicillin-streptomycin.

BM-MSC-derived and GA-MSC-derived exosomes were then isolated by differential ultracentrifugation, as described by Thery, et al.[162] Briefly, MSC-derived conditioned medium was collected and passed through a 22μm filter to remove dead cells and cellular debris, and centrifuged at 10,000 x gravity for 30 minutes, to remove large microvesicles (non-exosomal). The supernatant was collected and ultra-centrifuged at 100,000 x gravity for 90 minutes. The supernatant (exosome-free conditioned medium) was removed yielding a raw exosome pellet, which was resuspended in PBS. Exosomes were further purified by another ultra-centrifugation at 100,000 x gravity, for 90 minutes, with or without a 30% sucrose cushion depending on the experimental usage. Exosome pellets were then resuspended in PBS, NSC medium or lysis buffer depending on the experimental need.

Western Blot Analysis of GA-MSC-Derived Exosomes

In order to confirm that nano-vesicles isolated from MSC-derived conditioned medium were exosomes, I performed western blot for known exosomal markers. BM-MSC-derived and GA-MSC-derived exosomes were isolated by differential ultracentrifugation. Protein from BM-MSC-derived and GA-MSC-derived exosomes was extracted using membrane lysis buffer, and measured by Bradford protein assay. Western blot for the exosomal surface markers CD63 and GAPDH on MSC-derived exosomes, and the corresponding parental cell, were performed using anti-CD63 (Santa Cruz Biotechnology, Inc.) and anti-GAPDH (Sigma-Aldrich). Additionally, western blot for the non-exosomal surface markers CD16 and CD32 on MSC-derived exosomes, and the corresponding parental cell, were performed using anti-CD16 (Abcam) and anti-CD32 (Abcam).

Electron Microscopic Analysis of GA-MSC-Derived Exosomes

In order to more clearly confirm that nano-vesicles isolated from MSC-derived conditioned medium were exosomes, I performed electron microscopy analysis to

visualize morphology. BM-MSC-derived and GA-MSC-derived exosomes were isolated by differential ultracentrifugation. BM-MSC-derived and GM-MSC-derived exosomes were prepared for analysis by electron microscopy (EM) by the accepted protocol published by Thery, et al.[162] Briefly, MSC-derived exosomes were affixed to Formvar coated EM grids using 2% paraformaldehyde (PFA), and contrasted with 4% uranyl-oxalate. MSC-derived exosomes were then examined by electron microscopy on a JEM 1010 transmission electron microscope (JEOL) at an accelerating voltage of 80 Kv. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques). For gold particle (10nm) labeling, exosome grids were subjected to immuno-gold staining of the exosomal surface marker CD63 and the non-exosomal markers CD16 and CD32, utilizing gold-anti-rabbit (Sigma Aldrich), anti-CD63 (Santa Cruz Biotechnology, Inc.), anti-CD16 (Abcam), and anti-CD32 (Abcam). MSC-derived exosomes were then examined by electron microscopy as previously described.

Characterization of the Production System for GA-MSC-Derived Exosomes

In order to characterize the production system of MSC-derived exosomes, I utilized various growth conditions and quantified exosomes by ELISA. BM-MSCs and four GA-MSCs were expanded to 50%, 60%, 70%, 80%, 90% and 100% confluency, in eight 15cm dishes. Two of the dishes were incubated for 24 hours, two incubated for 48 hours, two incubated for 72 hours and two incubated for 96 hours. BM-MSC-derived and GA-MSC-derived exosomes were then isolated by differential ultracentrifugation as previously described. BM-MSC-derived and GA-MSC-derived exosomes from each time point in each group, were quantified by CD63 ELISA (System Biosciences), which utilizes standards calibrated by NanoSight. Briefly, BM-MSC-derived and GA-MSC-derived exosomes were affixed to anti-CD63 coated 96-well plates overnight, and subsequently analyzed after the addition of a secondary antibody, followed by an enzymatic colorimetric substrate. Later experiments utilized the NanoSight (NS300) system to not only quantify, but also measure the diameter of isolated exosomes. Briefly, BM-MSC-derived and GA-MSC-derived exosomes were resuspended in PBS, diluted 1:100, and passed

through the laser detector of the NS300. The NS300 utilizes laser light scattering to visualize the Brownian motion of nano-sized particles. This technology not only enables the quantification exosomes, but also produces a size distribution which includes statistical parameters.

Protein Characterization of GA-MSC-Derived Exosomes

In order to characterize the protein content of MSC-derived exosomes, I utilized protein array technology. BM-MSCs and GA-MSCs were expanded to 2.5 x 10^7 cells in MSC medium, washed with PBS and cultured in NSC medium for 48 hours. BM-MSC-derived and GA-MSC-derived conditioned medium (CM) was then collected, subjected to ultracentrifugation, the exosome-free conditioned medium (EF-CM) supernatant removed and saved, and the MSC-derived exosome pellets resuspended and lysed in neural stem cell (NSC) medium and lysis buffer. Immuno-blot was then performed using human growth factor and cytokine antibody array kits (Bio Ray), on BM-MSC-derived and GA-MSC-derived CM, BM-MSC-derived and GA-MSC-derived EF-CM and BM-MSC-derived and GA-MSC-derived exosomes, as well as the NSC medium/lysis buffer control. Briefly, protein from exosome samples were affixed to anti-growth factor and anti-cytokine coated array plates overnight, and subsequently analyzed after the addition of a secondary antibody, followed by an enzymatic substrate.

MicroRNA Characterization of GA-MSC-Derived Exosomes

In order to characterize the miRNA profile of MSC-derived exosomes, I utilized miRNA micro-array technology. BM-MSC-derived and GA-MSC-derived exosomes were isolated by differential ultracentrifugation, and exposed to RNase (1nM) for 15 minutes to eliminate free-floating extra-vesicular RNA elements. Remaining RNase was removed by PBS wash and subsequent centrifugation. Total RNA was then extracted by a combination of organic and solid-phase methods using the mirVana RNA isolation kit (Ambion). Additionally, total RNA was extracted from the parental cell line, and the miRNA profile for each sample was obtained using µParaflo® microfluidic biochip technology, through LC Sciences (Houston, TX).

2.2 Results

Characterization of GA-MSC-Derived Exosomes

One of the established methods for identifying exosomes is by western blot for tetraspanin membrane proteins and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both of which are commonly associated with exosomes. Consequently, we cultured and isolated nano-vesicles from BM-MSCs and GA-MSCs (see *experimental methods*). Western blot results show the presence of CD63, a member of the tetraspanin family, as well as GAPDH in the protein extracted from isolated nano-vesicles from BM-MSC and GA-MSCs (**Figure 18**). Additionally, the non-exosomal markers, CD16 and CD32, were absent in nano-vesicle isolates from BM-MSC and GA-MSCs, although they were present in parental MSCs.[163] These results indicate that the isolated MSC-derived nano-vesicles were exosomes.

In addition to harboring specific membrane protein constituents, exosomes are also characterized by specific size and shape criteria as outlined by Thery, et al.[162] However, the size of exosomes limits their visualization by conventional light microscopy, and electron microscopy (EM) is the gold standard for characterizing the morphology of exosomes. Therefore we used EM to further characterize the nano-vesicles isolated from BM-MSCs and GA-MSCs. EM of nano-vesicle isolates from BM-MSC and GA-MSCs demonstrate round nano-vesicles 40-100 nm in diameter, with a lipid bilayer, consistent with the known appearance of exosomes (**Figure 19**). To more precisely define these nano-vesicles as exosomes, we labeled the CD63 membrane protein with gold nano-particles. EM after immuno-gold staining against CD63, confirmed the presence of the tetraspanin exosomal marker in the membrane of these nano-vesicles. Furthermore, immuno-gold staining against CD16 and CD32 by EM, demonstrated the absence of these markers in the membrane of these nano-vesicles (**Figure 20**). These methods provided strong evidence that the nano-vesicles produced by BM-MSCs and GA-MSCs meet the criteria of exosomes.

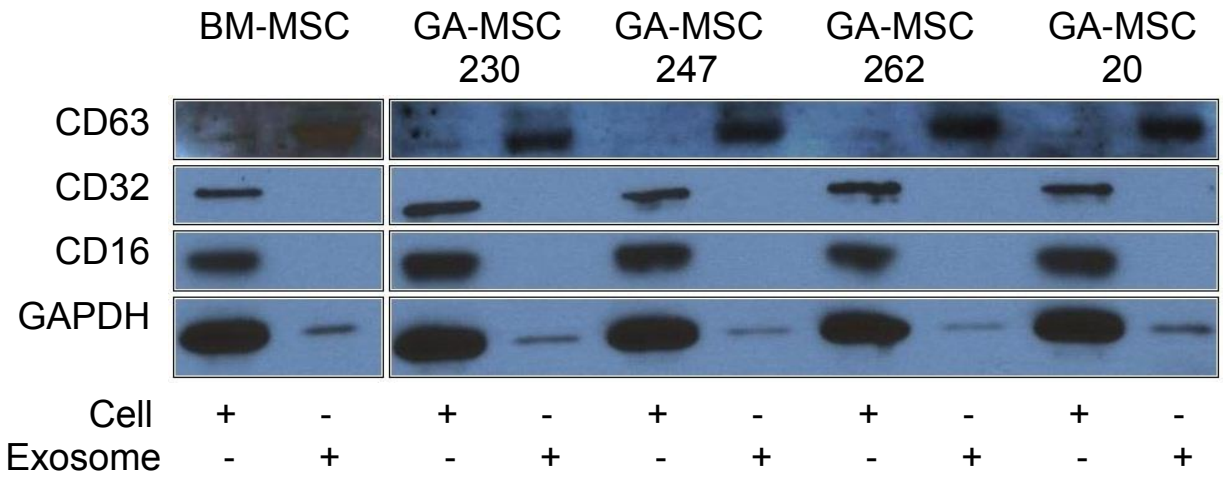


Figure 18. MSC-derived nano-vesicles express exosomal markers. Analysis of the exosomal markers CD63 and GAPDH, as well as the non-exosomal markers CD32 and CD16, in exosomes derived from BM-MSC and four GA-MSC lines. CD63 is expressed in all MSC-derived exosomes, and expression is higher than in the parental cell. GAPDH is also expressed in all MSC-derived exosomes, however expression is higher in the parental cell. Both CD32 and CD16 are not expressed in all MSC-derived exosomes, but are expressed in the parental cell.

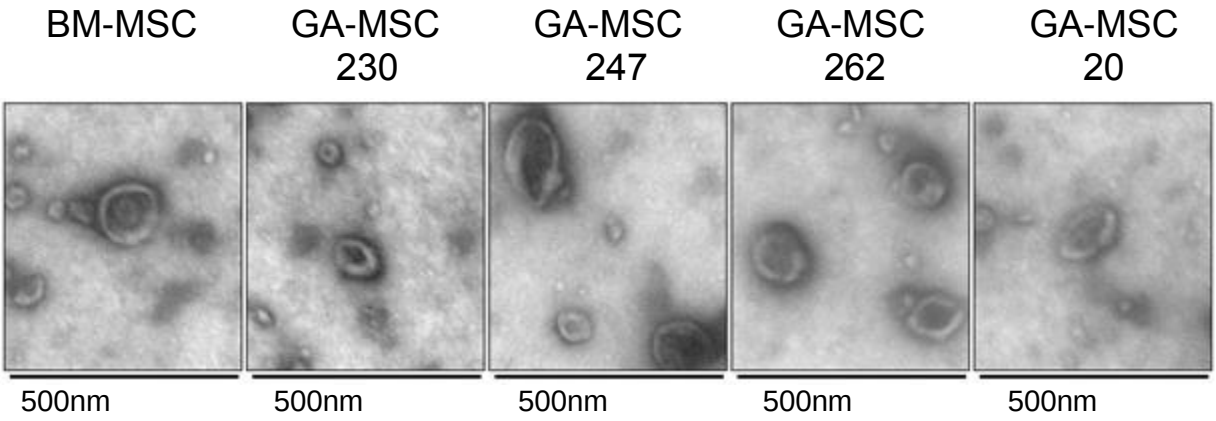


Figure 19. Morphology of MSC-derived nano-vesicles is identical to exosomes. Analysis of exosomes derived from BM-MSC and four GA-MSC lines, by scanning electron microscopy. Exosomes derived from all MSC lines exhibit the classical cupped-shape morphology with a distinct lipid bilayer, and are within the 40nm-100nm range.

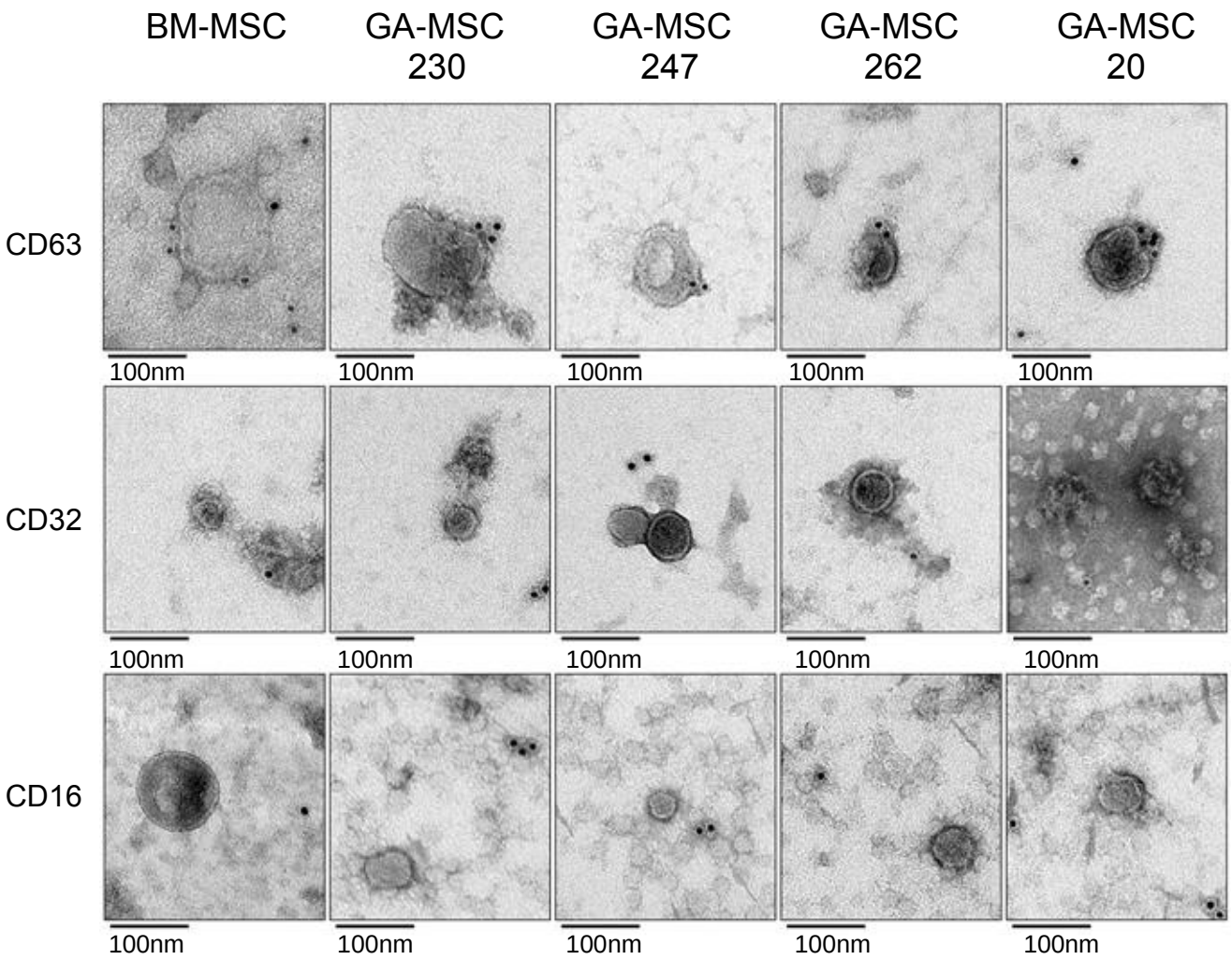


Figure 20. Exosome marker is expressed on MSC-derived nano-vesicles. Immuno-gold labeling of the exosomal marker CD63, and non-exosomal markers CD32 and CD16, of exsomes derived from BM-MSC and four GA-MSC lines. (Top Panel) Gold anti-body can bind to and label CD63 expressed on the membrane of all MSC-derived exosomes. (Middle/Bottom Panels) Gold anti-body cannot recognize any CD32 or CD16 markers in all MSC-derived exosomes.

The size of BM-MSC-derived and GA-MSC-derived exosomes was further verified by utilizing NanoSight technology, which can determine both the diameter and number of exosomes. Results from NanoSight analysis show the size distribution of BM-MSC-derived and GA-MSC-derived exosomes to be within the established 40-100nm range for exosomes (**Figure 21**). Interestingly, the average diameter of exosomes derived from BM-MSCs and GA-MSCs were not significantly different ($p > 0.05$, student's t-test). These data correlated with results of the western blot and electron microscopy analysis, and further supported the classification of the isolated BM-MSC-derived and GA-MSC-derived nano-vesicles as exosomes.

Production of GA-MSC-Derived Exosomes Under Cellular Stress

In order to understand the influence of culture conditions on exosomal production, we plated BM-MSCs and GA-MSCs at varying confluency and isolated exosomes at different time points (see *experimental methods*). These experiments aimed to mimic the tumor microenvironment by increasing the cell culture time, leading to decreased nutrients, as well as culturing cells at increasing levels of confluency, leading to more cell-to-cell contact. Under these conditions, BM-MSC and GA-MSCs initially increase their exosome production rate as nutrients are being used and cell-to-cell contact increases (**Figure 22**). However after 48 hours culture time and 70-80% confluency, BM-MSC-derived and GA-MSC-derived exosome production begins to decline. These results indicate that both BM-MSCs and GA-MSCs respond to cellular stress by decreasing exosome production, most likely for conservation of energy.

Growth Factor Content of GA-MSC-Derived Exosomes

GA-MSC-derived exosomes may alter the growth of GSCs via delivery of growth factors that can activate receptors on the recipient cell, or by transfer of growth factor receptors that are then incorporated into the membrane of the recipient cell. Therefore, I utilized protein array technology to analyze 33 growth factors and 8 growth factor receptors in BM-MSC-derived and GA-MSC-derived exosomes (**Table 3**). Specifically, BM-MSCs and GA-MSCs were cultured and protein was

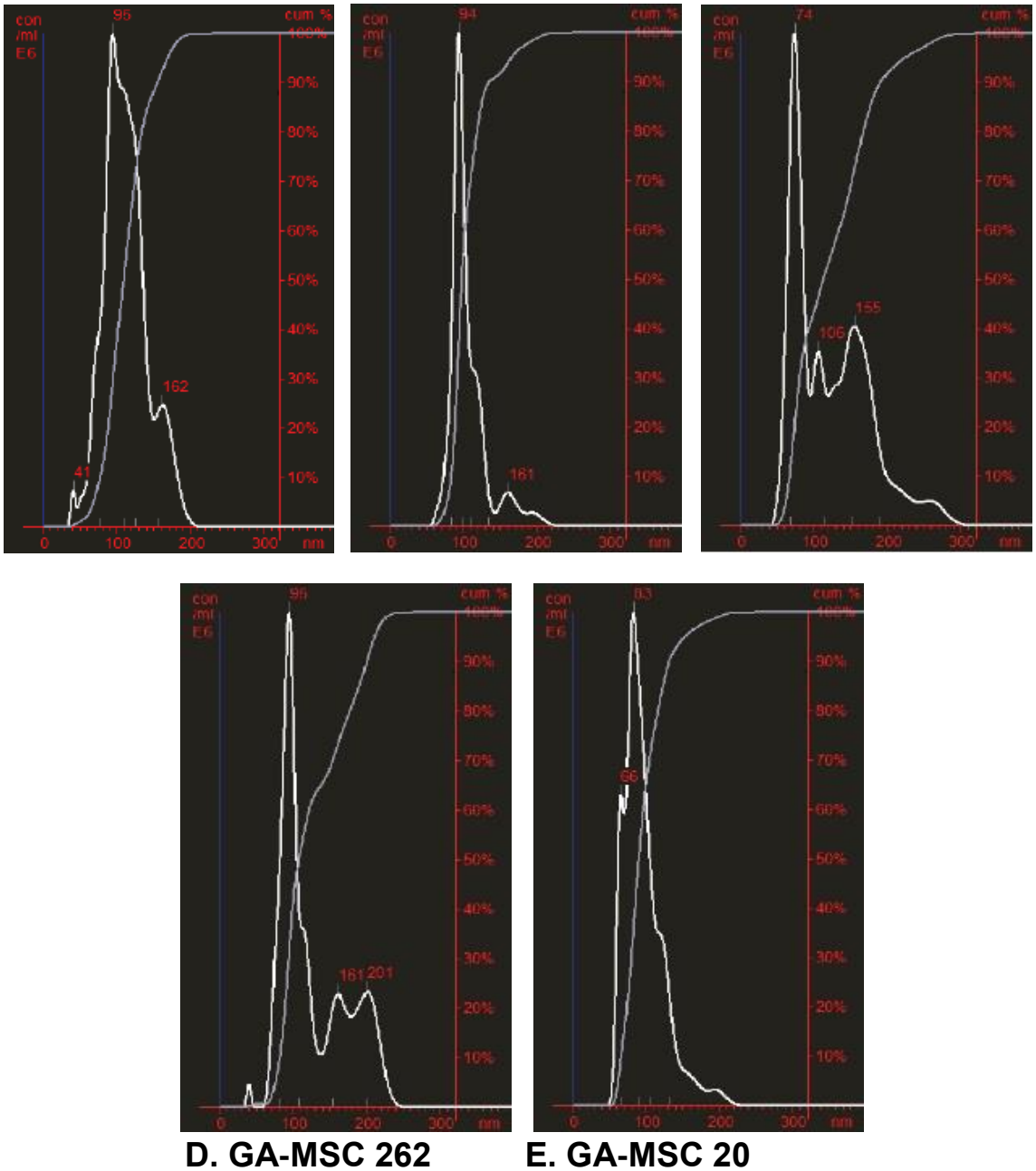


Figure 21. MSC-derived nano-vesicles exhibit exosome size distribution. (A) BM-MSC-derived exosomes have a mean diameter of 95nm. (B) GA-MSC 230-derived exosomes have a mean diameter of 94nm. (C) GA-MSC 247-derived exosomes have a mean diameter of 74nm. (D) GA-MSC 262 derived exosomes have a mean diameter of 95nm. (E) GA-MSC 262-derived exosomes have a mean diameter of 83nm.

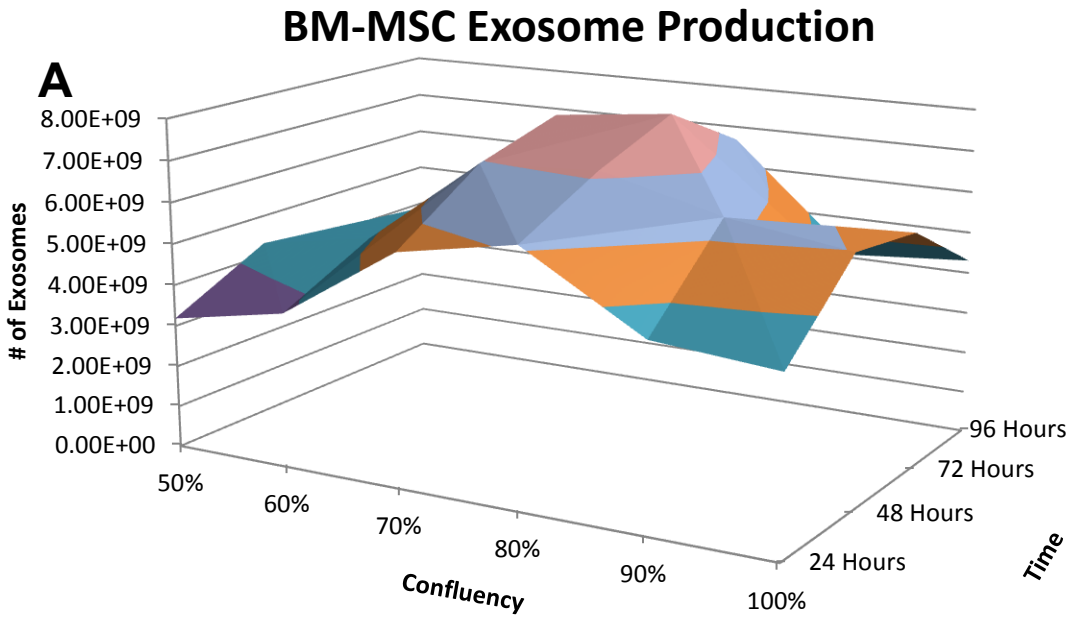
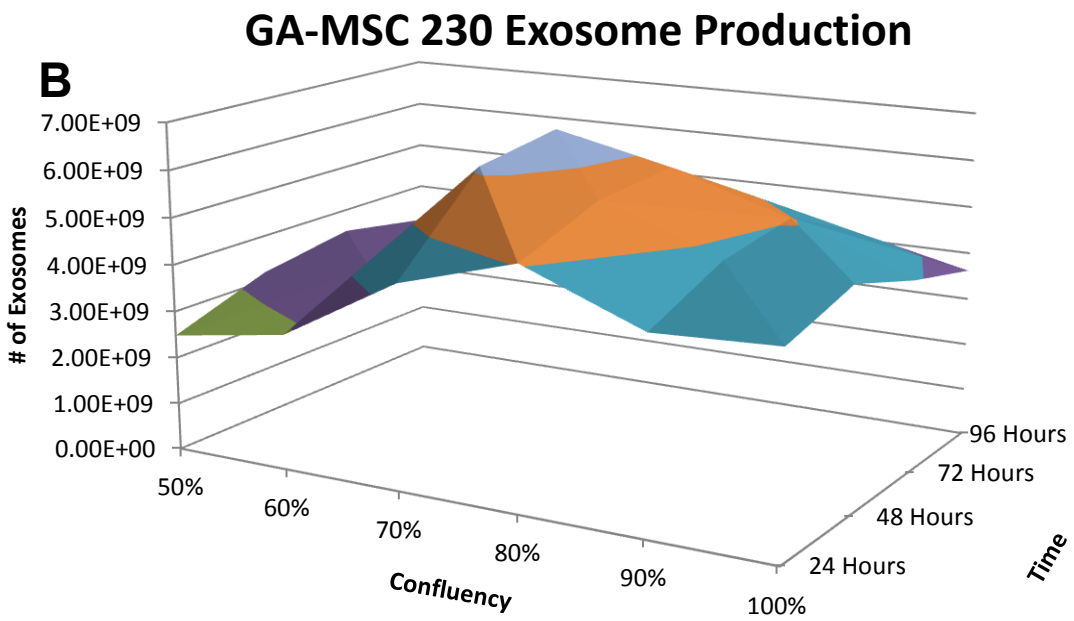


Figure 22. MSC-derived exosomes exhibit specific production systems. (A) Exosome production system for BM-MSC demonstrating a maximum of 7.87 x 10^9 exosomes produced at 80% confluency and 72 hours in culture.



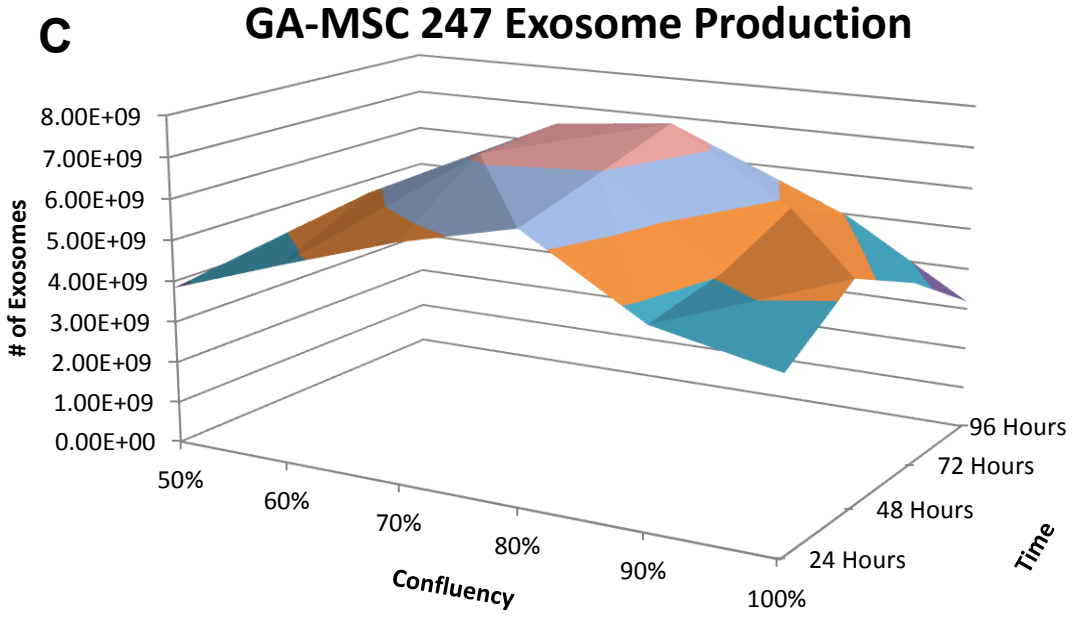
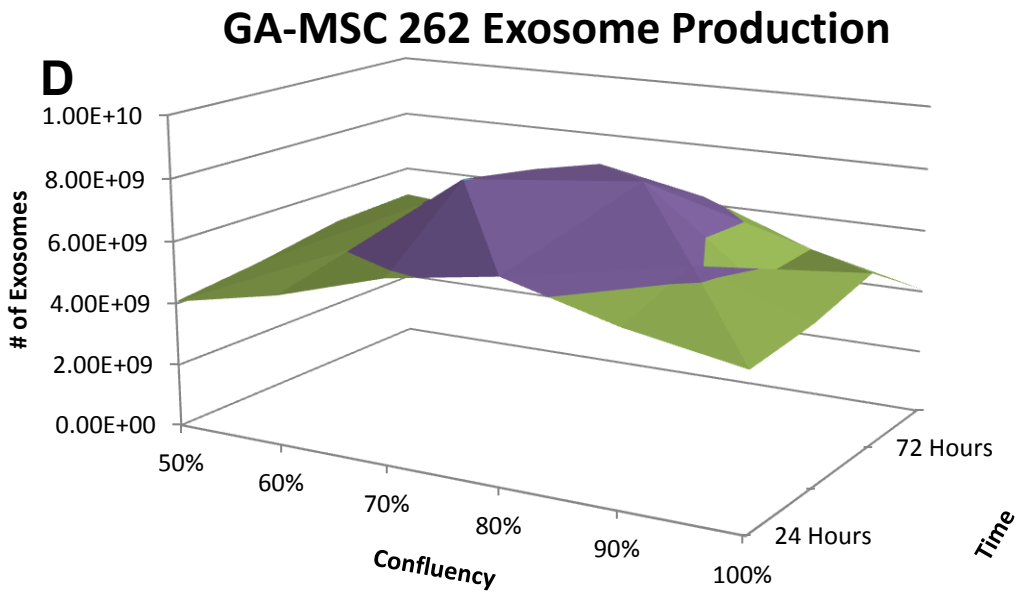


Figure 22. MSC-derived exosomes exhibit specific production systems. (B) Exosome production system of GA-MSC 230 demonstrating a maximum of 7.39 x 10^9 exosomes produced at 70% confluency and 72 hours in culture. (C) Exosome production system of GA-MSC 247 demonstrating a maximum of 7.57 x 10^9 exosomes produced at 80% confluency and 72 hours in culture.



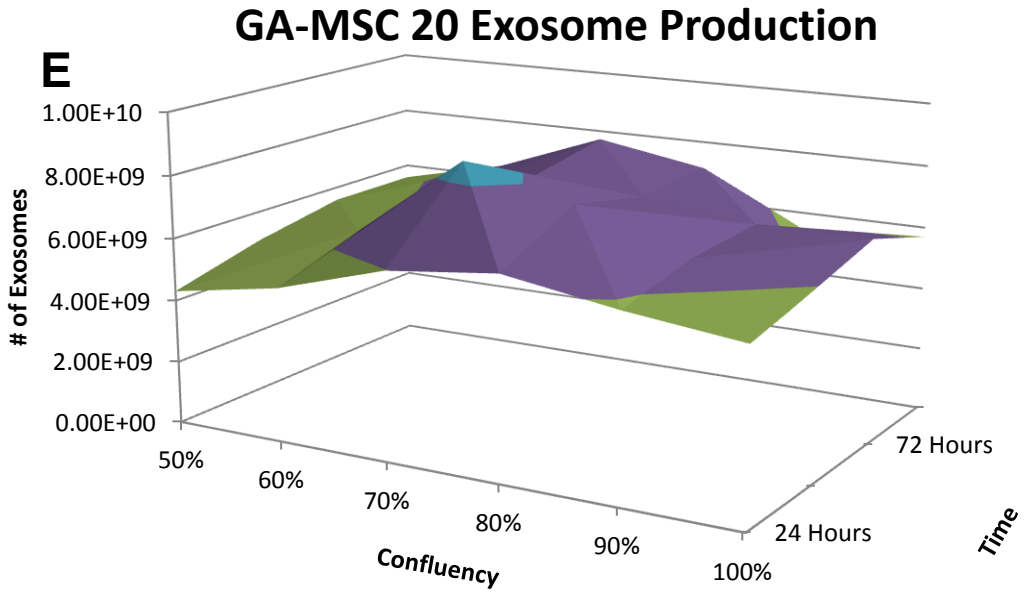


Figure 22. MSC-derived exosomes exhibit specific production systems. (D) Exosome production system of GA-MSC 262 demonstrating a maximum of 8.03 x 10^9 exosomes produced at 70% confluency and 48 hours in culture. (C) Exosome production system of GA-MSC 247 demonstrating a maximum of 8.51 x 10^9 exosomes produced at 70% confluency and 48 hours in culture.

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AREG	bFGF	b-NGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2	POS	POS	NEG	NEG	AREG	bFGF	b-NGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
3	G-CSF	GDNF	GM CSF	HB EGF	HGF	IGFBP 1	IGFBP 2	IGFBP 3	IGFBP 4	IGFBP 6	IGF-1	IGF-1 sR
4	G-CSF	GDNF	GM CSF	HB EGF	HGF	IGFBP 1	IGFBP 2	IGFBP 3	IGFBP 4	IGFBP 6	IGF-1	IGF-1 sR
5	IGF-2	M-CSF	M-CSF R	NT-3	NT-4	PDGF R alpha	PDGF R beta	PDGF AA	PDGF AB	PDGF BB	PLGF	SCF
6	IGF-2	M-CSF	M-CSF R	NT-3	NT-4	PDGF R alpha	PDGF R beta	PDGF AA	PDGF AB	PDGF BB	PLGF	SCF
7	SCF R	TGF alpha	TGF beta	TGF beta 2	TGF beta 3	VEGF	VEGF R2	VEGF R3	VEGF D	BLANK	BLANK	POS
8	SCF R	TGF alpha	TGF beta	TGF beta 2	TGF beta 3	VEGF	VEGF R2	VEGF R3	VEGF D	BLANK	BLANK	POS

Table 3. GA-MSC-derive exosomes do not contain major growth factors. Growth factor immuno-blot array for 33 major growth factors and 8 major growth factor receptors.

extracted from isolated exosomes and analyzed by immune-blot (see *experimental methods*). As controls, I utilized BM-MSC-derived and GA-MSC-derived conditioned medium (CM), BM-MSC-derived and GA-MSC-derived exosome-free conditioned medium (EF-CM), and neural stem cell (NSC) medium alone (**Figure 23**). GA-MSC-derived exosomes had the same growth factor profile as the neural stem cell medium alone, with the presence of four growth factors: granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), insulin-like growth factor-2 (IGF-II), and transforming growth factor-β (TGF- β). These data indicate that GA-MSC-derived exosomes did not contain any of the growth factors or growth factor receptors analyzed by the protein array. In contrast, several growth factors, such as platelet-derived growth factor-AA (PDGF-AA), insulin-like growth factor binding-protein-2 (IGFBP-2), and vascular endothelial growth factor (VEGF), are present in GA-MSC-derived CM (which contains a low concentration of exosomes), and matches the profile seen in the GA-MSC-derived EF-CM after ultra-centrifuge extraction of exosomes. Therefore, removing exosomes from GA-MSC-derived CM does not eliminate or decrease growth factor signatures. These data indicate that, whereas GA-MSC-derived CM and EF-CM contain soluble growth factors secreted by the cell, these growth factors and growth factors receptors are not present within BM-MSC-derived and GA-MSC-derived exosomes.

Profiling of Cytokines in GA-MSC-Derived Exosomes

GA-MSC-derived exosomes may also affect GSCs by delivering cytokines. Therefore, I utilized protein array technology to analyze 59 cytokines and 1 cytokine receptor in BM-MSC-derived and GA-MSC-derived exosomes (**Table 4**). Specifically, BM-MSCs and GA-MSCs were cultured and protein was extracted from isolated exosomes and analyzed by immune-blot (see *experimental methods*). As controls, I utilized BM-MSC-derived and GA-MSC-derived conditioned medium (CM), BM-MSC-derived and GA-MSC-derived exosome-free conditioned medium (EF-CM), and neural stem cell (NSC) medium alone (**Figure 24**). The cytokine protein arrays for BM-MSC-derived and GA-MSC-derived exosomes demonstrate no cytokines, which matches the profile seen for NSC medium alone. In contrast, BM-

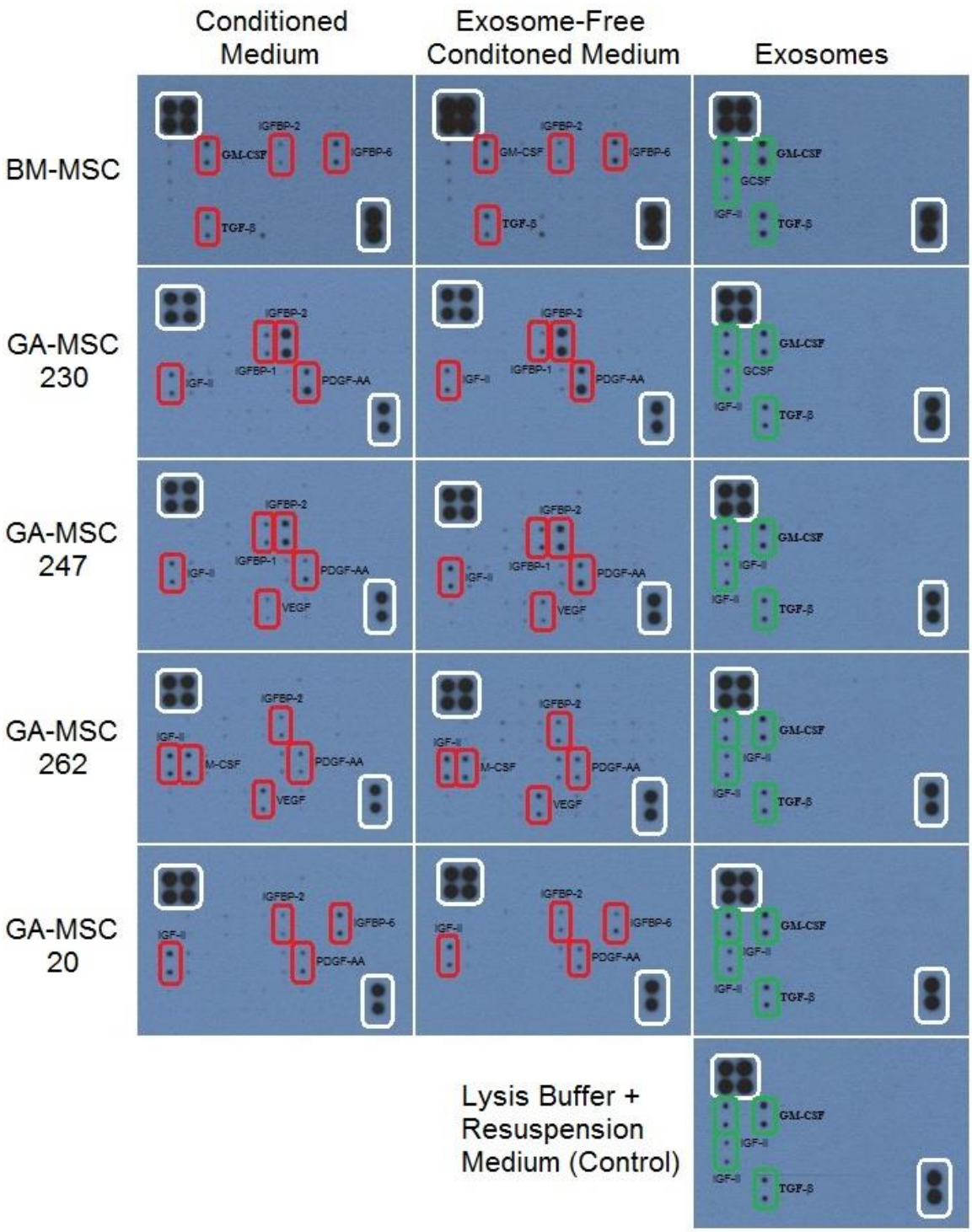


Figure 23. MSC-derived exosomes do not contain growth factors. Profiling of 33 growth factors and 8 growth factor receptors in exosomes derived from BM-MSC and four GA-MSC lines. The growth factor profiles for MSC-derived conditioned mediums and exosome-free conditioned mediums are identical, and demonstrate the expression of numerous growth factors. The growth factor profiles for MSC-derived exosomes is identical to the lysis buffer plus resuspension medium (control), and do not demonstrate the expression of any growth factors or growth factor receptors.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	BLANK	ANG	BDNF	BLC	BMP 4	BMP 6	CCL23	CNTF	EGF	Eotaxin 1
2	POS	POS	NEG	NEG	BLANK	ANG	BDNF	BLC	BMP 4	BMP 6	CCL23	CNTF	EGF	Eotaxin 1
3	Eotaxin 2	Eotaxin 3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM CSF	I-309	IFN gamma	IGFBP 1	IGFBP 2	IGFBP 4
4	Eotaxin 2	Eotaxin 3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM CSF	I-309	IFN gamma	IGFBP 1	IGFBP 2	IGFBP 4
5	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
9	PDGF BB	RANTES	SCF	SDF-1 alpha	TARC	TGF beta 1	TGF beta 3	TNF alpha	TNF beta	BLANK	BLANK	BLANK	BLANK	POS
10	PDGF BB	RANTES	SCF	SDF-1 alpha	TARC	TGF beta 1	TGF beta 3	TNF alpha	TNF beta	BLANK	BLANK	BLANK	BLANK	POS

Table 4. GA-MSC-derive exosomes do not contain major cytokines. Cytokine immuno-blot array for 59 major cytokines and 1 major cytokine receptor.

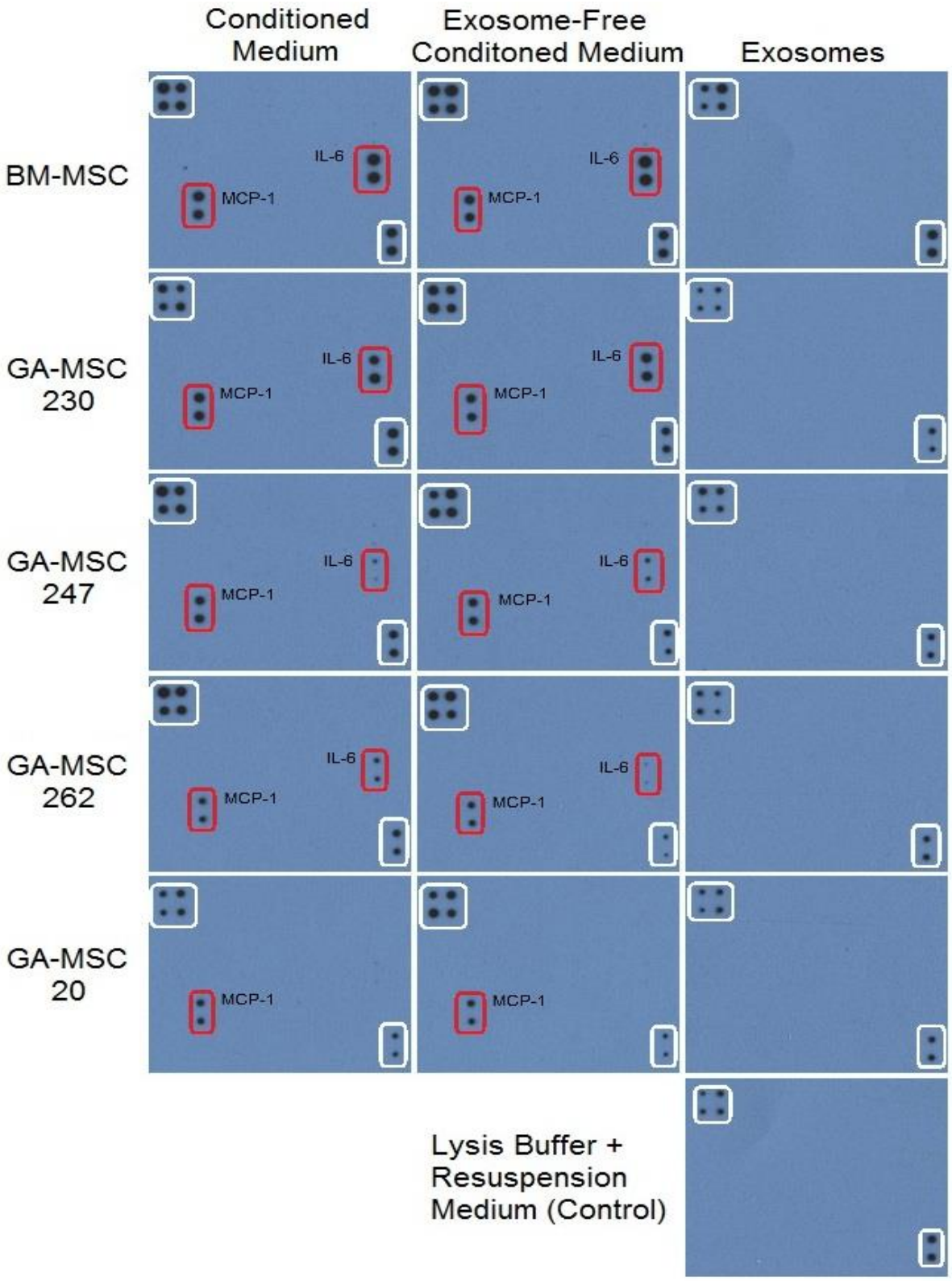


Figure 24. MSC-derived exosomes do not contain cytokines. Profiling of 59 cytokines and 1 cytokine receptor in exosomes derived from BM-MSC and four GA-MSC lines. The cytokine profiles for MSC-derived conditioned mediums and exosome-free conditioned mediums are identical, and demonstrate the expression of several cytokine. The cytokine profiles for MSC-derived exosomes is identical to the lysis buffer plus resuspension medium (control), and do not demonstrate the expression of any cytokine or cytokine receptors.

MSC-derived and GA-MSC-derived CM (which contains a low concentration of exosomes), contained several cytokines, such as interleukin-6 (IL-6) and monocyte chemo-attractant protein-1 (MCP-1), and has a similar profile as BM-MSC-derived and GA-MSC-derived EF-CM after ultra-centrifuge extraction of exosomes. Therefore, removing exosomes from BM-MSC-derived and GA-MSC-derived CM does not alter the cytokine signatures, indicating that these cytokines are not present within GA-MSC-derived exosomes.

MicroRNA Profiling of GA-MSC-Derived Exosomes

Exosomes are known to contain miRNA which, if present in GA-MSC-derived exosomes, have the potential to alter GSC growth by regulating genes at the level of transcription. Therefore, I utilized miRNA micro-array technology to analyze 2,019 miRNAs (miRBase 19.0) for their level of expression in GA-MSC-derived exosomes. Specifically, BM-MSCs and GA-MSCs were cultured and total RNA was extracted from isolated exosomes, as well as from the parental cell (see *experimental methods*). The resulting miRNA profiles for GA-MSC-derived exosomes demonstrate the presence of numerous miRNAs, distinctly different from that of the parental cell line with p-values < 0.001 by paired student's t-test (**Figure 25**). Interestingly, the miRNA profiles for GA-MSC-derived exosomes were significantly different from each other ($p < 0.001$, one-way analysis of variance) (**Figure 26**). However, when analyzed individually, the miRNA profiles between BM-MSC-derived exosomes and GA-MSC-247-derived exosomes, as well as between GA-MSC-230-derived exosomes and GA-MSC-247-derived exosomes, were not significantly different from each other ($p > 0.05$, student's t-test). Further analysis identified 37 miRNAs with an average expression level of ≥ 5000 hybridization intensity (top 0.1%), among all MSC-derived exosomes (**Table 5**), and were termed *highly expressed* miRNA. Although the overall miRNA profiles were significantly different among the group of MSC-derived exosomes, these highly expressed miRNA were consistent throughout the group. This indicates that the presence of these specific miRNAs may be conserved in all MSC-derived exosomes, and that any effects on GSC growth may be linked to these highly expressed miRNAs.

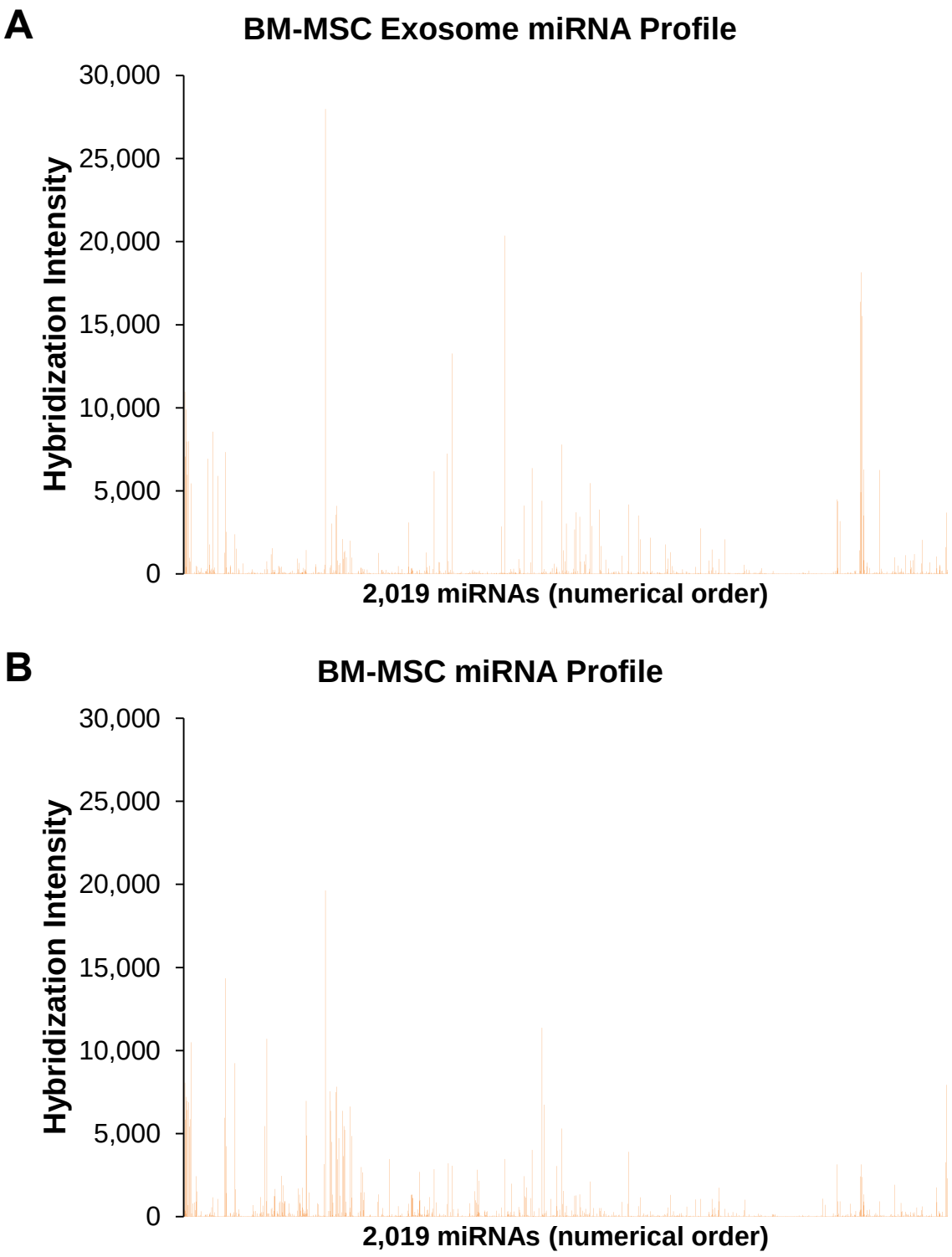


Figure 25. MSC-derived exosomes contain miRNA. (A/B) Profiling of 2,109 miRNA (miRBase 19.0) in BM-MSC and BM-MSC-derived exosomes. BM-MSC-derived exosomes expressed a significantly different miRNA profile compared to parental BM-MSC ($p < 0.001$, paired student's t-test).

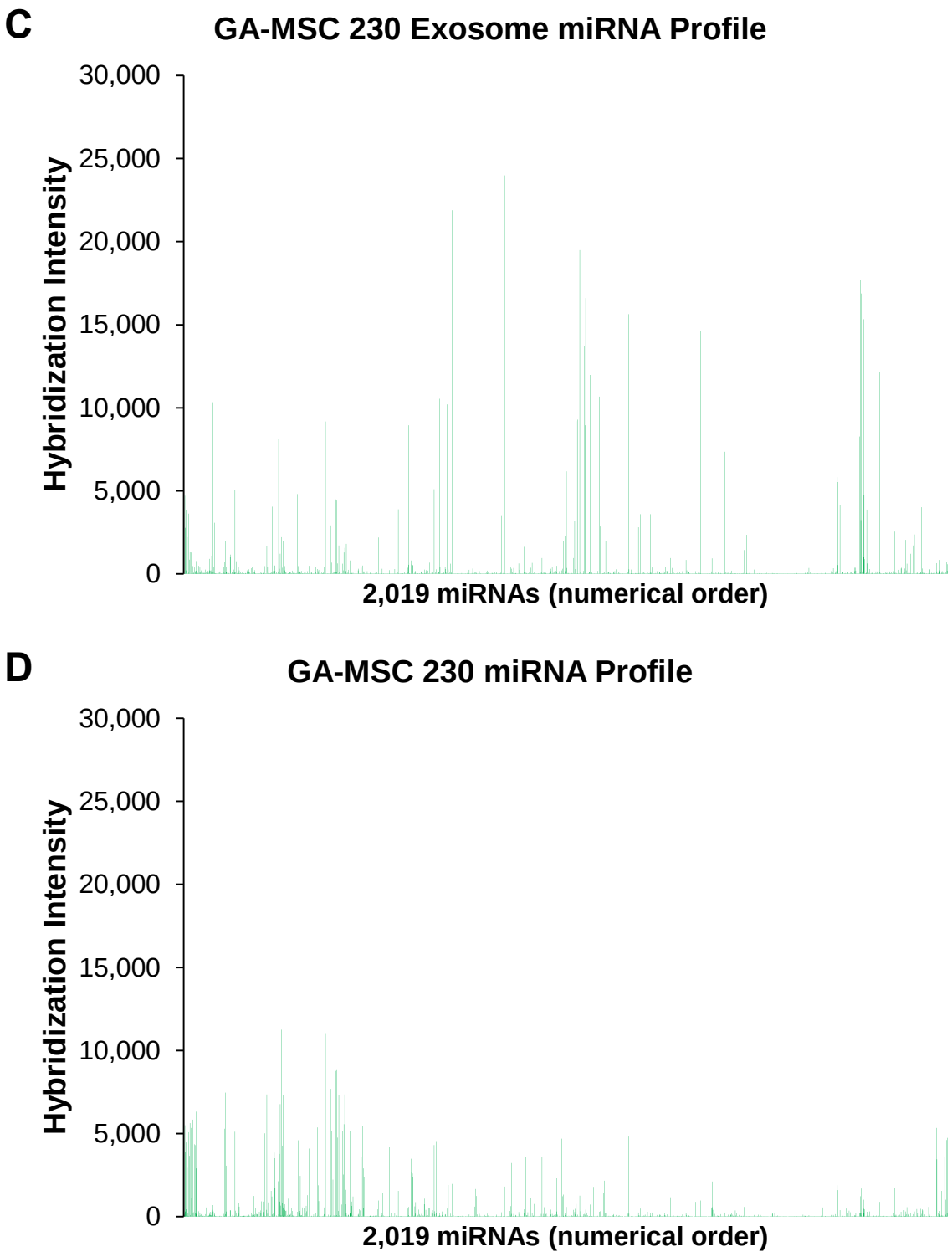


Figure 25. MSC-derived exosomes contain miRNA. (C/D) Profiling of 2,109 miRNA (miRBase 19.0) in GA-MSC 230 and GA-MSC 230-derived exosomes. GA-MSC 230-derived exosomes expressed a significantly different miRNA profile compared to parental GA-MSC 230 ($p < 0.001$, paired student's t-test).

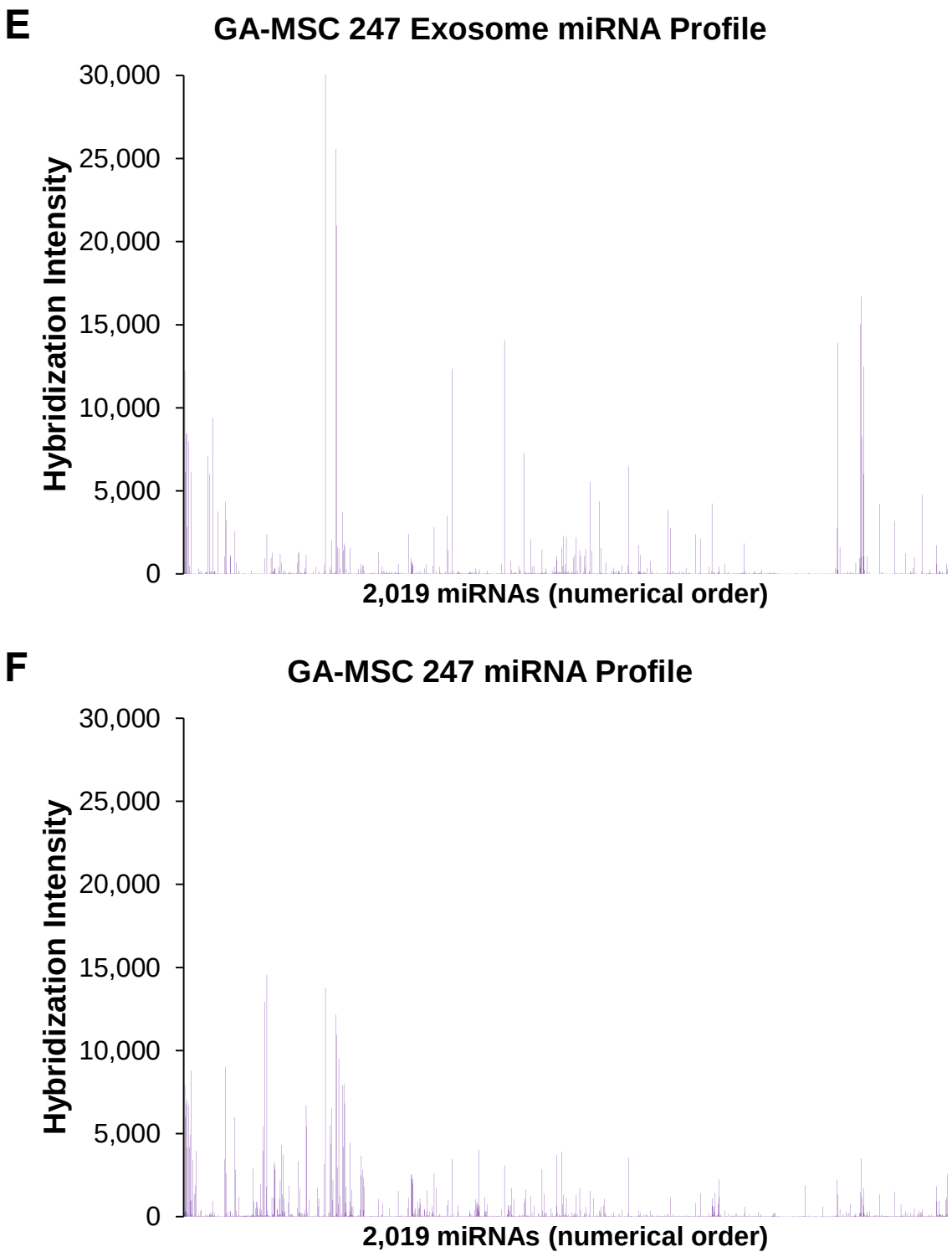


Figure 25. MSC-derived exosomes contain miRNA. (E/F) Profiling of 2,109 miRNA (miRBase 19.0) in GA-MSC 247 and GA-MSC 247-derived exosomes. GA-MSC 247-derived exosomes expressed a significantly different miRNA profile compared to parental GA-MSC 247 ($p < 0.001$, paired student's t-test).

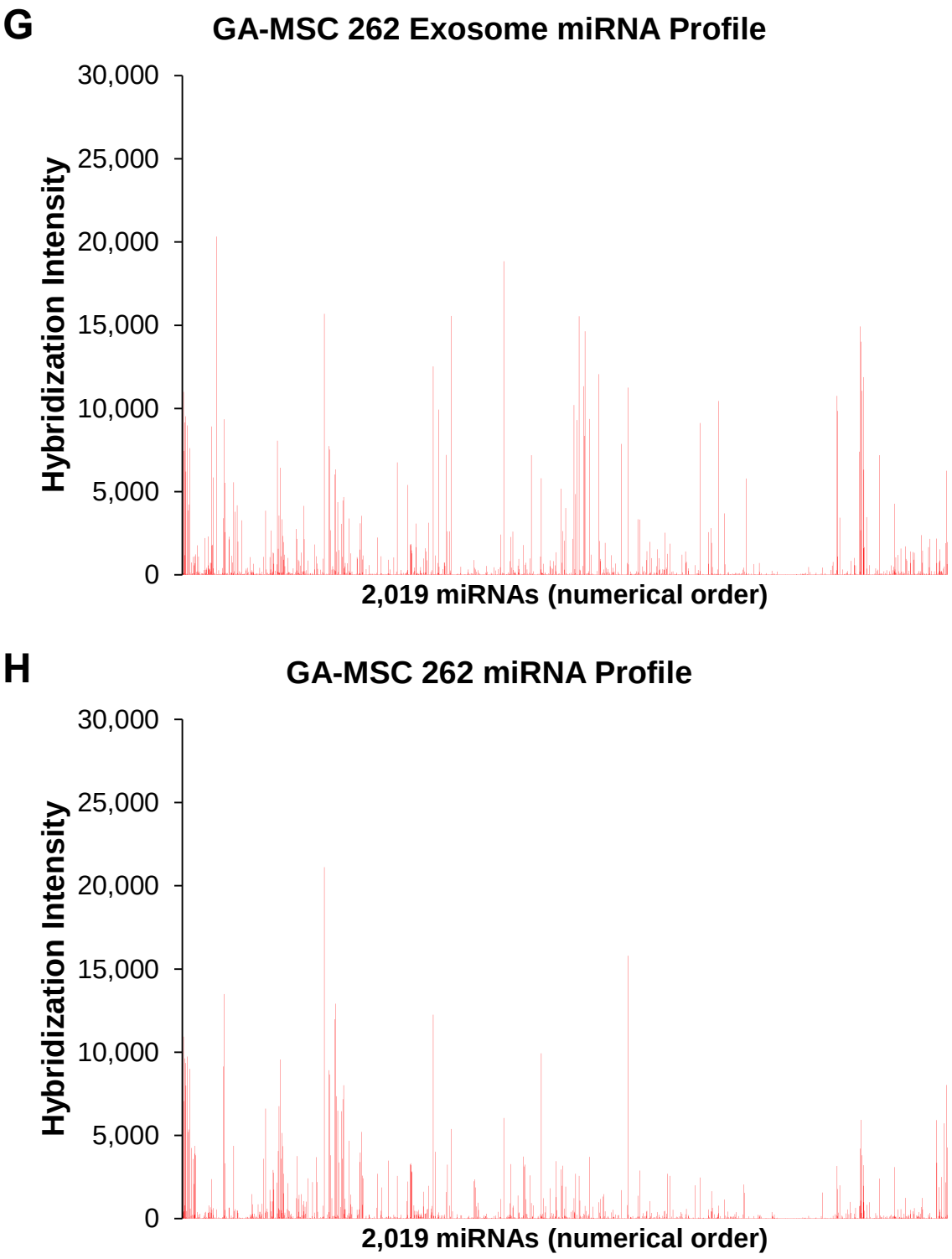


Figure 25. MSC-derived exosomes contain miRNA. (G/H) Profiling of 2,109 miRNA (miRBase 19.0) in GA-MSC 262 and GA-MSC 262-derived exosomes. GA-MSC 262-derived exosomes expressed a significantly different miRNA profile compared to parental GA-MSC 262 ($p < 0.001$, paired student's t-test).

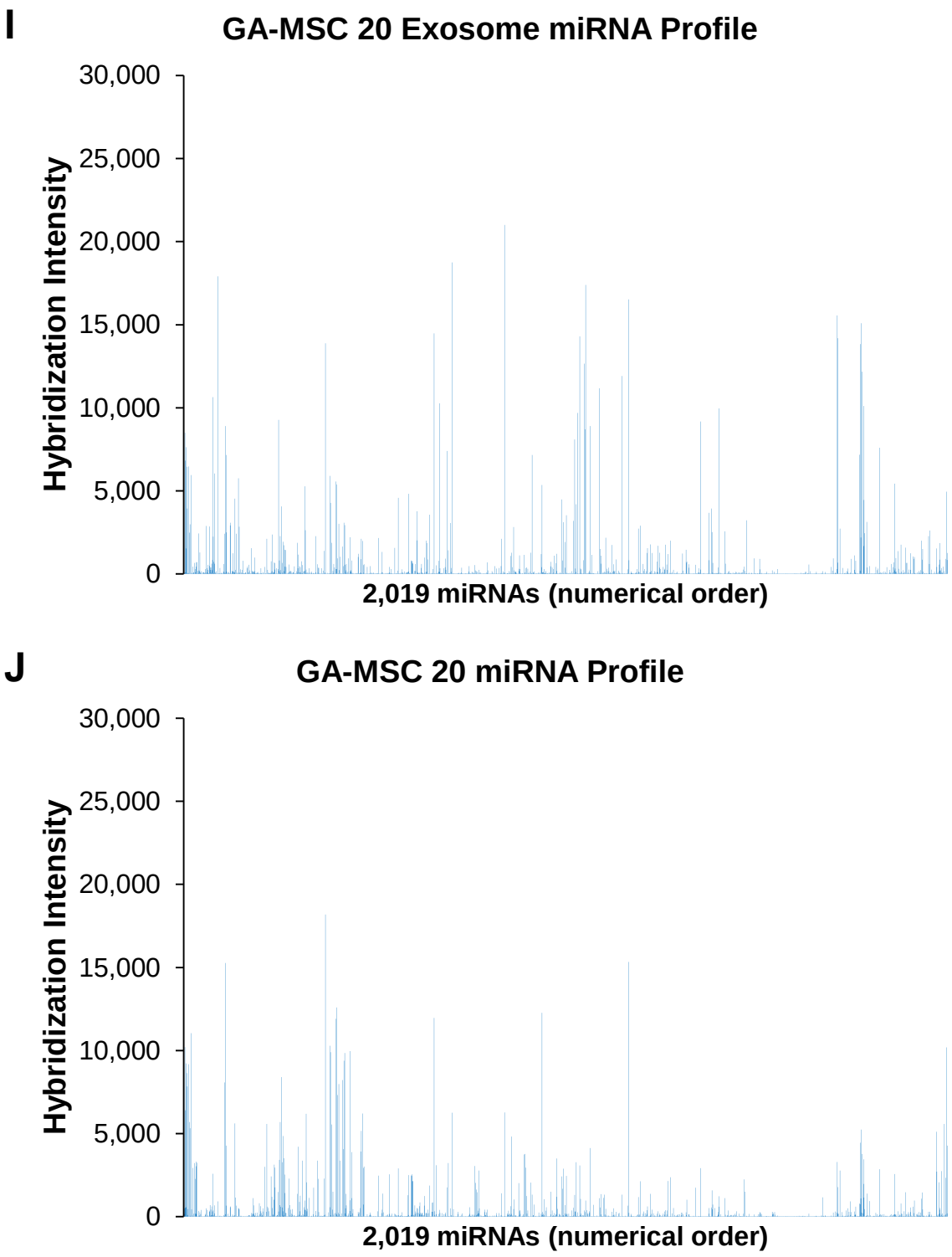


Figure 25. MSC-derived exosomes contain miRNA. (I/J) Profiling of 2,109 miRNA (miRBase 19.0) in GA-MSC 20 and GA-MSC 20-derived exosomes. GA-MSC 20-derived exosomes expressed a significantly different miRNA profile compared to parental GA-MSC 20 ($p < 0.001$, paired student's t-test).

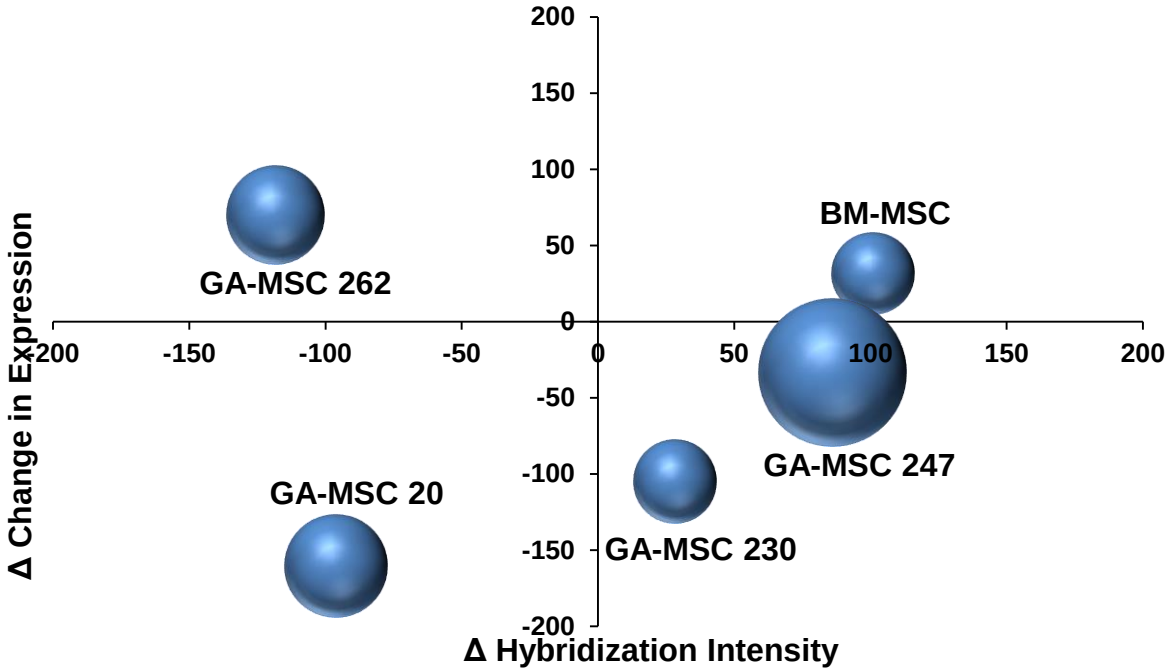


Figure 26. MicroRNA profiles of MSC-derived exosomes are different. Graphical representation of the difference in miRNA expression, between individual MSC-derived exosomes and the average miRNA expression for the group, versus the difference in change expression of miRNA (between the parental cell and exosome), between individual MSC-derived exosomes and the average fold change for the group. The change in miRNA profile standard deviation, between individual MSC-derived exosomes and the standard deviation for the group, is denoted by sphere size. MicroRNA profiles for the group of MSC-derived exosomes were significantly different from each other ($p < 0.01$, ANOVA). Individual comparison of miRNA profiles between BM-MSC-derived exosomes and GA-MSC 247-derived exosomes, as well as between GA-MSC 230-derived exosomes and GA-MSC 247-derived exosomes, were not significantly different from each other ($p > 0.05$, student's t-test).

Highly Expressed miRNA	Mean Expression Level	SD Above the Mean
hsa-miR-21-5p	26,723	17.98
hsa-miR-3960	19,651	13.16
hsa-miR-3665	16,364	10.92
hsa-miR-6089	16,163	10.79
hsa-miR-6087	15,568	10.38
hsa-miR-6090	12,200	8.08
hsa-miR-1246	11,941	7.91
hsa-miR-4497	10,837	7.16
hsa-miR-4668-5p	10,819	7.14
hsa-miR-4508	10,268	6.77
hsa-miR-6125	9,926	6.53
hsa-miR-1234-5p	9,568	6.29
hsa-miR-574-5p	9,568	6.29
hsa-let-7a-5p	9,482	6.23
hsa-miR-23a-3p	9,045	5.93
hsa-miR-4530	8,426	5.51
hsa-miR-4516	8,252	5.39
hsa-miR-23b-3p	8,244	5.39
hsa-miR-3613-3p	8,223	5.37
hsa-miR-4505	7,911	5.16
hsa-miR-574-3p	7,882	5.14
hsa-let-7c	7,793	5.08
hsa-miR-4787-5p	7,561	4.92
hsa-miR-638	7,482	4.87
hsa-let-7d-5p	7,272	4.73
hsa-miR-3656	7,110	4.62
hsa-let-7f-5p	7,008	4.55
hsa-miR-125b-5p	6,383	4.12
hsa-miR-3620-5p	6,373	4.11
hsa-let-7b-5p	6,046	3.89
hsa-miR-4492	5,726	3.67
hsa-miR-4507	5,398	3.45
hsa-miR-100-5p	5,282	3.37
hsa-miR-1587	5,269	3.36
hsa-miR-4484	5,069	3.22
hsa-miR-6085	5,046	3.21
hsa-miR-494	5,043	3.21

Table 5. MSC-derived exosomes contain highly expressed miRNA. MSC-derived exosomes contain 37 miRNA with average expression levels greater than 5000 hybridization intensity (> 3.0 standard deviations from the mean).

2.3 Discussion

In this chapter I show that GA-MSCs release exosomes, and that the production rate is dependent on both time in culture and cell-to-cell contact. I also show that the BM-MSC-derived and GA-MSC-derived exosomes do not contain significant amounts of growth factors or cytokines, but they do contain significant amounts of miRNA, including a specific group of miRNA that is highly expressed.

MSC-derived exosomes have been confirmed by the use of both surface markers, such as the tetraspanin proteins CD9 and CD81, as well as by electron microscopy.[32-33,164] A review of the most cited exosomal surface markers was published by Mathivana, et al., and shows that the tetraspanin family proteins were reliable markers for the detection of exosomes, as was the glycolytic enzyme GAPDH.[5] I show that GA-MSC-derived exosomes express both CD63 tetraspanin protein and GAPDH exosomal markers. Additionally, as in the seminal paper on exosome production by Trams, et al., I also visualized the characteristic morphology exhibited by GA-MSC-derived exosomes by electron microscopy. Most importantly, I was able to show by EM and immuno-gold staining that the CD63 surface marker was an integral part of the exosomal membrane.[15] These multiple lines of evidence prove that GA-MSCs produce classical exosomes.

The rate at which exosomes are produced has, to this point, been assumed constant, although exogenous factors such as time in culture and cell-to-cell contact could have a significant impact. The effect of environmental factors such as hypoxia, oxidative stress, and physical contact has been shown to affect the secretome profile of secreted proteins of many cells types.[165-167] Of these external factors, hypoxia has been the most extensively studied not only in secretomics, but also in the production of exosomes. For example, Ramteke, et al., demonstrated that exosomes secreted under hypoxic conditions by prostate cancer cells, enhance cell invasiveness and stemness, while Salomon, et al., showed that hypoxia-associated exosomes released from placenta-derived MSCs, mediate endothelial cell migration and blood vessel formation.[168-169] Also, work by Park, et al., revealed that hypoxia induced squamous cell carcinoma cells to produce exosomes capable of enhancing

angiogenesis and metastasis, while King, et al., demonstrated that hypoxic conditions mediated an increase in exosome secretion by breast cancer cells, and that this effect was facilitated by HIF-1α.[172-173] More relevant to my work, Kucharzewska, et al., showed that GBM-derived exosomes modified endothelial cells to improve tumor angiogenesis in a hypoxic environment.[73] In addition to hypoxic stress, other cellular stressors have also been implicated in influencing exosome secretion. This can be seen in work by Hedlund, et al., in which thermal and oxidative stress in leukemic T-cells and B-cells produces enhanced immunosuppressive exosomes.[174] These studies support the findings in which the controlled environmental factors, time in culture and cell-to-cell contact, affect the rate at which GA-MSC-derived exosomes are produced. To my knowledge this is the first evidence describing the effects of external stressors on exosome production rate, as the majority of research focuses on factors modifying exosomal content. Along this line, further investigation into the effects of other cellular stressors, along with paracrine signaling from neighboring GSCs, is necessary to gain a full understanding of GA-MSC-derived exosomes.

Exosomes have been shown to contain a variety of proteins, however, the use as a vehicle for delivering growth factors and cytokines is not well established. However, there is an association between exosomes and both growth factors and cytokines demonstrated in a variety of cell types. The content of exosomes can be altered by activation of growth factor receptors on the parental cell, as demonstrated by Genneback, et al., in which the treatment of cardiomyocytes with transforming growth factor-beta (TGF-β) and/or platelet derived growth factor-BB (PDGF-BB) resulted in cardiomyocyte-derived exosomes with significantly different mRNA content.[173] Conversely, exosomes can affect the expression of certain growth factor and receptors in recipient cells, as shown by Lee, et al., in which BM-MSC-derived exosomes down-regulate expression of vascular endothelial factor (VEGF) in breast cancer cells.[174] With respect to cytokines, Li and colleagues, show that stimulating liver non-parenchymal cells (LNPCs) with interferon-alpha (IFN-α) results in LNPC-derived exosomes with increased anti-hepatitis B virus (HBV) molecules, and Bretz, et al., demonstrate that stimulation of monocytes with exosomes derived from

malignant breast cancer ascites, induces the expression of interleukins -1-beta (IL-1β), -6 (IL-6), and tumor necrosis factor-alpha (TNF-α).[175-176] Furthermore, Huang, et al., shows that dendritic cells-derived exosomes contain epidermal growth factor (EGFR) in their membrane, and Hawari, et al, demonstrates that vascular endothelial cell-derived exosomes harbor the tumor necrosis factor receptor (TNFR) in their lipid bilayer, indicating the exosomal transfer of functional growth factor and cytokine receptors.[177-178] However, the packaging of these growth-promoting proteins inside exosomes, instead of in the exosomal membrane, has to my knowledge never been described. My results show the absence of a discrete set of growth factors and cytokines within the intra-exosomal compartment of GA-MSC-derived exosomes, which is logical given that the receptors for these proteins are extracellular and signaling proteins would not be able to produce an effect while trapped inside an exosome. To date, the only growth factor related cellular elements found to be packaged in exosomes are mRNA for transforming growth fact-beta-1 (TGF-β1) and insulin-like growth factor-1 (IGF-1) in endothelial cell-derived exosomes and BM-MSC-derived exosomes respectively, as described by Borges, et al., and Tomasoni, et al.[179-180] This results however, does not preclude the idea that growth promoting proteins could be in the exosomal membranes. In fact, some groups genetically modify exosomes to express certain growth factors and cytokines on their surface to enable binding to the extracellular domain of receptors on the recipient cell, in order to produce a desired effect. For example, Yu, et al., modified dendritic cell-derived exosomes to express membrane bound transforming growth fact-beta-1 (TGF-β1), while Zhang, et al., modified renal cancer cell-derived exosomes to express membrane bound interleukin-12 (IL-12).[181-182] The only known exosomal membrane proteins shown to produce growth promoting effects, are glycoproteins. Specifically, functional Wnt signaling proteins have been described in exosomes from both fibroblasts and BM-MSCs.[152,183] These membrane bound proteins were not evaluated in my analysis of the classical and most well described growth factors and cytokines, and further investigation into their presence in GA-MSC-derived exosomes is warranted.

Genetic material, such as DNA and RNA, have been extensively described in the exosomes of many cell types. Therefore, it is reasonable that GA-MSC-derived exosomes contain unique miRNA profiles. In addition to growth factor mRNA being present in exosomes from endothelial and mesenchymal stem cells, other DNA and RNA species are now accepted as classical exosomal constituents. Waldenstrom, et al., found that cardiomyocyte-derived exosomes contain both DNA and RNA that were functional in recipient fibroblasts.[184] However, DNA and RNA are general terms and include a variety of specific subtypes. In the realm of DNA species, Kahlert, et al., described the presence of double-stranded DNA, including mutations in KRAS and p53, in the exosomes derived from the serum of pancreatic cancer patients.[185] Additionally, the rare mitochondrial DNA (mtDNA) was discovered by Guescini, et al., in exosomes derived from myoblasts, astrocytes and GBM cells.[70,186] With regards to RNA, the presence of non-coding RNA species, such as miRNAs, are more extensively described in exosomes than mRNA. MicroRNA has the potential to produce longer lasting effects in recipient cells due its slow decay rate and the ability to regulate multiple genes at the transcription level. In contrast mRNA influence is relatively brief due to its faster decay rate as a result of translation into a protein. Consistent with other cell types, I showed that the GA-MSC-derived exosomes contained unique miRNA profiles as compared to the parental cell, including a specific group of miRNA that were highly expressed. These results are further supported by Wang, et al., Chen, et al., Koh, et al., and Xin, et al., who all described a wide variety of both precursor and mature functional miRNA in exosomes derived from BM-MSCs.[153,187-189] However, given the nature of mRNA and their potential as effectors on growth, the mRNA profiling of GA-MSC-derived exosomes is also necessary in future work.

One of the weaknesses in these experiments was not obtaining a full proteomic profile of GA-MSC-derived exosomes. Instead, I chose to focus on profiling 41 major growth factors and receptors, and 60 major cytokines and receptors. However, the list is not inclusive of all tumor-promoting proteins, and thus a full proteomic analysis would have improved the analysis. Likewise, I did not analyze the lipid component of GA-MSC-derived exosomes, which would be a more

complete characterization. Furthermore, I only characterized the miRNA within GA-MSC-derived exosomes, and did not evaluate other DNA or RNA species. Indeed, mRNA could have a significant impact on recipient cells, when delivered via exosomes. However, I chose to focus on miRNA due to their broad range of targets and potential to alter gene expression at the level of transcription.

Conversely, one of the strengths of these experiments was the use of multiple modalities to detect exosomes. Not only did I demonstrate GA-MSC-derived exosome production by western blot, but I also confirmed production by electron microscopy, immuno-gold labeling, and ELISA. Another strength of these experiments was the characterization of GA-MSC-derived production under cellular stress. This evaluation more accurately mimics the tumor microenvironment. The last strength of the experiments was the full characterization of all known miRNAs. Although many of the miRNAs have yet to be studied, this profiling provided a better understanding of exosome content that is capable of altering gene expression.

2.4 Conclusions

The results outlined in this chapter demonstrate for the first time the production of exosomes by GA-MSCs. These GA-MSC-derived exosomes express the accepted CD63 and GAPDH markers, and lack CD16 and CD32 non-exosomal markers. Importantly, GA-MSC-derived exosomes are also within the established 40-100nm range, and possess the characteristic lipid bilayer membrane. Moreover, the production of GA-MSC-derived exosomes varies with exposure to different levels of cellular stressors. Additionally, GA-MSC-derived exosomes do not contain 41 of the major growth factors, nor do they contain 60 of the major cytokines. However, GA-MSC-derived exosomes contain numerous miRNAs, with distinct profiles compared to the parental cell. Lastly, a specific group of miRNAs are highly expressed in all GA-MSC-derived exosomes. These results prove that GA-MSCs are capable of producing a varying amount of exosomes, depending on the environmental conditions, whose internal content have the potential to impact recipient cell biology.

Chapter III

GA-MSC-Derived Exosomes Increase GSC Tumorigenicity

GA-MSC-Derived Exosomes Increase GSC Tumorigenicity

Proof that exosomes play a role in the communication between GA-MSCs and GSCs requires demonstrating that GA-MSC-derived exosomes are taken up by GSCs, and that these exosomes can modify the biology of GSCs. To my knowledge this analysis has never been conducted in gliomas. Investigating the exosomal interaction between cells of the tumor microenvironment may establish a new intratumoral paracrine communication mechanism. Therefore, I hypothesized that GA-MSC-derived exosomes can be internalized by GSCs and can increase the tumorigenicity of GSCs. To test this hypothesis, I first established that GSCs are capable of taking up GA-MSC-derived exosomes. These results proved that intercellular exosomal interaction is feasible. I then showed that GSC proliferation and clonogenicity is increased by GA-MSC-derived exosomes *in vitro*, and that GA-MSC-derived exosomes increased the tumorigenicity of GSCs *in vivo*. These findings established that GA-MSCs in the glioma microenvironment can alter the growth and self-renewal of GSCs. Therefore, my studies indicate that GA-MSCs communicate with GSCs via exosomes.

3.1 Experimental Methods

GSC Internalization of GA-MSC-Derived Exosomes

In order to fluorescently label and track GA-MSC-derived exosomes, GA-MSCs were transduced with a commercially available GFP-CD63 lentiviral construct (System Biosciences), and expanded to 10^6 cells in MSC growth medium. Transduced GA-MSCs were then washed and incubated for 48 hours in serum-free NSC medium. GFP-labeled GA-MSC-derived exosomes were then isolated by differential ultracentrifugation (see *Chapter 2 Experimental Methods*), added to GSC cultures and allowed to incubate for 4 hours. Excess exosomes were subsequently removed by PBS wash, and effective internalization of GA-MSC-derived exosomes by GSCs was analyzed by fluorescent confocal microscopy.

Effects of GA-MSC-Derived Exosomes on GSC Proliferation

In order to isolate MSC-derived exosomes, BM-MSCs (Lonza, Inc.) and four GA-MSC lines (**Table 1**) were expanded to 5.0 x 10^7 cells in MSC growth medium. BM-MSCs and GA-MSCs were subsequently washed and incubated for 48 hours in serum-free NSC medium. Likewise, to isolate GSC-derived self-exosomes, four GSC lines (**Table 2**) were expanded to 1.0 x 10^7 cells in NSC growth medium. Both MSC-derived and GSC-derived exosomes were then isolated from the conditioned medium by differential ultracentrifugation and quantified by CD63 ELISA (see *Chapter 2 Experimental Methods*), The remaining supernatant after exosome extraction, *exosome-free conditioned medium* (EF-CM), was collected and utilized as a control. Additionally, neurospheres from four GSC lines (**Table 2**) were dissociated and placed in a 96 well plate at 2.5 x 10^3 cells/well. GSC-derived and MSC-derived exosomes were then added to GSC cultures at doses of 5.0 x 10^4 and 1.0 x 10^5 exosomes/µL at time zero and at 48 hours, and incubated for a total period of 96 hours. GSCs were also treated with NSC medium and GA-MSC-derived EF-CM. After 96 hours in culture, GSCs were assessed for viability using a colorimetric assay (water-soluble tetrazolium [WST-1], Roche), and the absorbance measured at 450nm. Absorbance of GSCs treated with MSC-derived exosomes was compared to

that of untreated GSC controls. All experiments were performed in quadruplicate and statistical analysis was performed utilizing the paired student's t-test.

Effects of GA-MSC-Derived Exosomes on GSC Clonogenicity

In order to isolate MSC-derived exosomes, BM-MSCs (Lonza, Inc.) and four GA-MSC lines (**Table 1**) were expanded to 5.0 x 10^7 cells in MSC growth medium. BM-MSCs and GA-MSCs were subsequently washed and incubated for 48 hours in serum-free NSC medium. Likewise, to isolate GSC-derived self-exosomes, four GSC lines (**Table 2**) were expanded to 1.0 x 10^7 cells in NSC growth medium. Both MSC-derived and GSC-derived exosomes were then isolated from the conditioned mediums by differential ultracentrifugation and quantified by CD63 ELISA (see *Chapter 2 Experimental Methods*), The remaining supernatant after exosome extraction, exosome-free conditioned medium (EF-CM), was collected and utilized as a control. GSC-derived and MSC-derived exosomes were then added to single-cell suspensions of four GSC lines (**Table 2**) in 96-well plates at a dose of 4.0 x 10^2 exosomes/µL at time zero, and at 1 week and 2 week time points. GSCs were also treated with NSC medium and GA-MSC-derived EF-CM. After 3 weeks of culture, quantification of GSC neurospheres was performed. Neurosphere formation of GSCs treated with GA-MSC-derived exosomes was compared to that of untreated GSC controls. All experiments were performed in quadruplicate and statistical analysis was performed utilizing the paired student's t-test.

Effects of GA-MSC-Derived Exosomes on GSC Tumorigenicity

In order to isolate MSC-derived exosomes, GA-MSC 262 and GA-MSC 20 were expanded to 5.0 x 10^7 cells in MSC growth medium. GA-MSCs were subsequently washed and incubated for 48 hours in serum-free NSC medium. Likewise, to isolate GSC-derived self-exosomes, GSC 262 and GSC 20 (matching pairs) were expanded to 1.0 x 10^7 cells in NSC growth medium. Both MSC-derived and GSC-derived exosomes were then isolated from the conditioned mediums by differential ultracentrifugation and quantified by CD63 ELISA (see *Chapter 2 Experimental Methods*), The remaining supernatant after exosome extraction,

exosome-free conditioned medium (EF-CM), was collected and utilized as a control. Additionally, GSC 262 and GSC 20 were expanded to 3.0 x 10^7 cells in preparation for pre-treatment and implantation. GSC-derived and GA-MSC-derived exosomes were then added to GSC cultures at a dose of 1.0 x 10^9 exosomes/mL. GSCs were also pre-treated with NSC medium and GA-MSC-derived EF-CM. After 96 hours of pre-treatment, excess exosomes were eliminated by PBS wash, and GSC neurospheres were dissociated. GSCs were then injected into the right frontal lobe of nude mice (n=15/group, 5.0 x 10^5 cells/mouse), utilizing a cranial bolt-guided, stereotactic system. A cohort of mice (n=9) were followed until moribund and then sacrificed. Another cohort of mice (n=6) were sacrificed at 20 and 40 days post implantation. After sacrifice, mouse brains were removed, embedded in paraffin, and sectioned tumors. Tumor volumes and survival results of the GSCs treated with GA-MSC-derived exosomes were compared to untreated GSC controls. Tumor volumes were calculated by adding multiple cross-sectional areas through the tumors after H&E staining of histologic sections. Statistical analysis was performed utilizing the paired student's t-test for tumor volume analysis, and log-rank (Mantel-Cox) test for survival analysis.

3.2 Results

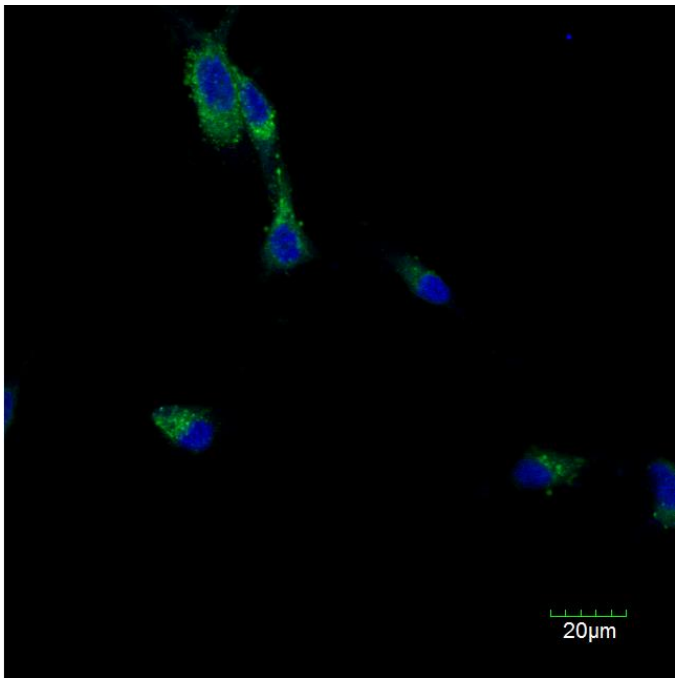
GSCs Internalize GA-MSC-derived exosomes

To confirm the localization of MSC-derived exosomes to the cytoplasm of GSCs, we labeled exosomes using a CD63-green fluorescent fusion protein. The robustness of the CD63 marker in GA-MSC-derived exosomes allowed the use of this CD63-GFP fusion protein for tracking. Additionally, I exploited the optical sectioning capabilities of fluorescent confocal microscopy for tracking of GA-MSC-derived exosomes, instead of fluorescent light microscopy. In these experiments, CD63-GFP expressing GA-MSCs were cultured, and GFP labeled exosomes were isolated and co-cultured with GSCs (see *experimental methods*). After 4 hours of co-culturing, fluorescent confocal microscopy revealed the presence of GFP-labeled GA-MSC-derived exosomes exclusively in the cytoplasm of GSCs, and not in the nucleus (**Figure 27**). Furthermore, GFP-labeled exosomes appeared to be grouped together within the cytoplasm of GSCs. Taken together, these results indicate that GA-MSC-derived exosomes can be internalized by GSCs.

GA-MSC-Derived Exosomes Increase GSC Proliferation

To determine the growth effects of MSC-derived exosomes on GSCs, I conducted *in vitro* co-culture experiments in which I assayed GSC proliferation after exposure to GA-MSC-derived exosomes. Specifically, GSCs and MSC were cultured, and exosomes isolated and co-cultured with GSCs (see *experimental methods*). Proliferation assays demonstrate that GA-MSC-derived exosomes significantly increased ($p < 0.01$, paired student's t-test) the proliferation of GSCs in a dose dependent manner (**Figure 28**). Importantly, the exosome-mediated promotion of growth in GSCs was significant with the treatment of exosomes derived from the matching GA-MSCs isolated from the same tumor specimen. Likewise, BM-MSC-derived exosomes also exerted growth-promoting effects on each GSC. However, in general the proliferation effects of the GA-MSC-derived exosomes were greater than that of BM-MSC-derived exosomes. As expected, and consistent with earlier results, treatment of GSCs with GA-MSC-derived EF-CM, also significantly

A



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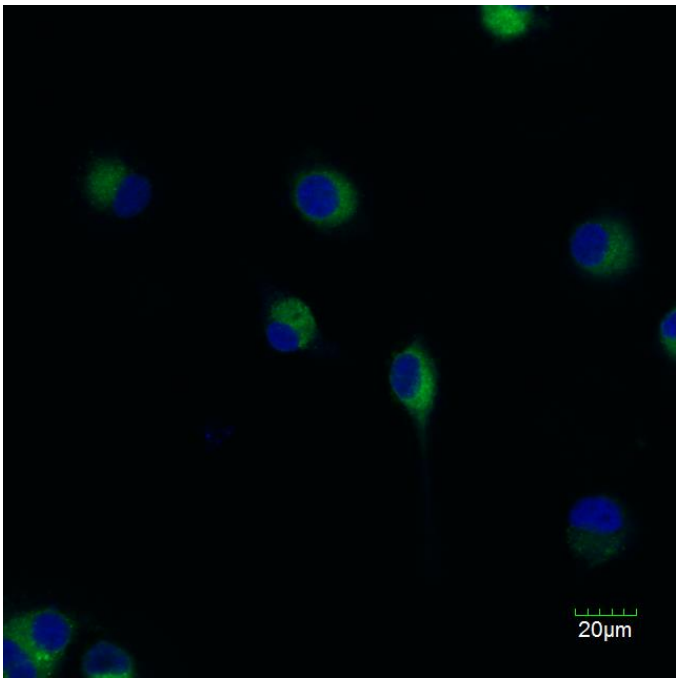

Figure 27. GSCs internalize fluorescently labeled MSC-derived exosomes. (A/B) Fluorescent confocal microscopy demonstrating the internalization of GFP-CD63 labeled BM-MSC-derived exosomes by GSC 262 and GSC 20, respectively. Fluorescently labeled exosomes (green) are localized to the cytoplasm of GSCs and are excluded from the nucleus (DAPI stained blue).

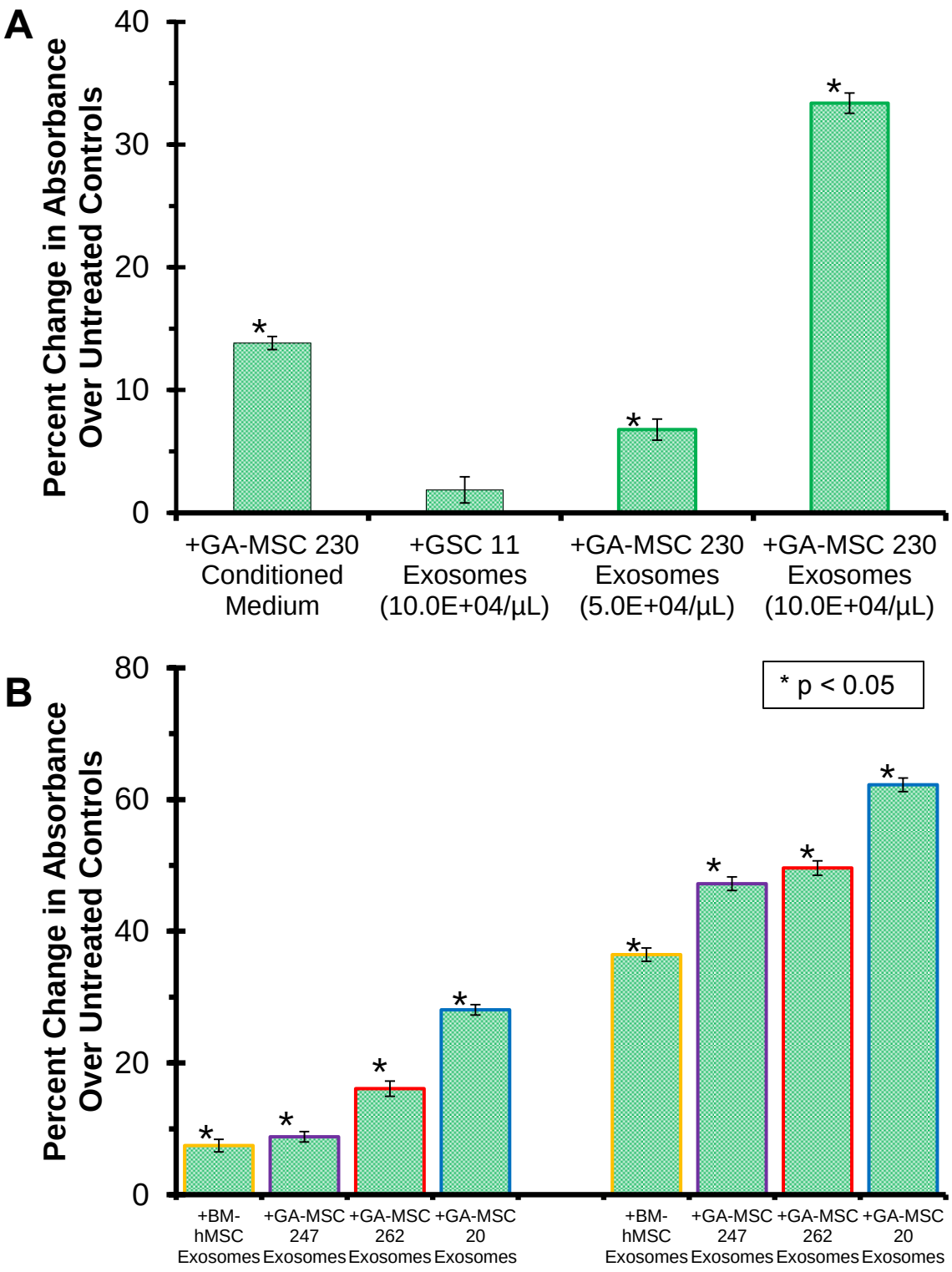

Figure 28. GA-MSC-derived exosomes increase GSC proliferation. (A) Proliferation assay for GSC 11 demonstrating a significant increase ($p < 0.01$, student's t-test) in viability with the treatment of GA-MSC 230-derived exosomes at two doses. As expected, treatment of GSC 11 with GA-MSC 230-derived exosome-free conditioned medium significantly increases viability, while treatment with GSC 11-derived self-exosomes does not have a significant effect. (B) Proliferation assay for GSC 11 demonstrating dose dependent significant increases ($p < 0.01$, student's t-test) in viability with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

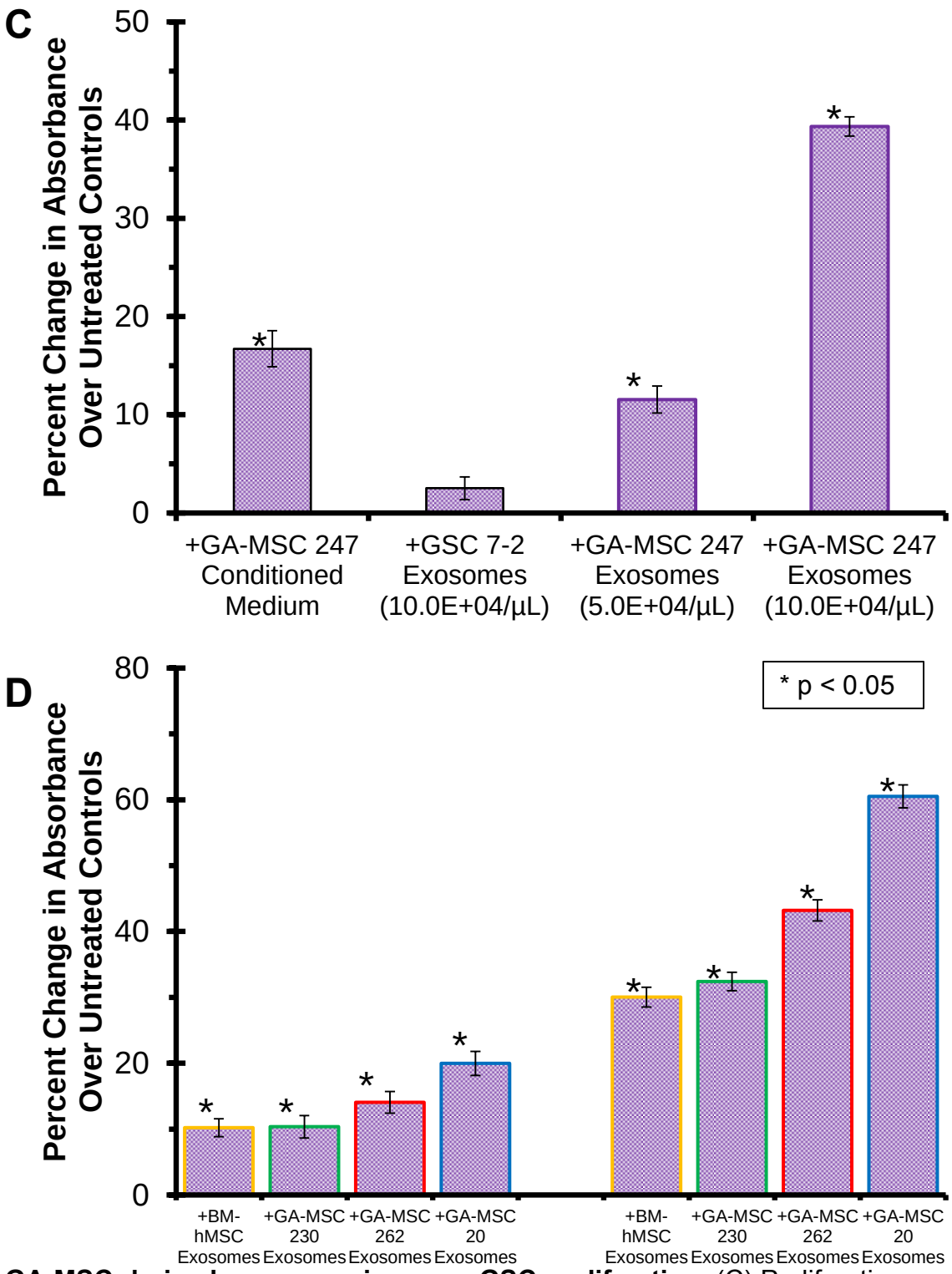

Figure 28. GA-MSC-derived exosomes increase GSC proliferation. (C) Proliferation assay for GSC 7-2 demonstrating a significant increase ($p < 0.01$, student's t-test) in viability with the treatment of GA-MSC 247-derived exosomes at two doses. As expected, treatment of GSC 7-2 with GA-MSC 247-derived exosome-free conditioned medium significantly increases viability, while treatment with GSC 7-2-derived self-exosomes does not have a significant effect. (D) Proliferation assay for GSC 7-2 demonstrating dose dependent significant increases ($p < 0.01$, student's t-test) in viability with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

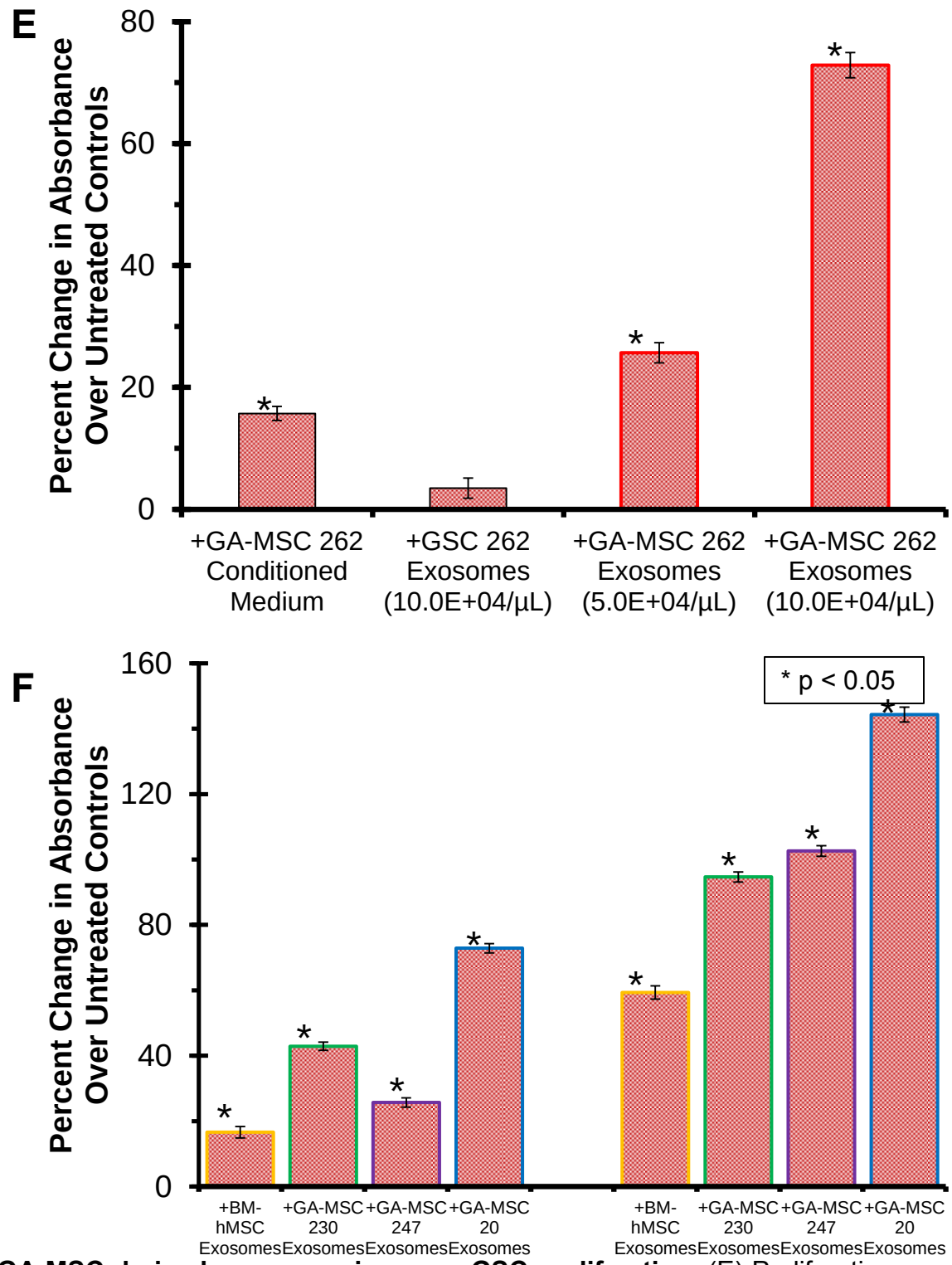

Figure 28. GA-MSC-derived exosomes increase GSC proliferation. (E) Proliferation assay for GSC 262 demonstrating a significant increase ($p < 0.01$, student's t-test) in viability with the treatment of GA-MSC 262-derived exosomes at two doses. As expected, treatment of GSC 262 with GA-MSC 262-derived exosome-free conditioned medium significantly increases viability, while treatment with GSC 262-derived self-exosomes does not have a significant effect. (F) Proliferation assay for GSC 262 demonstrating dose dependent significant increases ($p < 0.01$, student's t-test) in viability with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

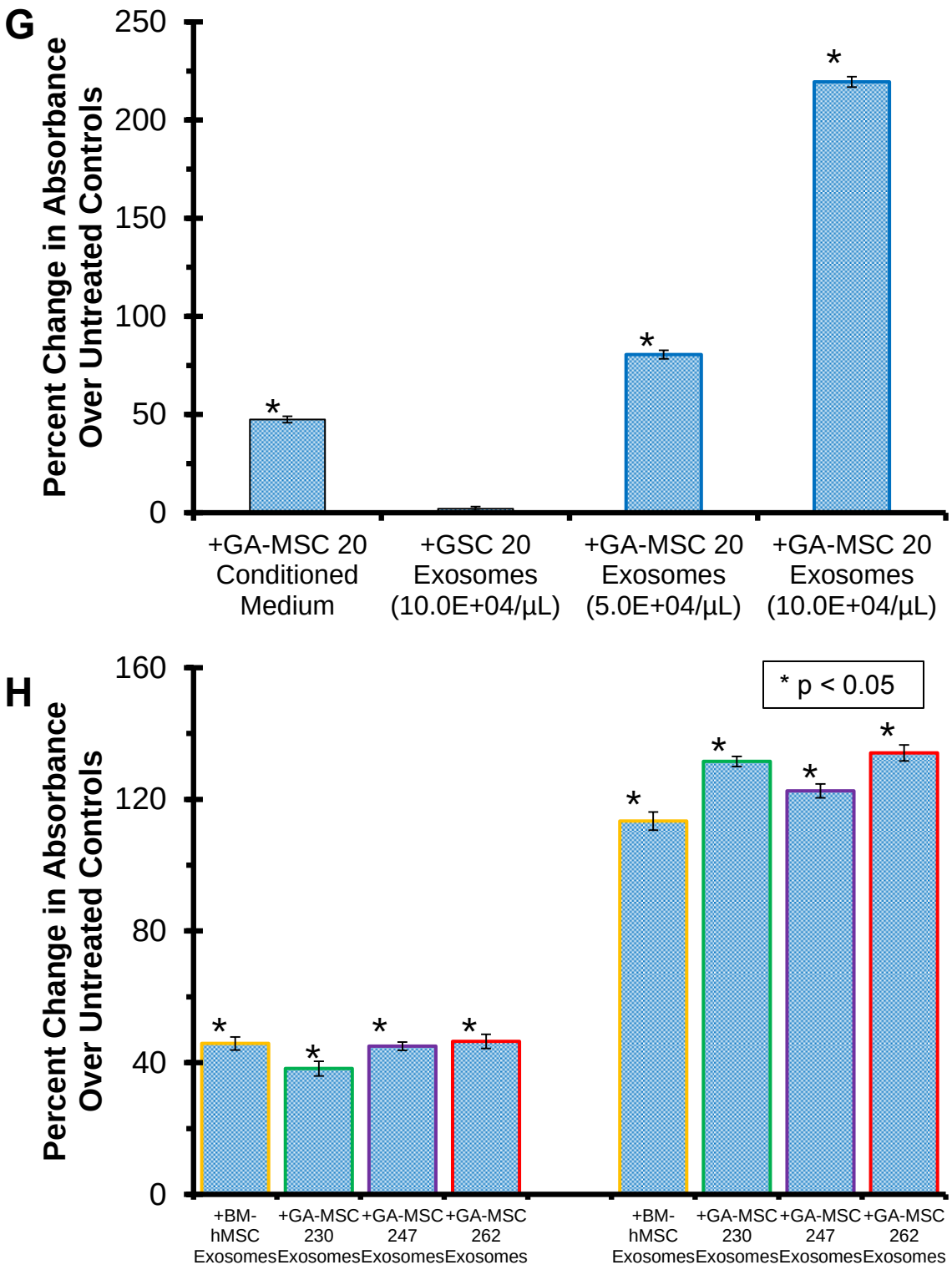

Figure 28. GA-MSC-derived exosomes increase GSC proliferation. (G) Proliferation assay for GSC 20 demonstrating a significant increase ($p < 0.01$, student's t-test) in viability with the treatment of GA-MSC 20-derived exosomes at two doses. As expected, treatment of GSC 20 with GA-MSC 20-derived exosome-free conditioned medium significantly increases viability, while treatment with GSC 20-derived self-exosomes does not have a significant effect. (H) Proliferation assay for GSC 20 demonstrating dose dependent significant increases ($p < 0.01$, student's t-test) in viability with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

increased GSC proliferation compared with untreated controls ($p < 0.01$, paired student's t-test). In contrast, treatment of GSCs with GSC-derived self-exosomes, did not have a significant effect on GSC proliferation ($p > 0.05$, paired student's t-test). This finding confirms previous reports that exosomes are recycled and do not exert any known autocrine effects on the cell from which they are derived, as GSC-derived self-exosomes had no effect on GSCs.[190] These results indicate that exosome-mediated growth effects are dependent on the cell-of-origin, and that BM-MSC-derived and GA-MSC-derived exosomes increase the proliferation of GSCs.

GA-MSC-Derived Exosomes Increase GSC Clonogenicity

I then evaluated the effects of MSC-derived exosomes on the ability of GSCs to self-replicate, by conducting *in vitro* co-culture experiments in which I assayed GSC clonogenicity after exposure to GA-MSC-derived exosomes. Specifically, GSCs and MSC were cultured, and exosomes isolated and co-cultured with GSCs (see *experimental methods*). Clonogenicity assays demonstrate that GA-MSC-derived exosomes significantly increased GSC neurosphere formation and thus clonogenicity ($p < 0.05$, paired student's t-test) (**Figure 29**). Importantly, the exosome-mediated promotion of self-renewal in GSCs was significant with the treatment of exosomes derived from the matching GA-MSCs isolated from the same tumor specimen Likewise, BM-MSC-derived exosomes also increased GSC clongenicity. As expected, treatment of GSCs with GA-MSC-derived EF-CM also increased GSC clonogenicity ($p < 0.05$, paired student's t-test). In contrast, and in agreement with the proliferation results, treatment of GSCs with GSC-derived self-exosomes did not have a significant effect on GSC clonogenicity ($p > 0.05$, paired student's t-test). These results indicate that BM-MSC-derived and GA-MSC-derived exosomes enhance the clonogenicity of GSCs.

GA-MSC-Derived Exosomes Enhance GSC Tumorigenicity

I next sought to determine whether the *in* vitro results also occurred *in* vivo. To assess the effects of GA-MSC-derived exosomes on GSCs *in vivo*, I utilized GSC xenografts in nude mice. Specifically, GSCs and MSC were cultured, and exosomes

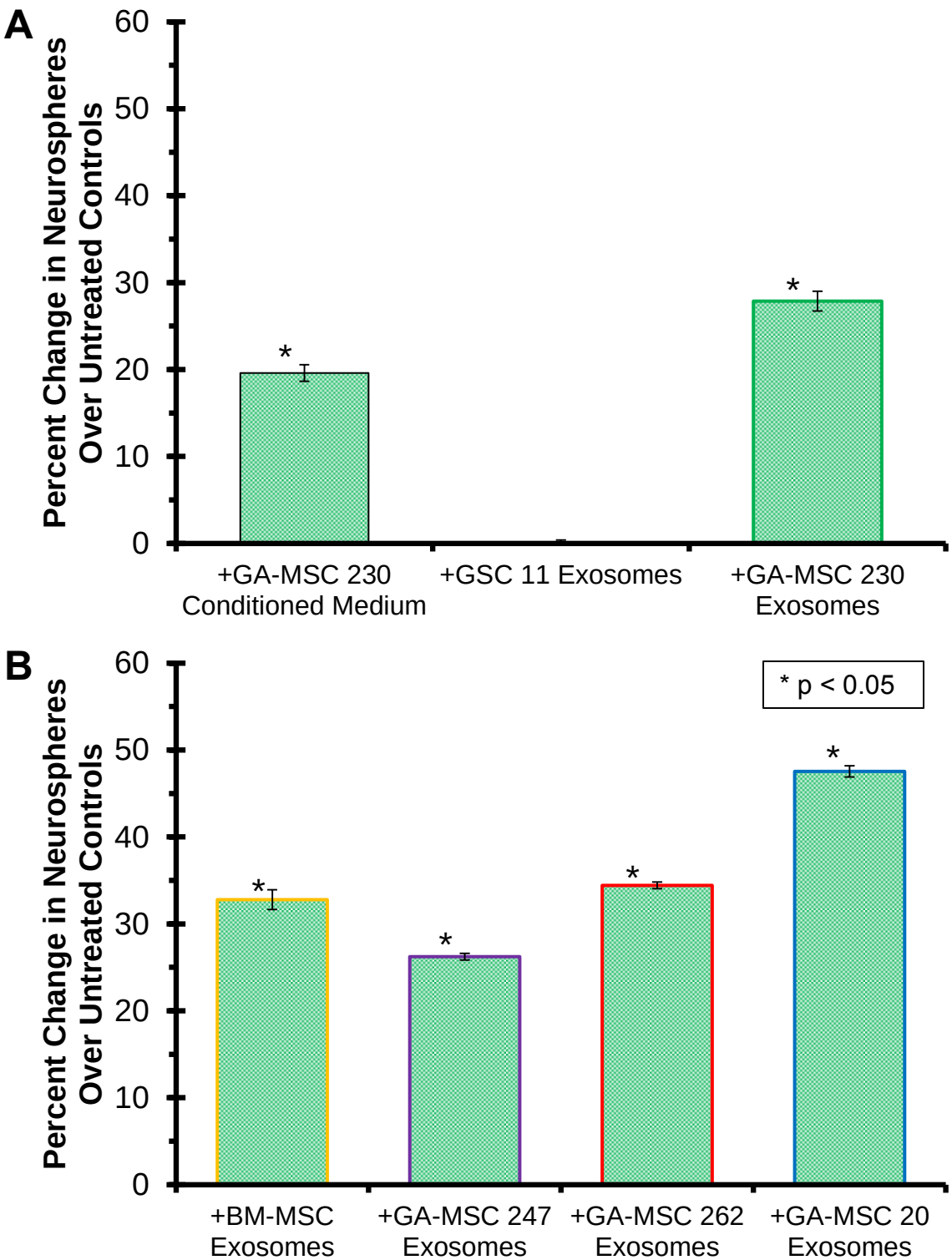

Figure 29 GA-MSC-derived exosomes increase GSC clonogenicity. (A) Clonogenic assay for GSC 11 demonstrating a significant increase ($p < 0.01$, student's t-test) in self-renewal with the treatment of GA-MSC 230-derived exosomes. As expected, treatment of GSC 11 with GA-MSC 230-derived exosome-free conditioned medium significantly increases self-renewal, while treatment with GSC 11-derived self-exosomes does not have a significant effect. (B) Clonogenic assay for GSC 11 demonstrating significant increases ($p < 0.01$, student's t-test) in self-renewal with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

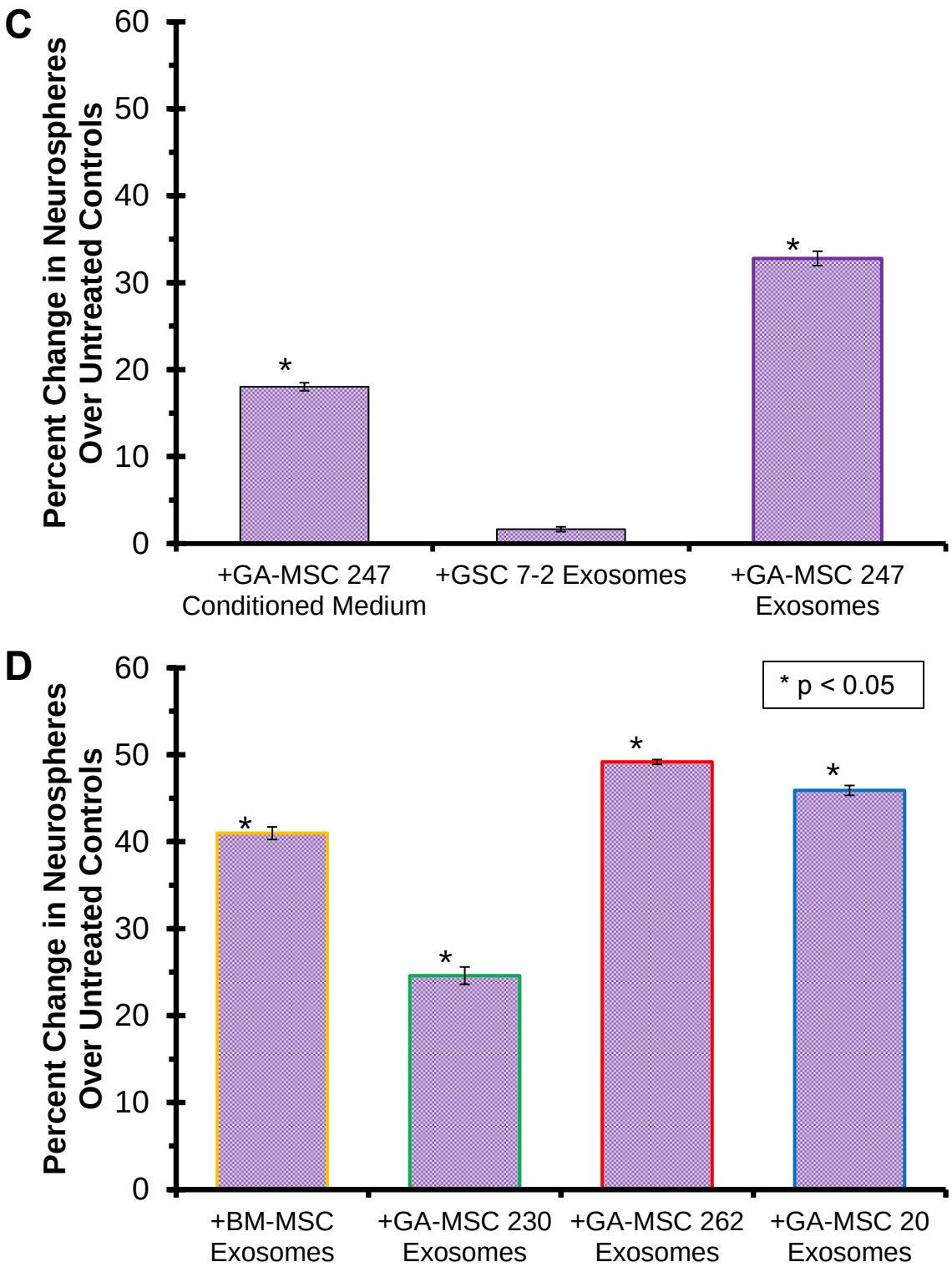

Figure 29 GA-MSC-derived exosomes increase GSC clonogenicity. (C) Clonogenic assay for GSC 7-2 demonstrating a significant increase ($p < 0.01$, student's t-test) in self-renewal with the treatment of GA-MSC 247-derived exosomes. As expected, treatment of GSC 7-2 with GA-MSC 247-derived exosome-free conditioned medium significantly increases self-renewal, while treatment with GSC 7-2-derived self-exosomes does not have a significant effect. (D) Clonogenic assay for GSC 7-2 demonstrating significant increases ($p < 0.01$, student's t-test) in self-renewal with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

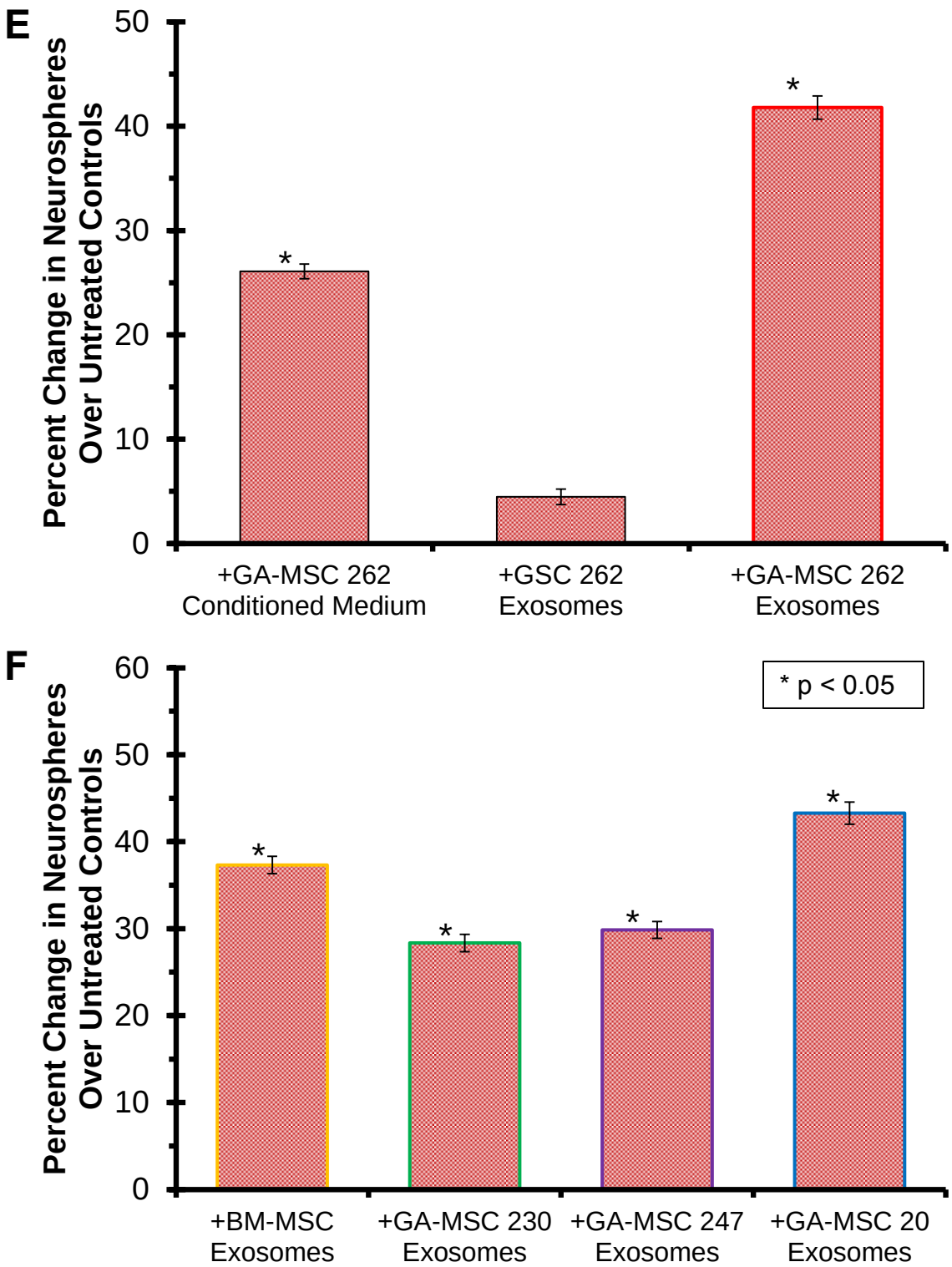

Figure 29 GA-MSC-derived exosomes increase GSC clonogenicity. (E) Clonogenic assay for GSC 262 demonstrating a significant increase ($p < 0.01$, student's t-test) in self-renewal with the treatment of GA-MSC 262-derived exosomes. As expected, treatment of GSC 262 with GA-MSC 262-derived exosome-free conditioned medium significantly increases self-renewal, while treatment with GSC 262-derived self-exosomes does not have a significant effect. (F) Clonogenic assay for GSC 262 demonstrating significant increases ($p < 0.01$, student's t-test) in self-renewal with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

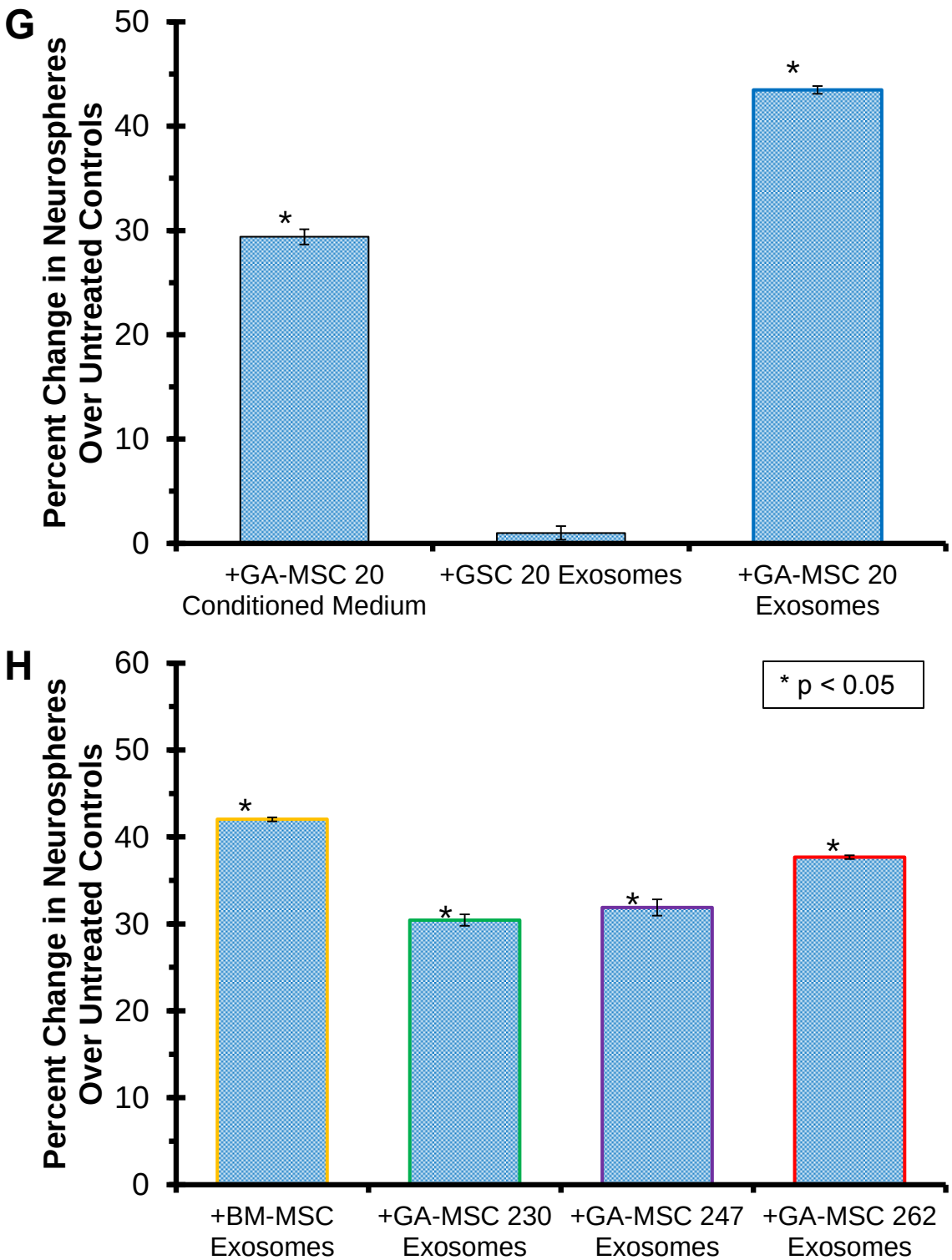

Figure 29 GA-MSC-derived exosomes increase GSC clonogenicity. (G) Clonogenic assay for GSC 20 demonstrating a significant increase ($p < 0.01$, student's t-test) in self-renewal with the treatment of GA-MSC 20-derived exosomes. As expected, treatment of GSC 20 with GA-MSC 20-derived exosome-free conditioned medium significantly increases self-renewal, while treatment with GSC 20-derived self-exosomes does not have a significant effect. (H) Clonogenic assay for GSC 20 demonstrating significant increases ($p < 0.01$, student's t-test) in self-renewal with treatment of exosomes derived from BM-MSC and three GA-MSC lines.

isolated and co-cultured with GSCs (see *experimental methods*). *In vivo* experiments demonstrated that pre-treatment of GSC 262 with GA-MSC 262-derived exosomes significantly decreased median survival from 56 to 45 days ($p < 0.05$, log-rank test) (**Figure30**). Furthermore, pre-treatment of GSC 20 with GA-MSC 20-derived exosomes significantly decreased median survival from 49 to 37 days ($p < 0.05$, log-rank test) (**Figure 30**). Additionally, in agreement with *in vitro* results, pre-treatment of GSC xenografts with GA-MSC-derived EF-CM also significantly decreased median survival ($p < 0.05$, log-rank test), while pre-treatment of GSCs with GSC-derived self-exosomes did not have a significant effect on median survival ($p > 0.05$, log-rank test).

Histologic analysis of brain specimens from mice implanted with GSC 262 pre-treated with GA-MSC 262-derived exosomes, demonstrated a significant increase in tumor volume when compared to untreated GSC controls at 40 days post-implantation, and not at 20 days post-implantation ($p < 0.01$, paired student's t-test) (**Figure 31 and Figure 32**). Similarly, histologic analysis of brain specimens from mice implanted with GSC 20 pre-treated with GA-MSC 20-derived exosomes, demonstrated a significant increase in tumor volume when compared to untreated GSC controls at both 20 and 40 days post-implantation ($p < 0.01$, paired student's t-test) (**Figure 31 and Figure 32**). As expected, pre-treatment of GSCs with GA-MSC-derived EF-CM also significantly increased tumor volume at 20 and 40 days post-implantation ($p < 0.05$, paired student's t-test). In contrast, and in agreement with *in vitro* results, treatment of GSCs with GSC-derived self-exosomes did not have a significant effect on tumor volume at 20 and 40 days post-implantation ($p > 0.05$, paired student's t-test). These histologic results corroborate with survival data and findings from *in vitro* experiments. Together, these results indicate that GA-MSC-derived exosomes significantly increase the tumorigenicity of GSCs.

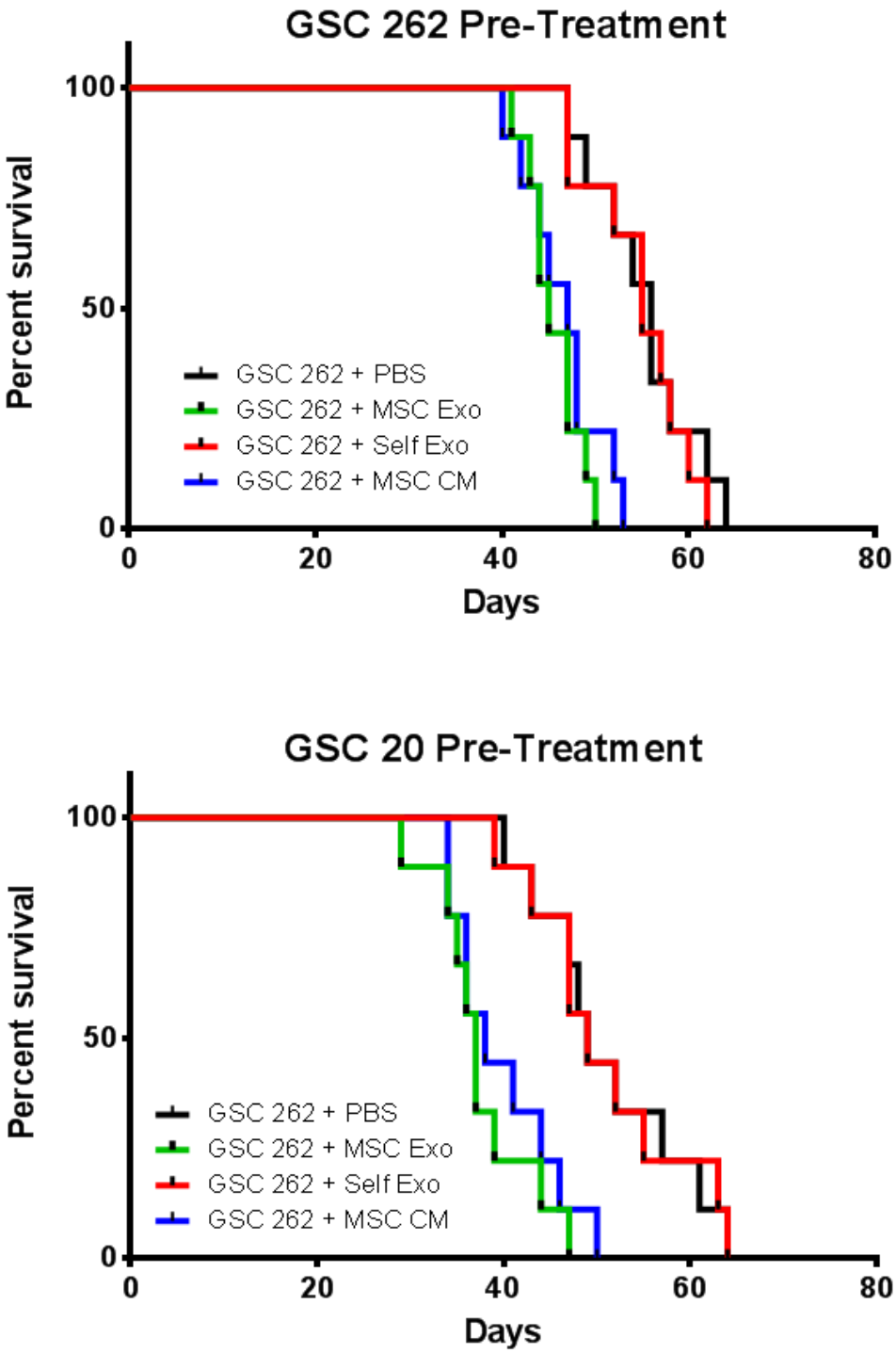

Figure 30. GA-MSC-derived exosomes decrease median survival. (A) Survival curve for mice with GSC 262 xenogafts, demonstrating a significant decrease ($p < 0.05$, log-rank test) in median survival from 56 to 45 days with pre-treatment of GA-MSC 262-derived exosomes. (B) Survival curve for mice with GSC 20 xenogafts, demonstrating a significant decrease ($p < 0.05$, log-rank test) in median survival from 49 to 37 days with pre-treatment of GA-MSC 20-derived exosomes.

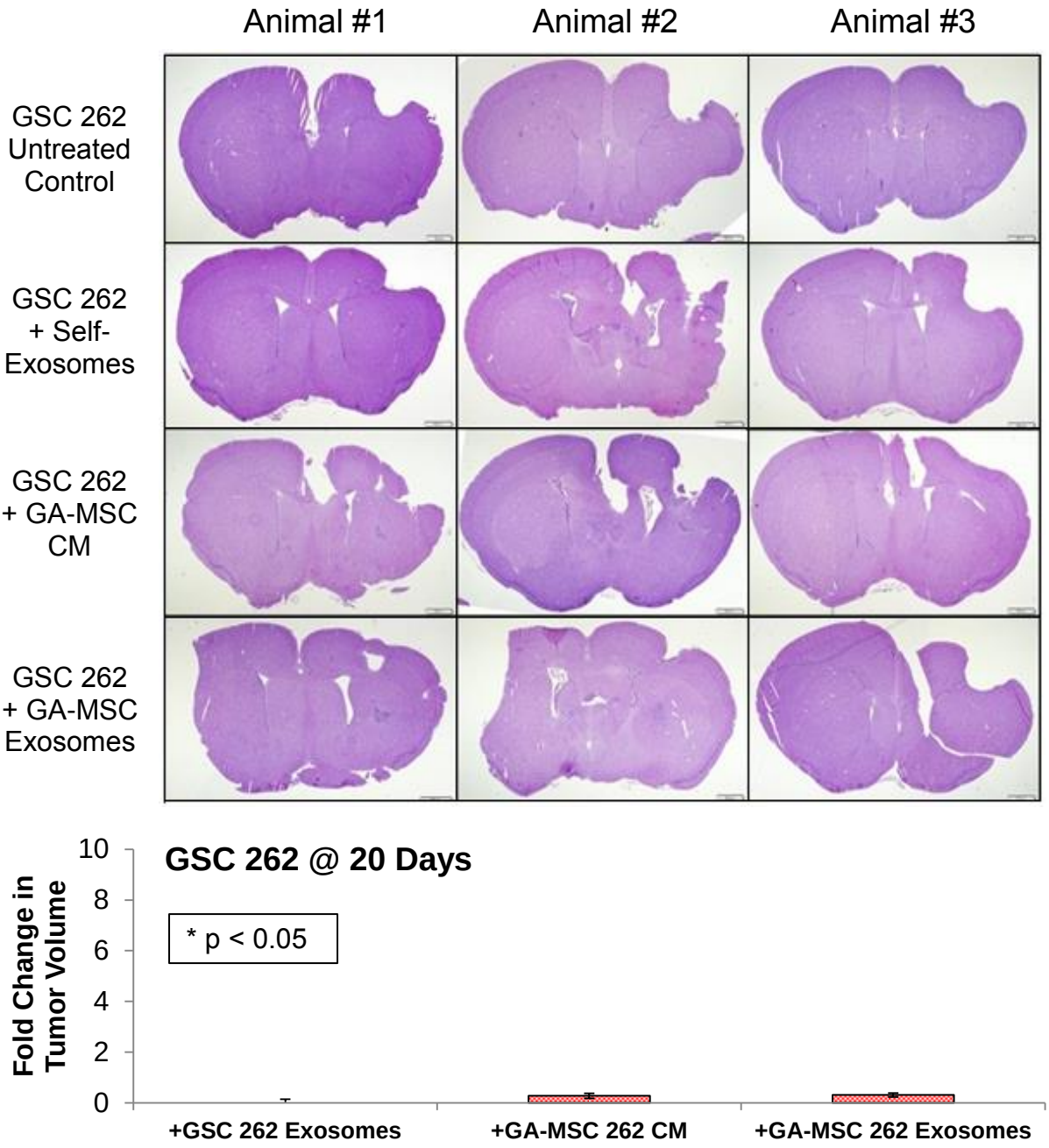

Figure 31. GA-MSC-derived exosomes increase tumor burden (20 days). (A/B) H&E staining and tumor volumes for GSC 262 xenografts 20 days post-implantation, demonstrating no significant difference in tumor burden between the untreated GSC 262 control group, and GSC 262 treated with GA-MSC 262-derived self exosomes.

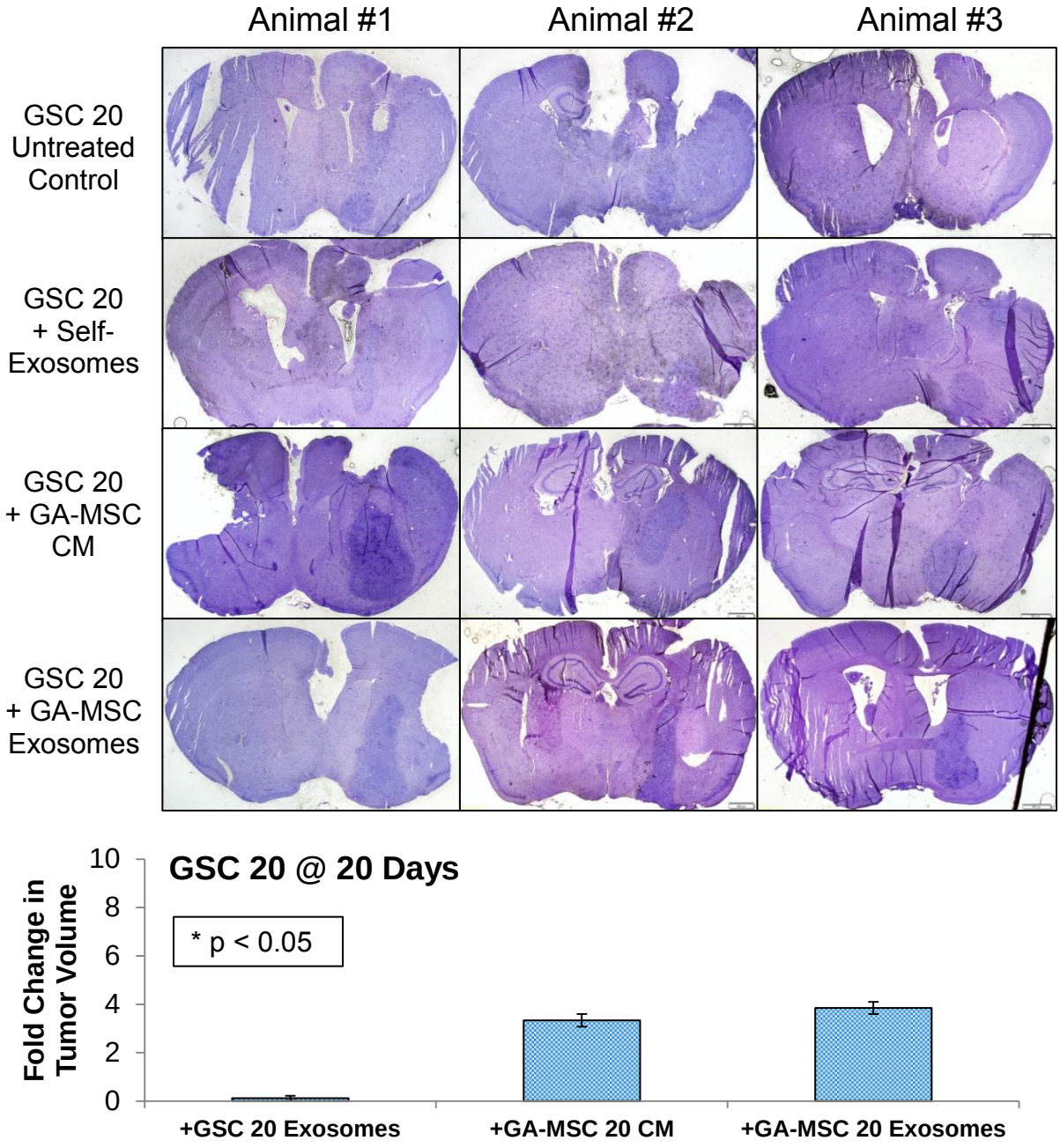

Figure 31. GA-MSC-derived exosomes increase tumor burden (20 days). (C/D) H&E staining and tumor volumes for GSC 20 xenografts 20 days post-implantation, demonstrating no significant difference in tumor burden between the untreated GSC 20 control group, and GSC 20 treated with GA-MSC 20-derived self exosomes.

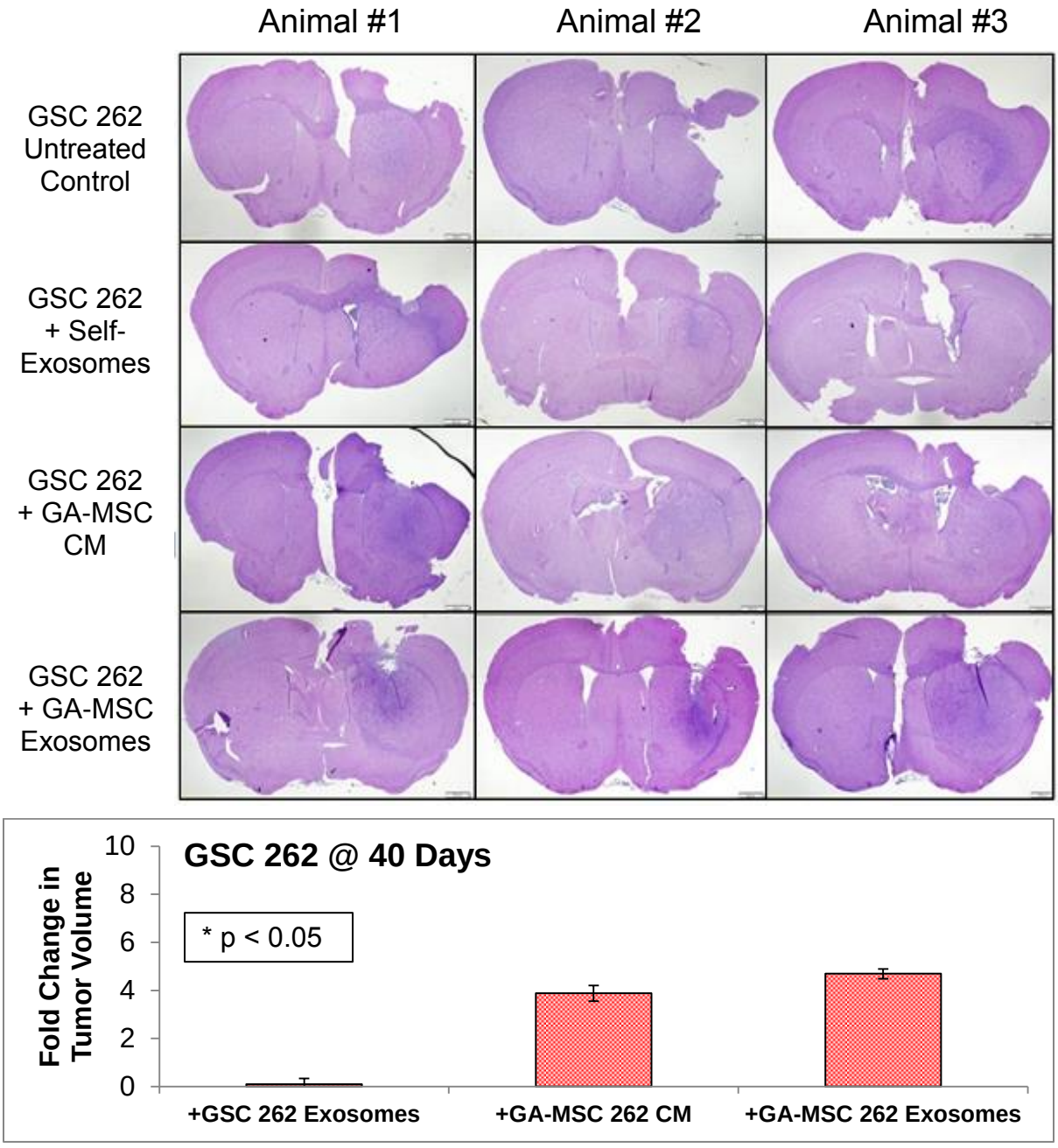

Figure 32. GA-MSC-derived exosomes increase tumor burden (40 days). (E/F) H&E staining and tumor volumes for GSC 262 xenografts 40 days post-implantation, demonstrating a significant difference in tumor burden between the untreated GSC 262 control group, and GSC 262 treated with GA-MSC 262-derived self exosomes.

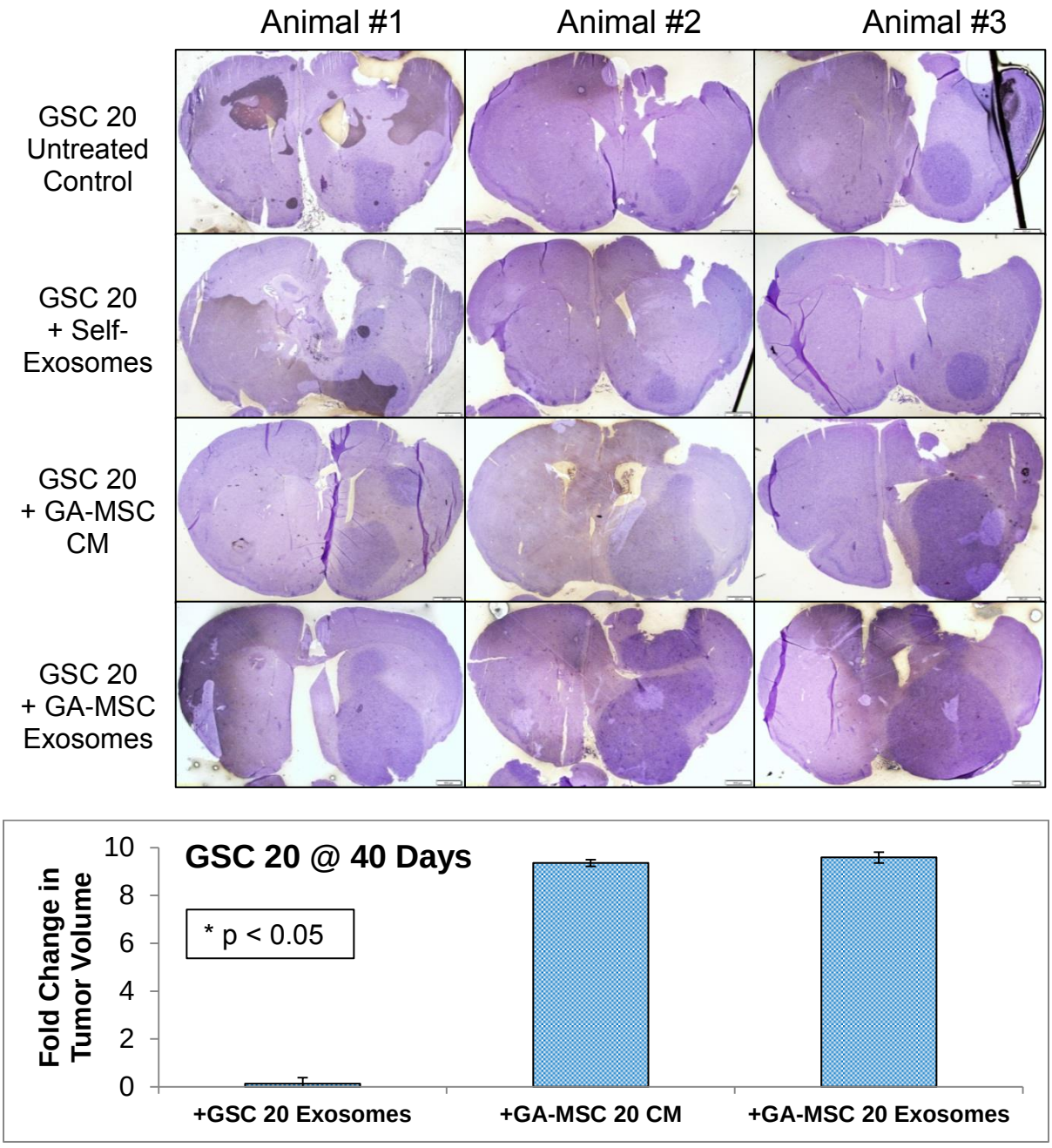

Figure 32. GA-MSC-derived exosomes increase tumor burden (40 days). (GH) H&E staining and tumor volumes for GSC 262 xenografts 20 days post-implantation, demonstrating no significant difference in tumor burden between the untreated GSC 20 control group, and GSC 20 treated with GA-MSC 20-derived self exosomes.

3.3 Discussion

In this chapter I show for the first time that GA-MSC-derived exosomes are taken up by GSCs, and that these exosomes increase proliferation and clonogenicity of GSCs *in vitro*, as well as increase tumorigenicity of GSCs *in vivo*.

Paracrine communication between cells of the tumor stromal cells and tumor propagating cells is well described, and recent studies have implicated exosomes as part of this system.[190-191] The majority of the literature focuses on the uptake and utilization of cancer stem cell-derived exosomes by the surrounding stromal cells of the microenvironment, although evidence supporting the internalization and usage of stromal-derived exosomes by cancer stem cells is mounting.[77-78,125-126] For example, Ekstrom, et al., showed that monocyte-derived exosomes, labeled with the fluorescent membrane dye PKH67, were taken up by MSCs into their cytoplasm, while Bijnsdorp, et al., revealed that prostate epithelial cells endocytosed PKH67-labeled colorectal cancer-derived exosomes.[192-193] More related to my research in gliomas, Skog, et al., demonstrated that GBM-derived PKH67-labeled exosomes, were internalized by brain microvascular endothelial cells.[80] These studies support the findings that fluorescent labeled exosomes can be visualized either attached to the membrane of within the cytoplasm of recipient cells. However, the tendency of this fluorescent dye, as with other lipophilic dyes, to leak out of the membrane and into surrounding cells suggests that they are not ideal for the labeling of exosomes.

To overcome the inadequacy of fluorescently labeling exosomes with the PKH67 membrane dye, other approaches were investigated. Evidence of a more reliable method for fluorescently labeling exosomes was first published by Wei, et al., who showed that lung cancer cell-derived exosomes could be labeled with a CD63-GFP fusion protein.[194] This method was then utilized by Suetsugu, et al., who demonstrated that CD63-labeled exosomes from breast cancer cells were taken up by normal lung tissue cells.[195] These two articles support the findings that GA-MSC-derived exosomes can be more precisely labeled with the CD63-GFP fusion protein, and that GA-MSC-derived exosomes can be more clearly tracked intracellularly in recipient GSCs to demonstrate internalization.

Although there are multiple studies exploring the intercellular communication between stromal cells and CSCs, few studies have investigated the role of stromal cell-derived exosomes in interactions with CSCs. Previous studies have indicated that stromal cell-derived exosomes can enhance the growth of CSCs in other cancer types, thereby supporting the results of my study. For example, Luga, et al., found that exosomes from breast cancer associated fibroblasts (CAFs) enhance the growth and metastasis of breast cancer cells via activation of the Wnt signaling pathway.[125] Similarly, Zhu, et al., showed that BM-MSC-derived exosomes increased the tumorigenicity of gastric carcinoma and colon cancer cells via the ERK1/2 signaling pathway.[155] Similarly, Roccaro, et al., reported that BM-MSC derived exosomes from patients with multiple myeloma, enhanced progression of multiple myeloma tumors via delivery of oncogenic proteins that affected cell adhesion and migration.[154] Consistent with these reports, I show that BM-MSC-derived exosomes promote the growth of CSCs in gliomas. My findings expand on the results of these studies by also showing that exosomes derived from a bona fide cell in the tumor microenvironment, GA-MSCs, also increase the growth of CSCs in gliomas. Furthermore, I also demonstrated that MSC-derived exosomes also increase the clonogenicity of CSCs in glioma, which was not investigated in these studies. Interestingly, GA-MSC-derived exosomes appeared to have more potent effects on GSC growth than BM-MSC-derived exosomes, suggesting that residence within the glioma niche enhances the growth promoting ability of MSCs. Therefore, to my knowledge, this is the first study to examine specifically the function of tumor-derived MSCs on CSC growth and self-renewal.

Interestingly, Roccaro, et al., also found that BM-MSC-derived exosomes from normal subjects without multiple myeloma, had an inhibiting effect on CSCs.[154] My experimental results did not indicate a tumor-inhibiting function of BM-MSC-derived exosomes, as described by Roccaro and colleagues.[154] Instead, BM-MSC-derived exosomes from normal subjects caused similar growth-promoting effects in GSCs as GA-MSC-derived exosomes. Nevertheless, these results define a glioma-promoting role of GA-MSC-derived exosomes and are supported by the literature. Furthermore, since we previously described the glioma-promoting properties of GA-

MSCs co-cultured with GSCs, it was not surprising that the exosomes released by GA-MSCs were also tumor-promoting in nature, and not tumor-inhibiting.[97] It is unclear why the experimental results of Roccaro, et al, differ from my investigation, however it is reasonable to consider that the role of BM-MSCs in multiple myeloma, a tumor of the bone, may be different from their role in other cancers.

To my knowledge, there are no studies describing a tumor-inhibiting role of stroma-derived exosomes. Instead, researchers are utilizing the robust functionality of the stroma-derived exosome system as a vector to deliver anti-tumor therapies.[74, 196] The reasoning behind this therapeutic approach, is that the tumor-promoting properties of modified stroma-derived exosomes are masked or overcome by the anti-tumor properties imparted by the therapeutic molecules. In any case, the enhancement of tumors by stroma-derived exosomes is being evaluated mechanistically in a variety of cancer types. Thus, these findings necessitate future investigation into the mechanism responsible for the glioma-promoting effects of GA-MSC-derived exosomes.

One of the weaknesses of this study was not conducting a time course experiment involving the tracking of fluorescently labeled GA-MSC-derived exosomes. This would have allowed the visualization of the exosome internalization process by GSCs, thus providing better insight and understanding of this intercellular communication pathway. Another weakness was not performing a limiting dilution for the study of clonogenicity *in vivo*. This experiment would involve implanting fewer and fewer GSCs pre-treated with GA-MSC-derived exosomes, in order to determine the least amount of cells that can form a tumor. Results from this experimental method would yield *in vivo* proof of clonogenic enhancement in GSCs, whereas tumor burden yields *in vivo* proof of only proliferative enhancement. Nevertheless, I did study, and show increases in, GSC growth and tumorigenicity *in vivo*.

Conversely, one of the strengths of this study was the use of matched pairs of GSCs and GA-MSCs that were isolated from the same patient tumor specimen. To my knowledge the use of such pairs in glioma studies is unique and has never been incorporated in research studies. This novel approach ensures that I investigated the

communication between cells actually present in the tumor niche, rather than utilizing commercially available BM-MSCs or the U87 glioblastoma cell line. However, I did utilize BM-MSCs in order to demonstrate the tumor-promoting function of MSC-derived exosomes in general. Another strength was the use of GSCs treated with GA-MSC-derived exosome-free conditioned medium as a positive control in demonstrating increases in GSC growth and self-renewal. Normal GA-MSC-derived conditioned medium contains a low concentration of exosomes, and therefore should not be used as a control. Furthermore, I utilized GSCs treated with GSC-derived self-exosomes as a negative control to demonstrate that the tumor-promoting properties are not universal to all exosomes, but are specific to the cell of origin. The last strength of this study was pre-treating GSCs with GA-MSCs in culture before *in vivo* implantation. Since I was investigating the exosomal communication between GA-MSCs and GSCs, this pre-treatment protocol ensured that GA-MSC-derived exosomes only interacted with GSCs. Conversely, co-injection of GSC and GA-MSC-derived exosomes would allow for exosomes to interact with other cells in the tumor niche.

3.4 Conclusions

The results of this investigation demonstrate for the first time that GA-MSC-derived exosomes can be tracked utilizing both fluorescent membranes dye and GFP-CD63 fusion protein. Furthermore, GSCs can internalize and organize GA-MSC-derived exosomes intracellularly. Most importantly I demonstrate that GA-MSC-derived exosomes significantly increase the proliferation of GSCs *in vitro* in a dose dependent manner. Additionally, GA-MSC-derived exosomes can also increase neurosphere formation, and thus clonogenicity, of GSCs *in vitro*. Moreover, GA-MSC-derived exosomes significantly increase the tumorigenicity of GSCs *in vivo*, thereby increasing tumor burden and decreasing median survival. These results prove that GA-MSC-derived exosomes alter the biology of GSCs.

Chapter IV

MicroRNA in GA-MSC-Derived Exosomes Promote Glioma Growth

MicroRNA in GA-MSC-Derived Exosomes Promote Glioma Growth

Evidence that GA-MSCs promote the growth of gliomas via exosomal miRNA requires identification of oncogenic miRNAs in GA-MSC-derived exosomes and demonstration that the miRNAs are functional in GSCs. To my knowledge such an analysis has never been conducted in gliomas. By investigating the role of exosomal miRNAs in the evolution of glioma, I hope to define a new intratumoral paracrine communication system. Therefore, I hypothesized that miRNA delivered via GA-MSC-derived exosomes increase the tumorigenicity of GSCs. To test this hypothesis, I first identified a specific group of miRNAs which were both highly expressed and highly enriched in GA-MSC-derived exosomes. I then compared expression levels for predicted gene targets of this group of exosomal miRNAs in untreated GSCs with those in GSCs after treatment with GA-MSC-derived exosomes. These results established a functional connection between miRNAs in GA-MSC-derived exosomes and recipient GSCs. I then showed that the growth and self-renewal of GSCs are increased by over-expression of these exosomal miRNAs in GSCs *in vitro*. These findings established that GA-MSCs alter the proliferation and clonogenicity of GSCs by secreting exosomes carrying specific miRNA. In the future, I aim to demonstrate that miRNA in GA-MSC-derived exosomes increase the tumorigenicity of GSCs *in vivo*.

4.1 Experimental Methods

Identification of Key MicroRNAs in GA-MSC-Derived Exosomes

In order to identify miRNAs that were not only highly expressed in MSC-derived exosomes (**Table 5**), but also highly enriched, I utilized the miRNA profiles obtained from earlier experiments (see *Chapter 2 Experimental Methods*). Out of the 2,019 miRNA analyzed, I identified a sub-population of miRNA that had significantly different average levels of expression between the MSC-derived exosomes and the parental cell ($p < 0.05$, paired student's t-test). This sub-population was termed *enriched* in MSC-derived exosomes. The change in expression for each enriched miRNA was then calculated by taking the difference in average expression levels between the MSC-derived exosome and the parental cell. MicroRNA that had expression changes greater than 2 standard deviations from the mean (top 2.5%) were termed *highly enriched* miRNA.

In order to identify miRNA in the enriched subpopulation that were also the most highly expressed in MSC-derived exosomes, an expression-to-enrichment ratio (E:R ratio) was calculated, by multiplying the change in expression between the exosome and the parental cell, by the ratio of expression levels between the exosome and the parental cell.

$$\text{E:R ratio} = (\text{Exosome} - \text{Cell}) \times (\text{Exosome} / \text{Cell})$$

The E:R ratio yielded two groups of miRNAs at the extremes of the distribution. One group of miRNA was both highly expressed and highly enriched in MSC-derived exosomes when compared to the MSC parental cell. Another group of miRNA was both highly expressed and highly enriched in parental MSCs, and thus had low expression and was depleted from MSC-derived exosomes.

Identification of Nucleotide Motifs in Exosome Enriched and Depleted MicroRNA

In order to determine if specific nucleotide motifs in miRNA correlate with preferential packaging into exosomes, pre-miRNA (stem-loop) and mature miRNA sequences were obtained from the miRBase database for the highly enriched and

highly expressed miRNA in MSC-derived exosomes.[197] Pre-miRNA and mature-miRNA sequences were initially analyzed for their degree of guanine-cytosine to uracil-adenine (G-C:U-A) ratio. Additionally, pre-miRNA and mature-miRNA sequences were evaluated to identify specific sub-sequences that were common among the highly expressed and highly enriched miRNA groups. Statistical analysis was performed utilizing the paired student's t-test.

Gene Expression Profiling of GSCs Treated with GA-MSC-Derived Exosomes

In order to isolate MSC-derived exosomes, GA-MSC 262 and GA-MSC 20 were expanded to 5.0 x 10^6 cells in MSC growth medium. GA-MSCs were subsequently washed and incubated for 48 hours in serum-free NSC medium. GA-MSC-derived exosomes were then isolated from the conditioned mediums by differential ultracentrifugation and quantified by CD63 ELISA (see *Chapter 2 Experimental Methods*). GA-MSC-derived exosomes were then added to GSC cultures (1.0 x 10^9 exosomes/mL). GSCs were also treated with NSC medium as a control. After 48 hours of treatment, excess exosomes were removed by PBS wash, and total RNA was isolated from both groups of GSCs (see *Chapter 2 Experimental Methods*). Total RNA from both groups of GSCs was then analyzed for gene expression profiling utilizing Illumina next-generation sequencing technology, through LC Sciences. Gene expression profiles from untreated GSCs and GSCs after treatment with GA-MSC-derived exosomes were statistically compared by paired student's t-test.

Gene Target Analysis of Exosome Enriched and Depleted MicroRNA

I identified predicted gene targets for the 8 highly enriched and highly expressed miRNA in MSC-derived exosomes, utilizing the miRTarget 2.0 database (> 80 target score).[198] I then assessed the expression level of the predicted gene targets for the 8 highly expressed and highly enriched miRNA in GSCs after treatment with GA-MSC-derived exosomes, by querying the relative gene expression profile. Specifically, the level of expression for a given miRNA gene target in untreated GSCs controls was compared to that of GSCs treated with GA-

MSC-derived exosomes. This analysis yielded an average fold change for each predicted gene target of the 8 highly expressed and highly enriched miRNA. By the same method, I also performed analysis for the predicted gene targets of the 8 miRNA that are depleted from GA-MSC-derived exosomes. The average fold change among the predicted gene targets for highly enriched and highly expressed miRNA in GA-MSC-derived exosomes, was then compared to that of the depleted miRNA by performing un-paired student's t-test.

Over-Expression of MicroRNAs in GSCs

To test the effects of highly expressed and highly enriched exosomal miRNA on GSCs, each miRNA was over-expressed in GSCs by lentiviral (LV) transduction. Initially, plasmids of LV-GFP-miRNA constructs for each specific miRNA, as well as LV-GFP-miRNA-scramble construct (control), were isolated from expanded bacterial stocks (Qiagen) using a plasmid maxi kit (Qiagen). Lentiviral vectors were generated by transfecting human embryonic kidney 293FT (HEK-293FT) cells with individual miRNA plasmids, along with a viral packaging plasmid and a viral envelope plasmid. Resulting lentiviral (LV) vectors were isolated by ultracentrifugation of conditioned medium from HEK-293 cells. Viral titers were then calculated by inoculation of HEK-293 cells with limiting dilutions of complete LV-GFP-miRNA viral vectors, and assessing for GFP expression. Each LV-GFP-miRNA viral vector was then transduced in GSC 262 and GSC 20 (1.0×10^{6} cells) at a multiplicity of infection (MOI) of 3, and cultured under puromycin selection (1nM) for 72 hours. Integration of the GFP-miRNA construct was assessed by fluorescent light microscopy. RNA was then isolated from each of the GFP-miRNA transduced cell lines (see *Chapter 2 Experimental Methods*) for verification of miRNA over-expression by qRT-PCR. Additionally, RNA was also isolated from GSCs treated with NSC medium, as well as from GSCs treated with GA-MSC-derived exosomes for a period of 48 hours. The level of miRNA expression determined by qRT-PCR for the GFP-miRNA expressing GSCs was then compared to that of untreated GSC controls and GSCs after treatment with GA-MSC-derived exosomes. Statistical analysis was performed utilizing paired student's t-test.

Functional Verification of Over-Expressed miRNA in GSCs

GSCs stably expressing the GFP-miR construct were expanded to 10^6 cells, and protein isolated by the addition of cell lysis buffer (see *Chapter 2 Experimental Methods*). Additionally, GSCs were expanded to 1.0 x 10^6 cells and treated with NSC medium, GA-MSC-derived exosomes (1.0 x 10^9 exosomes/mL), and GA-MSC-derived exosomes plus anti-miRNA (Qiagen) at 1nM. After 48 hours of culture, excess exosomes were removed by PBS wash, and protein was isolated by the addition of cell lysis buffer (see *Chapter 2 Experimental Methods*). Specific predicted gene targets for each over-expressed miRNA were then assessed for degree of down-regulation by western blot analysis. Results were compared with the degree of miRNA gene target expression in GSCs after treatment with NSC medium, after treatment with GA-MSC-derived exosomes, and after treatment with GA-MSC-derived exosomes plus anti-miR inhibitors (Qiagen) at 1nM.

Effects of Exosomal MicroRNAs on GSC Proliferation

In order to isolate MSC-derived exosomes, GA-MSC 262 and GA-MSC 20 were expanded to 5.0 x 10^6 cells in MSC growth medium. GA-MSCs were subsequently washed and incubated for 48 hours in serum-free NSC medium. MSC-derived and GSC-derived exosomes were then isolated from the conditioned mediums by differential ultracentrifugation and quantified by CD63 ELISA (see *Chapter 2 Experimental Methods*). GSC 262 and GSC 20 were then placed in 16 wells of a 96-well plate (2.5 x 10^3 cells/well). Four the wells were treated with NSC medium. Another four wells were treated with GA-MSC-derived exosomes (1.0 x 10^4 exosomes/µL). Another four wells were treated with GA-MSC-derived exosomes (1.0 x 10^4 exosomes/µL) plus anti-miRNA (Qiagen) by lipofection at 1nM. The last four wells were treated with anti-miRNA (Qiagen) by lipofection at 1nM. Additionally, neurospheres from GSCs expressing the GFP-miRNA constructs were dissociated and placed into another four wells of the 96-well plate (2.5 x 10^3 cells/well). Finally, neurospheres of GSCs expressing the GFP-miRNA-scramble construct (control) were placed into another four wells of the 96-well plate (2.5 x 10^3 cells/well). After 96 hours of incubation, GSC viability was assessed using a colorimetric assay WST-1

(Roche), and the absorbance measured at 450nm. The absorbance for each experimental treatment group of GSCs was compared to that of untreated GSC controls, as well as to that of GSCs expressing the GFP-miRNA-scramble construct. All experiments were performed in quadruplicate and statistical analysis was performed utilizing the paired student's t-test.

Effects of Exosomal MicroRNAs on GSC Clonogenicity

In order to isolate MSC-derived exosomes, GA-MSC 262 and GA-MSC 20 were expanded to 5.0 x 10^6 cells in MSC growth medium. GA-MSCs were subsequently washed and incubated for 48 hours in serum-free NSC medium. MSC-derived and GSC-derived exosomes were then isolated from the conditioned mediums by differential ultracentrifugation and quantified by CD63 ELISA (see *Chapter 2 Experimental Methods*). Neurospheres from GSC 262 and GSC 20 were then dissociated and placed into single cell suspensions in each well of four 96-well plates. One 96-well plate was treated with NSC medium. Another 96-well plate was treated with GA-MSC-derived exosomes (4.0 x 10^2 exosomes/µL). Another 96-well plate was treated with GA-MSC-derived exosomes (4.0 x 10^2 exosomes/µL) plus anti-miRNA (Qiagen) by lipofection at 1nM. The last 96-well plate was treated with anti-miRNA (Qiagen) by lipofection at 1nM. Additionally, neurospheres from GSCs expressing the GFP-miRNA constructs were dissociated and placed into single cell suspension in each well of another 96-well plate. Finally, neurospheres from GSCs expressing the GFP-miRNA-scramble construct (control) were dissociated and placed into single cell suspension in each well of another 96-well plate. After 3 weeks of incubation, GSC neurospheres in all 96-well plates were quantified. Percentage of neurosphere formation in each GSCs experimental group was compared to that of untreated GSC controls, as well as to that of GSCs expressing the GFP-miRNA-scramble construct. All experiments were performed in quadruplicate and statistical analysis was performed utilizing paired student's t-test.

Effects of Exosomal MicroRNAs on GSC Tumorigenicity

GA-MSC 262 and GA-MSC 20 were expanded to 5.0 x 10^7 cells in MSC growth medium. Exosomes were isolated from conditioned medium of GA-MSC 262 and GA-MSC 20 by differential ultracentrifugation, and quantified by CD63 ELISA. Subsequently, GSC 262 and GSC 20 were expanded to 3.0 x 10^7 cells in preparation for pre-treatment and implantation. GSCs were then pre-treated with either NSC medium, GA-MSC-derived exosomes (1.0 x 10^9 exosomes/mL), GA-MSC-derived exosomes (1.0 x 10^9 exosomes/mL) plus anti-miR (Qiagen) by lipofection at 1nM, and anti-miR (Qiagen) by lipofection at 1nM. Additionally, GSCs expressing the GFP-miRNA construct, and GSCs expressing the GFP-miRNA-scramble construct, we expanded to 7.5 x 10^6 cells each. After 96 hours of pre-treatment, excess exosomes were eliminated by PBS wash, and GSC neurospheres were dissociated. GSCs were then injected into the right frontal lobe of nude mice (n=15/group, 5.0 x 10^5 cells/mouse), utilizing a cranial bolt-guided, stereotactic system. A cohort of mice (n=9) were followed until moribund and then sacrificed. Another cohort of mice (n=6) were sacrificed at 20 and 40 days post implantation. After sacrifice, mouse brains were removed, embedded in paraffin, and sectioned tumors. Tumor volumes and survival results of the GSCs treated with GA-MSC-derived exosomes were compared to untreated GSC controls. Tumor volumes were calculated by adding multiple cross-sectional areas through the tumors after H&E staining of histologic sections. Statistical analysis was performed utilizing the paired student's t-test for tumor volume analysis, and log-rank test for survival analysis.

4.2 Results

Identification of Key MicroRNA in GA-MSC-Derived Exosomes

To identify potential miRNA in MSC-derived exosomes that could be causing the increase in GSC tumorigenicity, I first evaluated the miRNA profiles of exosomes from BM-MSCs and four GA-MSC lines (see *Chapter 2 Results*). I identified a sub-population of miRNA that were highly enriched in MSC-derived exosomes when compared to the parental cell, with changes in expression greater than 4000 hybridization intensity (> 2 standard deviations from the mean) (**Table 6**). I then calculated the expression-to-enrichment ratio (E:R ratio) for each miRNA, and graphed the distribution of the E:R ratios (**Figure 33**). From one extreme of this distribution, I identified a group of 8 miRNAs that were the most highly expressed and highly enriched (> 3 standard deviations from the mean) in MSC-derived exosomes when compared to the parental cells (**Table 7**). This cutoff correlated with average miRNA expression levels greater than 5000 hybridization intensity. Conversely, from the other extreme of the distribution, I identified a group of 8 miRNA that were the most highly expressed and highly enriched (> 1 standard deviation from the mean) in the parental cells when compared to MSC-derived exosomes (**Table 7**). The group of miRNA that are highly expressed and highly enriched in the parental cells are thus relatively depleted from MSC-derived exosomes. This level of enrichment and depletion suggests the existence of a mechanism which targets miRNAs into, or out of, MSC-derived exosomes.

Interestingly, the nucleotide composition of both the enriched and depleted miRNAs in GA-MSC-derived exosomes are significantly different from each other. Specifically, the Glutamine-Cytosine to Adenine-Uracil ratio (G-C:A-U) for the group of enriched miRNAs is 2.12:1 in pre-miRNA, and 3.20:1 in mature miRNA (**Table 8**). Conversely, the G-C:U-A ratio for the group of depleted miRNAs is 0.65:1 in pre-miRNA, and 0.72:1 in mature miRNA (**Table 8**). Thus, there is significantly greater amount of G-C nucleotides, compared to U-A nucleotides, in miRNA that are enriched in GA-MSC-derived exosomes ($p < 0.01$). Furthermore, analysis of the stem-loop miRNA sequences in both the enriched and depleted miRNA in GA-MSC-

Highly Enriched miRNA	Expression Level Cell	Expression Level Exosome	Δ Expression	Standard Deviation
hsa-miR-3960	4,136	19,651	15516	7.710
hsa-miR-6087	2,757	15,568	12811	6.365
hsa-miR-3665	4,030	16,364	12334	6.128
hsa-miR-6089	3,903	16,163	12260	6.091
hsa-miR-1246	563	11,941	11377	5.652
hsa-miR-6090	2,398	12,200	9803	4.869
hsa-miR-4508	644	10,268	9624	4.780
hsa-miR-4497	1,994	10,837	8843	4.392
hsa-miR-574-5p	1,483	9,568	8085	4.015
hsa-miR-1234-5p	1,558	9,568	8011	3.978
hsa-miR-6125	2,153	9,926	7773	3.860
hsa-miR-4530	657	8,426	7769	3.858
hsa-miR-4505	258	7,911	7653	3.800
hsa-miR-3620-5p	268	6,373	6105	3.030
hsa-miR-3656	1,059	7,110	6050	3.003
hsa-miR-4516	2,450	8,252	5802	2.880
hsa-miR-4787-5p	1,764	7,561	5797	2.877
hsa-miR-638	1,686	7,482	5796	2.877
hsa-miR-4492	176	5,726	5550	2.755
hsa-miR-4507	185	5,398	5213	2.587
hsa-miR-574-3p	2,743	7,882	5139	2.550
hsa-miR-1587	198	5,269	5071	2.516
hsa-miR-6085	457	5,046	4589	2.276
hsa-miR-4484	868	5,069	4201	2.084

Table 6. MSC-derived exosomes contain highly enriched miRNA. MSC-derived exosomes contain 24 miRNA with a change in expression level from the parental cell greater than 4000 hybridization intensity (> 2.0 standard deviations from the mean).

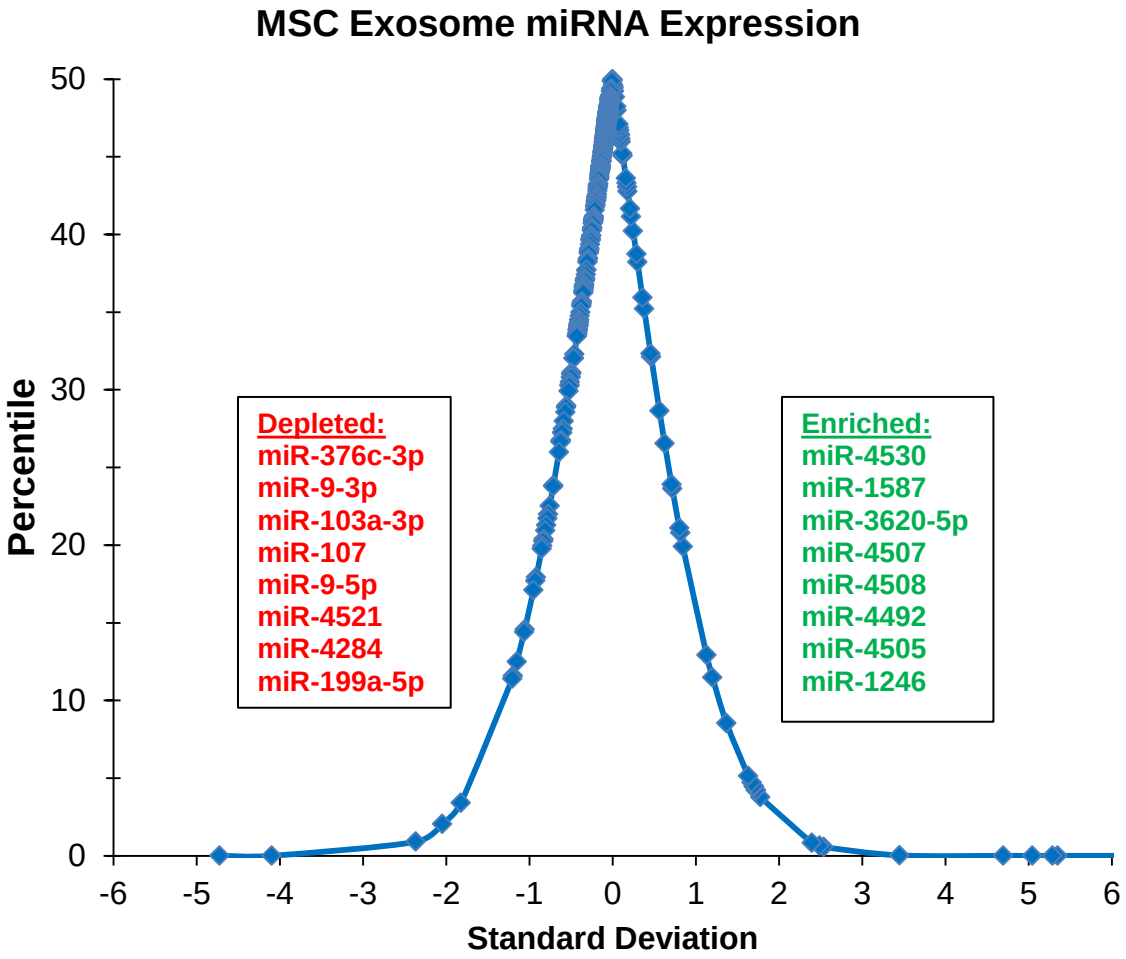

Figure 33. GA-MSC-derived exosomes contain highly expressed and highly enriched miRNA. Distribution of the E:R ratio for miRNA in MSC-derived exosomes. Exosome-enriched miRNA were both highly expressed compared to exosome-depleted miRNA, which were both highly expressed in the parental MSC.

A

Enriched miRNA	Expression Level Cell	Expression Level Exosome	Δ Expression	E:R ratio	Standard Deviation
hsa-miR-1246	563	11,941	11377	241097	8.438
hsa-miR-4505	258	7,911	7653	234683	8.212
hsa-miR-4492	176	5,726	5550	180857	6.314
hsa-miR-4508	644	10,268	9624	153480	5.349
hsa-miR-4507	185	5,398	5213	151764	5.288
hsa-miR-3620-5p	268	6,373	6105	144924	5.047
hsa-miR-1587	198	5,269	5071	134920	4.695
hsa-miR-4530	657	8,426	7769	99625	3.450

B

Depleted miRNA	Expression Level Cell	Expression Level Exosome	Δ Expression	E:R ratio	Standard Deviation
hsa-miR-376c-3p	2,130	149	-1981	-28384	-1.063
hsa-miR-9-3p	1,361	57	-1304	-30875	-1.150
hsa-miR-103a-3p	3,450	334	-3116	-32169	-1.196
hsa-miR-107	3,719	383	-3337	-32436	-1.205
hsa-miR-9-5p	2,994	151	-2843	-56255	-2.045
hsa-miR-4521	966	14	-952	-65330	-2.365
hsa-miR-4284	2,729	64	-2666	-114478	-4.098
hsa-miR-199a-5p	2,599	50	-2549	-132188	-4.722

Table 7. MSC-derived exosomes contain enriched and depleted miRNA. (A) MSC-derived exosomes contain 8 miRNA which are the most highly expressed and highly enriched when compared to the parental cell (> 3.0 standard deviations from the mean). (B) MSC-derived exosomes are depleted of 8 miRNA which are the most highly expressed and highly enriched in the parental MSC (> 1.0 standard deviations from the mean).

A

Enriched miRNA	Pre-miRNA Sequence		Mature miRNA Sequence	
	G-C %	U-A%	G-C %	U-A%
miR-1246	43.84	56.16	42.11	57.89
miR-1587	60.38	39.62	66.67	33.33
miR-3620-5p	70.89	29.11	81.82	18.18
miR-4492	76.25	23.75	94.12	5.88
miR-4505	68.49	31.51	72.22	27.78
miR-4507	67.31	32.69	75.00	25.00
miR-4508	80.00	20.00	93.75	6.25
miR-4530	76.79	23.21	77.78	22.22
Average	67.99	32.01	75.43	24.57
Std Dev	11.59		16.60	
p-value	0.002		0.002	

B

Depleted miRNA	Pre-miRNA Sequence		Mature miRNA Sequence	
	G-C %	U-A%	G-C %	U-A%
miR-9-3p	32.91	67.09	28.57	71.43
miR-9-5p	32.91	67.09	34.78	65.22
miR-103a-3p	48.72	51.28	47.83	52.17
miR-107	46.91	53.09	50.00	50.00
miR-199a-5p	50.70	49.30	50.00	50.00
miR-376c-3p	28.79	71.21	38.10	61.90
miR-4284	32.53	67.47	30.43	69.57
miR-4521	41.67	58.33	54.55	45.45
Average	39.39	60.61	41.78	58.22
Std Dev	8.87		10.00	
p-value	0.005		0.027	

Table 8. Exosomal miRNAs have varying nucleotide ratios. (A) MicroRNAs enriched in GA-MSC-derived exosomes contain significantly higher percentages of G-C nucleotides compared to U-A nucleotides. (B) MicroRNAs depleted in GA-MSC-derived exosomes contain significantly lower percentages of G-C nucleotides compared to U-A nucleotides.

derived exosomes, reveals three conserved 4 nucleotide-long sub-sequences that are specific to each group. The combination of the sub-sequences GGCU, GGAC, and CAGG are found in 100% of the miRNA enriched in MSC-derived exosomes, and not found together in any of the miRNA depleted from MSC-derived exosomes (**Table 9**). Conversely, the combination of the sub-sequences UGUU, UGUA, and UGUG are found in 100% of the miRNA depleted in MSC-derived exosomes, and are not found together in any of the miRNA enriched in MSC-derived exosomes (**Table 9**). This suggests that specific nucleotide motifs, as well as certain levels of G-C:U-A enrichment, may lead to particular miRNAs being preferentially packaged into MSC-derived exosomes.

Verification of Exosomal MicroRNA Functionality

In order to determine whether the enriched miRNA in GA-MSC-derived exosomes are capable of affecting gene expression in GSCs, I first identified predicted gene targets for this group of miRNAs. Specifically, utilizing the miRTarget 2.0 database, I identified 251 unique predicted gene targets for the 7 of the 8 highly expressed and highly enriched miRNA in MSC-derived exosomes (no predicted gene targets for miR-4508) (**Table 10**).[198] Conversely, utilizing the miRTarget 2.0 database, I identified 245 unique predicted gene targets for all 8 of the miRNAs that are depleted from MSC-derived exosomes (**Table 10**).[198] Expression levels for the predicted gene targets in the two groups were then obtained from the gene expression profiling performed on GSC 262 and GSC 20. The expression level of the predicted gene targets in untreated GSCs were compared with that of GSCs treated with GA-MSC-derived exosomes. The fold change in expression of the 251 predicted gene targets for the 7 enriched miRNA, was significantly more ($p < 0.01$) than the average fold change in expression of the 245 predicted gene targets for the 8 depleted miRNA (**Figure 34**). These results indicate that highly expressed and highly enriched miRNA in GA-MSC-derived exosomes are capable of down-regulating their predicted gene targets in GSCs. These findings also indicate that the predicted gene targets for depleted miRNA were not as down-regulated in GSCs after treatment with GA-MSC-derived exosomes, due to their low expression level.

A

Enriched miRNA	Stem Loop Sequence
miR-1246	UGUAUCCUUGAAUGGAUUUUUGGAGCAGGAGUGGACACCUGACCCAAAGGAAAUCAAUCCAUAGGCUAGCAAU
miR-1587	UUUGGGCUGGGCUGGGUUGGGCAGUUCUUCUGCUGGACUCACCUGUGACCAGC
miR-3620-5p	GUGAGGUGGGGGCCAGCAGGGAGUGGGCUGGGCUGGGCUGGGCCAAGGUACAAGGCCUCACCCUGCAUCCCGCACCCAG
miR-4492	CUGCAGCGUGCUUCUCCAGGCCCCGCGCGCGGACAGACACACGGACAAGUCCCGCCAGGGGCUGGGCGCGCGCCAGCCGG
miR-4505	GGAGGCUGGGCUGGGACGGACACCCGGCCUCCACUUUCUGUGGCAGGUACCUCCUCCAUGUCGGCCCGCCUUG
miR-4507	UCUGGGCUGAGCCGAGCUGGGUUAAGCCGAGCUGGGUUGGGCUGGGCUGGGU
miR-4508	AGGACCCAGCGGGGCUGGGCGCGCGGAGCAGCGCUGGGUGCAGCGCCUGCGCCGGCAGCUGCAAGGGCCG
miR-4530	CGACCGCACCCGCCCGAAGCUGGGUCAAGGAGCCCAGCAGGACGGGAGCGCGGCGC
miR-1246	UGUAUCCUUGAAUGGAUUUUUGGAGCAGGAGUGGACACCUGACCCAAAGGAAAUCAAUCCAUAGGCUAGCAAU
miR-1587	UUUGGGCUGGGCUGGGUUGGGCAGUUCUUCUGCUGGACUCACCUGUGACCAGC
miR-3620-5p	GUGAGGUGGGGGCCAGCAGGGAGUGGGCUGGGCUGGGCUGGGCCAAGGUACAAGGCCUCACCCUGCAUCCCGCACCCAG
miR-4492	CUGCAGCGUGCUUCUCCAGGCCCCGCGCGCGGACAGACACACGGACAAGUCCCGCCAGGGGCUGGGCGCGCGCCAGCCGG
miR-4505	GGAGGCUGGGCUGGGACGGACACCCGGCCUCCACUUUCUGUGGCAGGUACCUCCUCCAUGUCGGCCCGCCUUG
miR-4507	UCUGGGCUGAGCCGAGCUGGGUUAAGCCGAGCUGGGUUGGGCUGGGCUGGGU
miR-4508	AGGACCCAGCGGGGCUGGGCGCGCGGAGCAGCGCUGGGUGCAGCGCCUGCGCCGGCAGCUGCAAGGGCCG
miR-4530	CGACCGCACCCGCCCGAAGCUGGGUCAAGGAGCCCAGCAGGACGGGAGCGCGGCGC
miR-1246	UGUAUCCUUGAAUGGAUUUUUGGAGCAGGAGUGGACACCUGACCCAAAGGAAAUCAAUCCAUAGGCUAGCAAU
miR-1587	UUUGGGCUGGGCUGGGUUGGGCAGUUCUUCUGCUGGACUCACCUGUGACCAGC
miR-3620-5p	GUGAGGUGGGGGCCAGCAGGGAGUGGGCUGGGCUGGGCUGGGCCAAGGUACAAGGCCUCACCCUGCAUCCCGCACCCAG
miR-4492	CUGCAGCGUGCUUCUCCAGGCCCCGCGCGCGGACAGACACACGGACAAGUCCCGCCAGGGGCUGGGCGCGCGCCAGCCGG
miR-4505	GGAGGCUGGGCUGGGACGGACACCCGGCCUCCACUUUCUGUGGCAGGUACCUCCUCCAUGUCGGCCCGCCUUG
miR-4507	UCUGGGCUGAGCCGAGCUGGGUUAAGCCGAGCUGGGUUGGGCUGGGCUGGGU
miR-4508	AGGACCCAGCGGGGCUGGGCGCGCGGAGCAGCGCUGGGUGCAGCGCCUGCGCCGGCAGCUGCAAGGGCCG
miR-4530	CGACCGCACCCGCCCGAAGCUGGGUCAAGGAGCCCAGCAGGACGGGAGCGCGGCGC

Table 9. Exosomal miRNA contain specific nucleotide sequences. (A) MicroRNAs enriched in GA-MSC-derived exosomes contain 3 specific nucleotide motifs (CAGG, GGAC, and GGCU) in their stem-loop sequences, which are not present to a high degree in depleted miRNAs.

B

Depleted miRNA	Stem Loop Sequence
miR-9-3p	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAUAACCCCA
miR-9-5p	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAUAACCCCA
miR-103a-3p	UACUGCCCUCGGCUUCUUUACAGUGCUGCCUUGUUGCAUAUGGAUCAAGCAGCAUUGUACAGGGCUAUGAAGGCAUUG
miR-107	CUCUCUGCUUUCAGCUUCUUUACAGUGUUGCCUUGUGGCAUGGAGUUCAAGCAGCAUUGUACAGGGCUAUCAAAGCACAGA
miR-199a-5p	GCCAACCCAGUGUUCAGACUACCUGUUCAGGAGGCUCUCAAUGUGUACAGUAGUCUGCACAUUGGUUAGGC
miR-376c-3p	AAAAGGUGGAUAUUCCUUCUAUGUUUAUGUUAUUUAUGGUUAAACAUAGAGGAAAUUCCACGUUUU
miR-4284	AUACAUACAUGUACACACACAUGUCAUCCACACACAUACAUAUAUAUAUGUUUGUAUGGAUAUGUGUGUGUGUAUGUGUGUGUAU
miR-4521	UCGGCUAAGGAAGUCCUGUGCUCAGUUUUGUAGCAUCAAAACUAGGAUUUCUCUUGUUAC
miR-9-3p	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAUAACCCCA
miR-9-5p	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAUAACCCCA
miR-103a-3p	UACUGCCCUCGGCUUCUUUACAGUGCUGCCUUGUUGCAUAUGGAUCAAGCAGCAUUGUACAGGGCUAUGAAGGCAUUG
miR-107	CUCUCUGCUUUCAGCUUCUUUACAGUGUUGCCUUGUGGCAUGGAGUUCAAGCAGCAUUGUACAGGGCUAUCAAAGCACAGA
miR-199a-5p	GCCAACCCAGUGUUCAGACUACCUGUUCAGGAGGCUCUCAAUGUGUACAGUAGUCUGCACAUUGGUUAGGC
miR-376c-3p	AAAAGGUGGAUAUUCCUUCUAUGUUUAUGUUAUUUAUGGUUAAACAUAGAGGAAAUUCCACGUUUU
miR-4284	AUACAUACAUGUACACACACAUGUCAUCCACACACAUACAUAUAUAUAUGUUUGUAUGGAUAUGUGUGUGUGUAUGUGUGUGUAU
miR-4521	UCGGCUAAGGAAGUCCUGUGCUCAGUUUUGUAGCAUCAAAACUAGGAUUUCUCUUGUUAC
miR-9-3p	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAUAACCCCA
miR-9-5p	CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCUAGAUAACCGAAAGUAAAAAUAACCCCA
miR-103a-3p	UACUGCCCUCGGCUUCUUUACAGUGCUGCCUUGUUGCAUAUGGAUCAAGCAGCAUUGUACAGGGCUAUGAAGGCAUUG
miR-107	CUCUCUGCUUUCAGCUUCUUUACAGUGUUGCCUUGUGGCAUGGAGUUCAAGCAGCAUUGUACAGGGCUAUCAAAGCACAGA
miR-199a-5p	GCCAACCCAGUGUUCAGACUACCUGUUCAGGAGGCUCUCAAUGUGUACAGUAGUCUGCACAUUGGUUAGGC
miR-376c-3p	AAAAGGUGGAUAUUCCUUCUAUGUUUAUGUUAUUUAUGGUUAAACAUAGAGGAAAUUCCACGUUUU
miR-4284	AUACAUACAUGUACACACACAUGUCAUCCACACACAUACAUAUAUAUAUGUUUGUAUGGAUAUGUGUGUGUGUAUGUGUGUGUAU
miR-4521	UCGGCUAAGGAAGUCCUGUGCUCAGUUUUGUAGCAUCAAAACUAGGAUUUCUCUUGUUAC

Table 9. Exosomal miRNA contain specific nucleotide sequences. (B) MicroRNAs depleted in GA-MSC-derived exosomes contain 3 specific nucleotide motifs (UGUG, UGUA, and UGUU) in their stem-loop sequences, which are not present to a high degree in enriched miRNAs.

Table 10. MicroRNAs enriched in GA-MSC-derived exosomes down-regulate predicted gene targets in GSCs. Expression levels of predicted gene targets for miRNA enriched and depleted in GA-MSC-derived exosomes.

Gene Targets for miRNAs Enriched in Cell (245)	Fold Change	Gene Targets for miRNAs Enriched in Exosome (251)	Fold Change
Average	-0.53	Average	-3.79
Std Dev	3.55	Std Dev	3.48
Max	12.14	Max	4.43
q3	5.15	q3	0.67
Median	-1.84	Median	-3.09
q1	-4.08	q1	-10.40
Min	-6.32	Min	-17.70

ABCC5	-2.44	ABCG4	-5.92	CYLD	8.84	CTSB	-9.30	ITPRIPL2	-1.14	JRK	-3.27	PAN3	-3.12	PAX5	-5.20	SNX25	-1.90	SMG7	-6.13
ACOT7	5.04	ABR	-2.13	CYYR1	-2.42	CX3CL1	-4.65	JHDM1D	-3.35	KCNS1	-4.50	PBX3	9.76	PCDH7	-3.22	SNX7	0.00	SPOCK2	-2.51
ACTL6A	3.10	ACCN2	-4.07	DCBLD2	-1.86	DAGLA	-3.21	KCNJ2	-3.15	KIAA0930	-3.95	PCDH15	0.00	PCSK6	-4.22	SPOPL	-2.12	ST3GAL1	-3.46
ACVR2B	-2.13	ACPP	-3.53	DCUN1D4	4.27	DDX6	-2.76	KIAA0247	-3.62	KIAA1045	-2.47	PCDHAC2	-2.55	PIGX	-2.60	SPTLC2	-3.75	ST3GAL2	-1.88
ADIPOR1	-2.28	ACSF3	-7.94	DDHD2	-2.56	DENND1A	-2.34	KIAA0317	-2.85	KIAA1143	-1.49	PCGF2	-1.50	PLEKHG4B	-3.37	SREK1IP1	-2.82	STX16	-3.66
AGAP1	-0.67	ACVRL1	-17.04	DDX18	-2.24	DIEXF	-2.99	KIAA1328	-3.64	KIAA1324	-3.59	PCGF5	-1.58	PLXNA4	-2.15	SRGAP1	-3.48	STYX	-3.59
AGFG1	6.34	ADAP1	2.22	DICER1	9.24	DNAJB14	-3.37	KIAA1467	-3.70	KIAA1755	-3.69	PCMTD1	-3.80	POTEM	-0.56	STARD13	-4.31	SUV39H1	-2.21
ALDH8A1	3.92	ADARB2	-2.06	DOK6	-1.95	DNAL1	-5.85	KIAA1804	-2.60	KLHL14	-3.09	PCSK2	0.62	PPARD	-15.59	SYNJ1	-1.86	SYNGAP1	-2.59
ANO1	3.99	ADD1	-5.41	DR1	1.81	DPYSL5	-1.86	KIAA1826	-1.32	KLHL5	-9.76	PDK4	-2.47	PPP1R16B	-11.54	TBC1D15	-2.81	SZT2	-1.95
ANO3	-3.52	ADRB3	-3.10	DSCC1	0.17	DYNC1I1	-2.88	KIF5C	-2.98	KLK4	0.02	PDLIM5	0.00	PRAF2	-3.02	TCEA1	0.77	TAGLN	-1.08
AP1S2	-2.23	AGL	-12.41	DTD1	2.89	EEFSEC	-2.88	KITLG	11.91	KSR2	-2.82	PFKM	-2.23	PSAP	-6.49	TGFB2	4.29	TLN2	2.21
AP1S3	-2.55	AKNA	-3.51	E2F7	-2.03	EIF4H	-2.11	KLF5	-0.48	LMF1	-4.07	PHC3	-2.70	PSAPL1	-0.64	THUMPD3	-1.53	TM2D2	-2.19
AR	-1.23	ANTXR2	-3.09	EIF2C4	-3.05	EMG1	-0.15	KLLN	-3.28	LMLN	-15.44	PHYHIPL	-2.27	PSEN1	-0.01	TIAM1	-2.37	TMC7	-2.10
ARFRP1	-2.22	ARHGAP1	-3.18	EIF4E	-0.99	ENSA	-9.51	LARP1	-2.02	LPHN1	-4.65	PLA2R1	2.75	PSMA5	-2.09	TMEM120B	-1.44	TMEM123	-1.94
ARL8B	-2.06	ARHGDIA	1.01	ENAH	-0.97	ERBB4	-3.23	LECT1	2.35	LRRIQ3	-1.89	PLIN5	-2.94	PTCD3	-1.83	TMPRSS15	-4.31	TMEM184A	-3.00
ARMC1	-2.16	ARL2BP	-2.02	ENPEP	-5.26	ERC1	-4.82	LEP	-0.65	LYNX1	-3.72	PNN	-0.14	PTGIS	-2.09	TNC	0.68	TOB2	-3.27
ATF6	-2.70	ARRB1	-7.15	ENTPD1	3.32	ERLIN2	-5.69	LHFP	0.59	LZTS1	-0.96	PODXL	3.06	PTPRT	-3.72	TNKS2	-3.11	TOM1L2	-3.25
ATL2	-1.83	ASCC3	-2.88	ERMN	-2.32	EVC	-1.90	LRP6	-2.78	MARVELD1	-4.25	POLI	-3.04	RAB11A	-2.80	TNPO1	-0.49	TP53I11	-5.87
AXIN2	0.91	ATF1	-1.55	ESRRG	1.79	FAM45A	-3.53	LRRTM1	-2.95	MCCC2	1.60	POU2F1	-2.36	RAB6B	-2.38	TNRC6B	7.72	TP73	-4.05
BCAP29	9.80	ATOH8	-1.54	FAM107B	-1.42	FAM53C	-5.19	LYVE1	-3.31	MECP2	-2.29	PPM1A	7.87	RAP1GAP2	-3.40	TRIM25	-2.58	TRMT11	-2.30
BCL10	-2.27	ATP10B	-2.72	FAM115C	-2.74	FANCF	-2.73	MAGI1	8.08	MEN1	-9.94	PPP1R2	-2.51	RASA1	-3.09	TSPAN6	-2.48	TSPAN18	-5.81
BICD2	-1.96	ATP6V0A2	-3.93	FAM176A	-0.20	FBLIM1	-5.48	MAGT1	-2.31	MFNG	-4.16	PPP6R2	-1.74	RASGEF1A	-1.92	TTC8	8.99	TSPAN9	-12.31
BNIP3L	-1.92	BCAM	-2.67	FAM91A1	-3.11	FBXO11	-16.52	MAP3K11	-2.31	MGLL	-13.80	PRDM1	2.09	REEP6	-2.35	UBE3C	-2.31	TTYH2	-3.27
BTLA	8.01	BEND4	-13.30	FBN1	-1.59	FBXO41	-2.11	MAPK1	-0.19	MGRN1	-4.83	PRDM6	-3.04	RFTN2	-4.05	VGLL3	-1.54	TTYH3	-2.58
C10orf47	-5.77	BEND6	-3.86	FBXW2	2.89	FOSL2	2.71	MARCH6	-1.68	MLKL	-9.05	PRPF40A	-2.80	RNFT2	-1.33	VPS26A	-1.52	TUSC5	-4.14
C11orf9	-4.07	BNIPL	-1.82	FGF2	-1.56	FOXK1	-2.88	MBLAC2	-3.82	MLLT1	-1.59	PRTG	-3.31	RPL36A	-2.43	WDR1	-0.22	TYRO3	-1.00
C1orf21	-3.68	BRWD1	-4.44	FGFRL1	0.52	FUT3	-17.70	MCTP2	6.00	MLX	-2.77	PTCHD3	0.48	RRP7A	-2.40	WNK3	-1.38	UBR2	-3.12
C21orf91	3.30	BTBD9	-11.33	FIGN	-3.29	FUT6	-6.82	MDGA2	0.00	MPPED2	-15.66	PTPLB	-2.72	RTKN2	-2.91	YAF2	0.00	USP30	-1.55
C9orf150	-3.63	C17orf103	-3.81	FLRT3	7.51	FXN	-4.21	MED26	-1.92	MRPS25	-3.34	QTRTD1	-2.45	RXRA	-1.74	YOD1	-2.67	VAMP1	-4.00
CALB2	1.20	C19orf54	-1.31	FOXM1	-2.77	GBP4	-2.58	MTMR12	-2.83	MS4A2	-1.83	RAB14	-3.49	SALL1	-2.87	ZAK	-2.46	VAT1	-3.28
CAPN2	-1.13	C1orf144	-1.95	FSTL1	-0.34	GFER	-1.73	MTMR6	-2.54	MSR1	-1.36	RAB3B	-1.96	SARM1	-3.32	ZBTB41	-2.23	VSX2	0.93
CAPZA1	-1.54	C1orf95	-3.02	GABARAPL	-1.80	GIPC3	-2.32	MYBL1	-1.66	MUT	-1.94	RAB3IP	-0.03	SCN3A	-1.07	ZCCHC2	-2.94	WDR48	-1.49
CASK	1.28	C2orf71	-3.90	GABRA1	5.87	GK5	-3.91	N4BP1	-4.01	MYOT	-0.46	RALGAPA1	-2.24	SDC3	-3.70	ZFPM2	-1.67	WDTC1	-3.07
CCDC50	-2.56	C6orf168	-2.60	GABRB2	2.20	GLRB	-5.07	NAA16	4.65	N4BP3	-2.46	RBM24	6.53	SEMA6A	-1.04	ZFR2	2.09	WWP1	-3.20
CCNJ	0.00	CACNA1E	-9.51	GALNT3	-1.91	GNAI2	-2.77	NAA20	0.00	NAA60	-4.13	RETSAT	-2.97	SEPHS1	-8.68	ZHX1	4.43	YY1AP1	-5.39
CD109	-4.01	CACNG8	-4.28	GGPS1	2.01	GPBP1L1	-2.02	NDEL1	-2.61	NAT8L	-1.89	RIC3	0.00	SEPN1	-3.35	ZMYM2	4.38	ZBTB8A	-2.91
CD80	-5.30	CC2D1B	-2.03	GNPDA2	-2.74	GPR161	-2.48	NEDD9	1.72	NCOR1	-3.18	RLIM	-3.48	SEPT6	-4.66	ZNF20	5.91	ZC3H7B	-0.82
CDK17	-1.35	CD84	-16.90	GNPNAT1	-2.56	GPRIN1	-1.14	NEIL1	-2.83	NCS1	-5.61	RNASEL	-2.97	SGOL1	-5.84	ZNF292	-1.71	ZCCHC24	-3.54
CDK6	-0.88	CFLAR	-4.27	GOLPH3	-2.67	GRIK3	-3.72	NF1	3.11	NDOR1	-6.54	SAMD5	-1.83	SH3BP2	-3.10	ZNF440	-3.70	ZDHHC8	-2.88
CELSR1	-1.63	CHRNA4	-4.36	GON4L	-1.58	GSG1L	4.43	NID1	-3.28	NFIC	-6.68	SGMS2	-6.32	SHROOM3	-1.83	ZNF586	2.40	ZFP41	-2.24
CHIC1	-1.20	CHST6	-3.81	GTPBP3	5.69	H3F3B	-2.12	NOVA1	-1.93	NFIX	-1.97	SHROOM4	-3.90	SKIL	-5.97	ZNF664	11.53	ZFP90	3.49
CHMP4C	-3.53	CLCC1	-1.97	HERPUD1	0.80	HAS2	-1.43	NSL1	12.14	NKIRAS2	-9.43	SIRT1	0.00	SLC16A7	-2.99	ZNF704	10.10	ZMIZ2	-3.10
CLOCK	-1.39	CLDN19	1.16	HIC2	-2.79	HCN1	-5.18	NTNG1	3.26	NPTXR	2.39	SLC12A2	-2.55	SLC22A15	-4.73	ZNF763	-3.40	ZNF155	-14.98
CNOT6L	-2.78	CLIP3	-8.90	HIPK2	-2.58	HOOK3	-2.50	NUS1	-1.45	ODZ3	0.10	SLC20A2	1.51	SLC28A3	-4.58	ZNF776	-3.16	ZNF267	-0.64
COL4A3BP	1.08	CMTM3	-4.34	HS6ST1	-0.74	HPSE2	-7.70	NXT2	-3.71	OLFML2A	-0.55	SLC35B3	0.60	SLC35E1	-2.30	ZNF814	-1.84	ZNF320	-3.36
CPEB3	4.30	CPLX2	-8.74	HSPA12A	1.29	IBA57	-2.28	ONECUT1	-3.16	OPRM1	-1.80	SLC4A1	-3.81	SLC35E2	-2.40			ZNF37A	-3.47
CRIM1	-1.52	CPNE5	-4.12	IFI44	-2.45	IFNAR1	-3.12	ONECUT2	-3.02	OSBP2	0.42	SLC4A8	-3.31	SLC45A2	-6.26			ZNF445	-2.63
CSDA	-1.30	CREBL2	-2.59	IPMK	-1.99	IL17REL	-1.25	OSBPL3	-0.55	PACS1	-2.70	SLC50A1	10.51	SLC46A1	-4.16			ZNF518B	-1.43
CTNNA1	-2.49	CRP	-1.06	ITGB1	3.20	INSIG2	-3.18	OTUD6B	-2.91	PACSIN1	-4.09	SNAPC3	3.90	SLC6A9	-10.29			ZNF594	-2.02
CUL3	-1.25	CRTC1	-4.40	ITM2B	-2.66	IRGQ	-2.87	PABPC5	-1.19	PANK1	-4.11	SNRK	-5.47	SLX4	-2.06			ZZZ3	-2.74

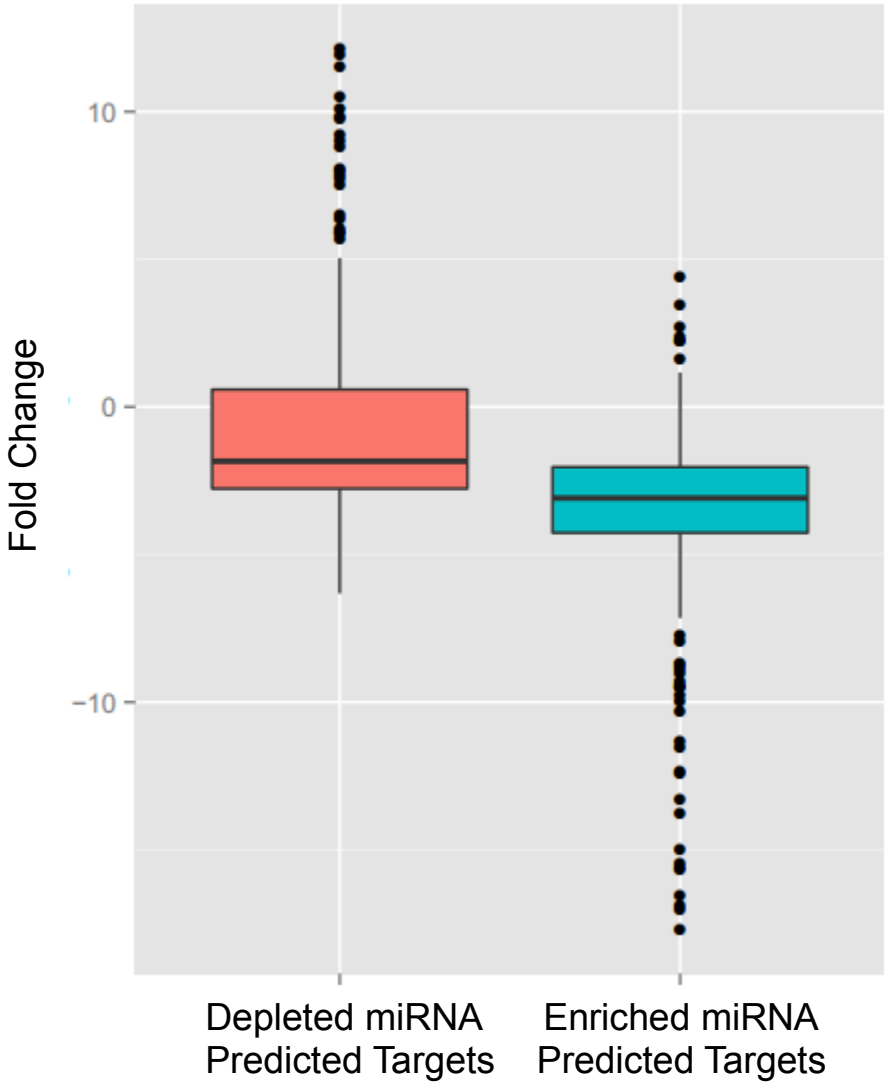

Figure 34. Predicted gene targets of exosome enriched miRNA are down-regulated after treatment of GSCs with GA-MSC-derived exosomes. Change in expression level for predicted targets of exosome enriched miRNA, versus that of exosome depleted miRNA, demonstrating a significant difference ($p < 0.001$, Wilcoxon test) after treatment of GSCs with GA-MSC-derived exosomes for 48 hours. The average and median fold change of predicted targets for exosome depleted miRNA was -0.53 and -1.84, respectively. The average and median fold change of predicted targets for exosome enriched miRNA was -3.79 and -3.09, respectively.

MicroRNA in GA-MSC-Derived Exosomes Increase GSC Proliferation

To determine the direct influence of the GA-MSC-derived exosome enriched miRNA on GSC proliferation, I evaluated effects on proliferation after the over-expression of these miRNA in GSCs. Specifically, I utilized lentiviral transduction to over-express each of the 7 enriched exosomal miRNAs that had predicted gene targets in GSC 262 and GSC 20. Over-expression of miRNA was verified by qRT-PCR analysis of RNA isolated from lentiviral transduced GSCs (**Figure 35**). Importantly, increases in expression of the 7 enriched exosomal miRNA were also seen in qRT-PCR analysis of GSCs after treatment with GA-MSC-derived exosomes (**Figure 35**). Of the 7 miRNAs evaluated, only the over-expression of miR-1587 and miR-3620-5p produced significant increases ($p < 0.05$) in proliferation of GSCs, which were similar to that of GSCs after treatment with GA-MSC-derived exosomes (**Figure 36**). GSC proliferation was also increased with the transduction of miR-1246, however the affect was not significant ($p > 0.05$). Interestingly, GSC proliferation was also increased after the transduction of the LV-GFP-miR-scramble construct, however not to the degree of GSCs after treatment with GA-MSC-derived exosomes. These results indicate that both miR-1587 and miR-3620-5p are capable of increasing GSC proliferation.

MicroRNA in GA-MSC-Derived Exosomes Increase GSC Clonogenicity

To determine the direct influence of the GA-MSC-derived enriched miRNA on GSC clonogenicity, I evaluated the effects on clonogenicity after over-expression of these miRNA in GSCs. Specifically, I utilized lentiviral transduction to over-express each of the 7 enriched exosomal miRNA that had predicted gene targets in GSC 262 and GSC 20. Of the 7 miRNAs evaluated, only the over-expression of miR-1587 produced a significant increase ($p < 0.05$) in clonogenicity of GSCs, which was similar to that of GSCs after treatment with GA-MSC-derived exosomes (**Figure 37**). GSC proliferation was also increased with the transduction of miR-3620-5p, however the affect was not significant ($p > 0.05$). Interestingly GSC clonogenicity was not affected after transduction with the LV-GFP-miR-scramble construct. These results indicate that only miR-1587 is capable of increasing GSC clonogenicity.

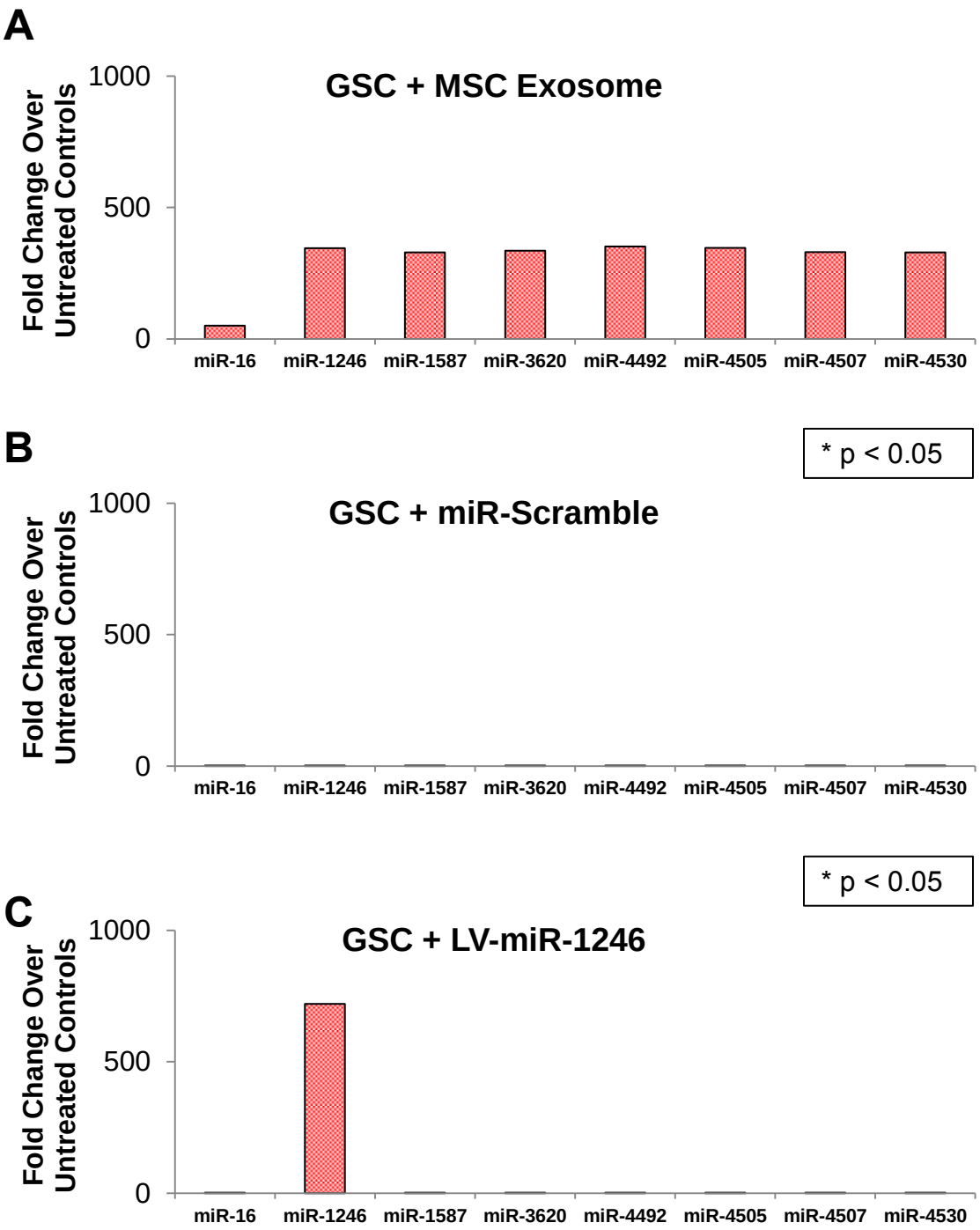

Figure 35. Lentiviral transduction of miRNA constructs increases expression in GSCs. (A) qRT-PCR data for GSC 262 demonstrating significant fold increases in all enriched exosomal miRNA after delivery of GA-MSC 262-derived exosomes. (B) qRT-PCR data for GSC 262 demonstrating no significant increases in enriched exosomal miRNA after transduction with the LV-miR-scramble construct. (C) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-1246 after transduction with the LV-miR-1246 construct.

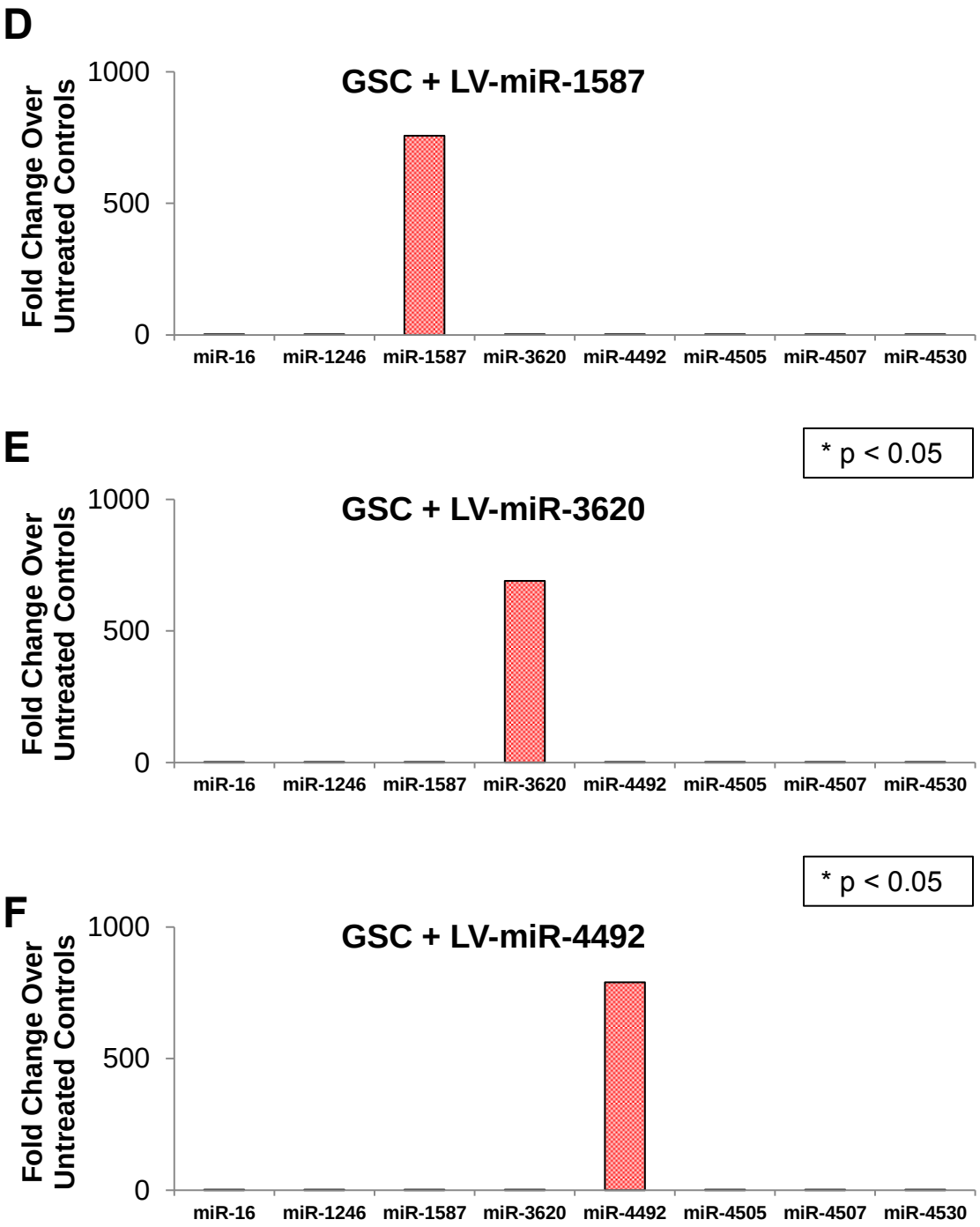

Figure 35. Lentiviral transduction of miRNA constructs increases expression in GSCs. (D) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-1587 after transduction with the LV-miR-1587 construct. (E) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-3620-5p after transduction with the LV-miR-3620-5p construct. (F) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-4492 after transduction with the LV-miR-4492 construct.

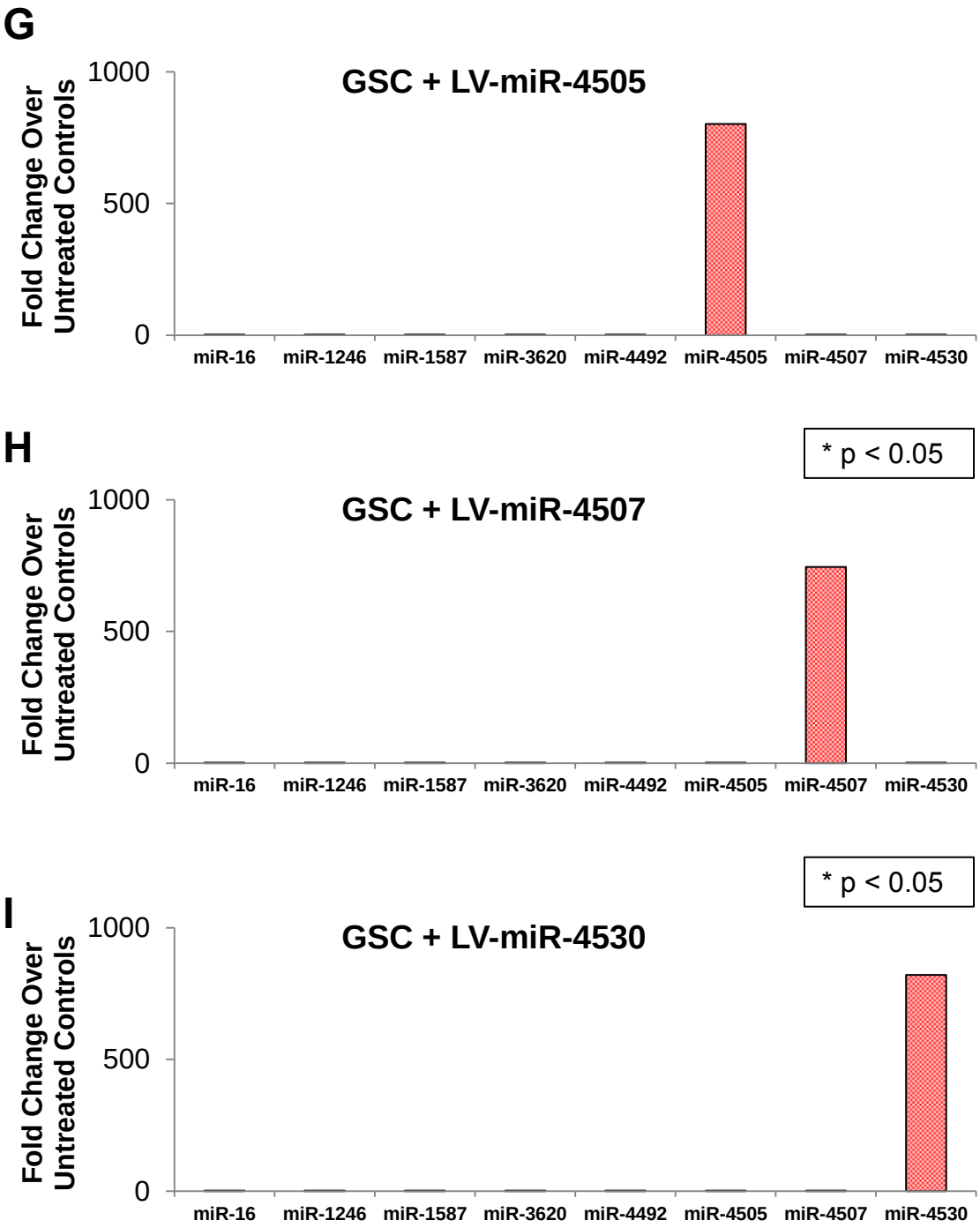

Figure 35. Lentiviral transduction of miRNA constructs increases expression in GSCs. (G) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-4505 after transduction with the LV-miR-4505 construct. (H) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-4507 after transduction with the LV-miR-4507 construct. (I) qRT-PCR data for GSC 262 demonstrating a significant increase in miR-4530 after transduction with the LV-miR-4530 construct.

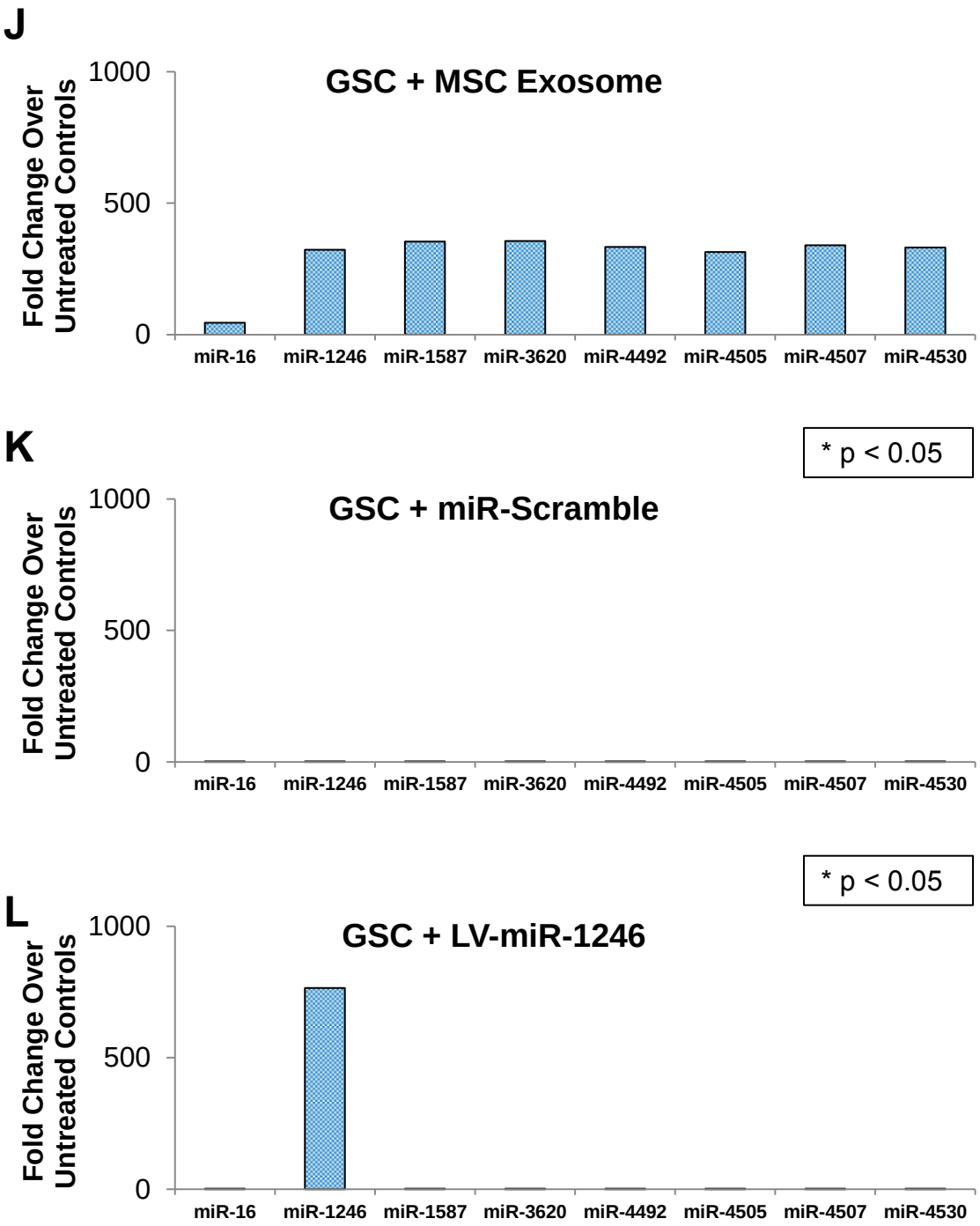

Figure 35. Lentiviral transduction of miRNA constructs increases expression in GSCs. (A) qRT-PCR data for GSC 20 demonstrating significant fold increases in all enriched exosomal miRNA after delivery of GA-MSC 262-derived exosomes. (B) qRT-PCR data for GSC 20 demonstrating no significant increases in enriched exosomal miRNA after transduction with the LV-miR-scramble construct. (C) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-1246 after transduction with the LV-miR-1246 construct.

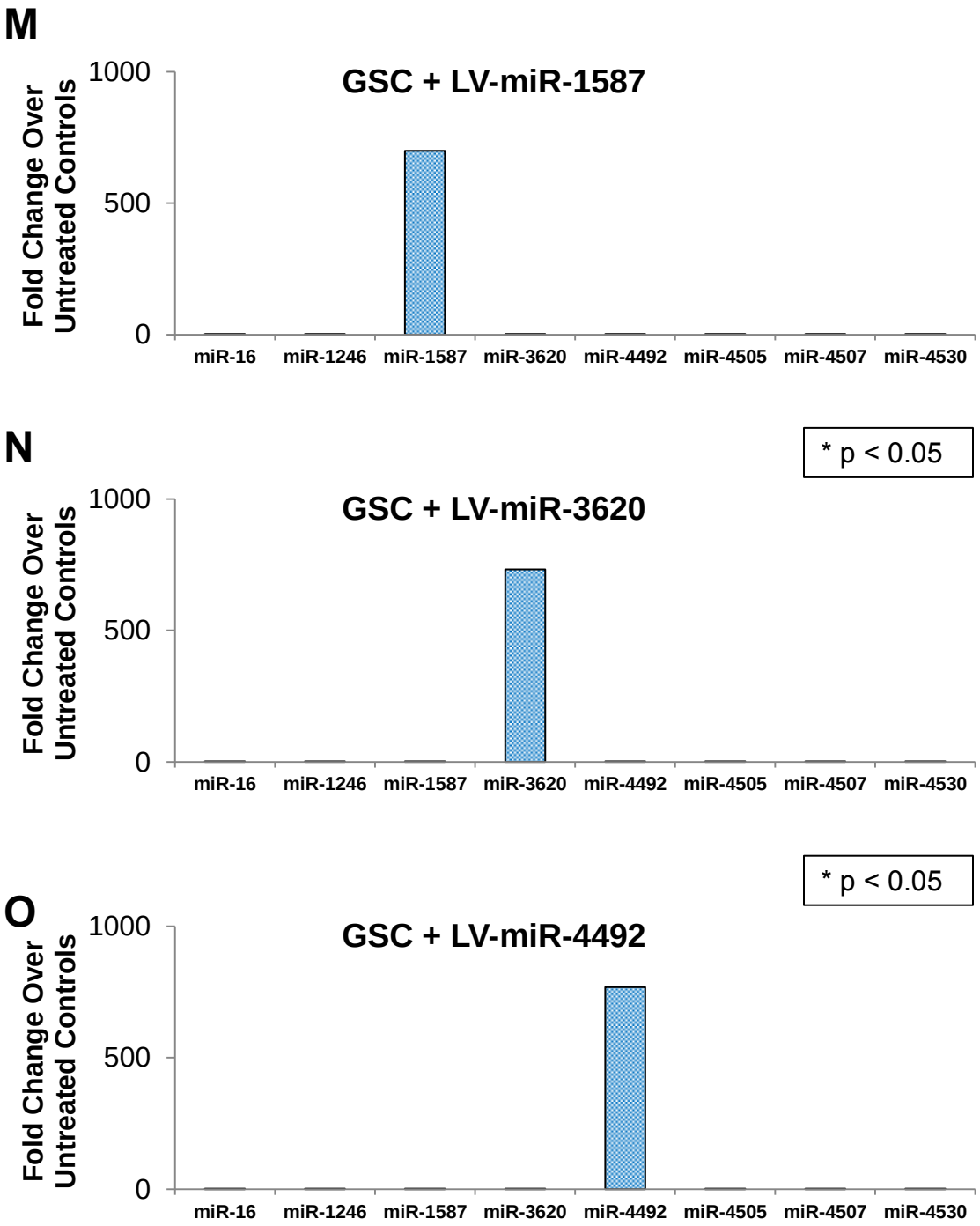

Figure 35. Lentiviral transduction of miRNA constructs increases expression in GSCs. (D) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-1587 after transduction with the LV-miR-1587 construct. (E) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-3620-5p after transduction with the LV-miR-3620-5p construct. (F) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-4492 after transduction with the LV-miR-4492 construct.

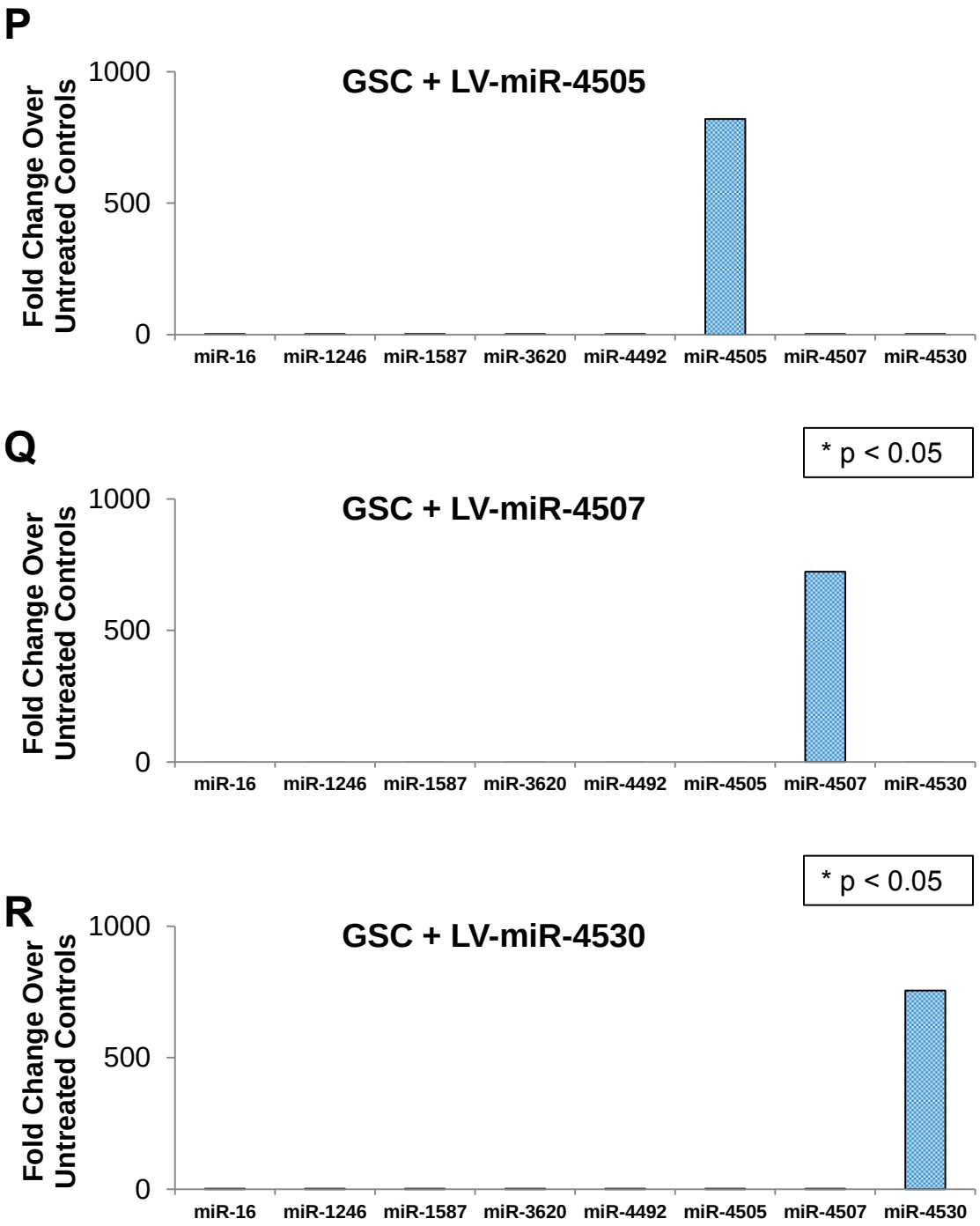

Figure 35. Lentiviral transduction of miRNA constructs increases expression in GSCs. (G) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-4505 after transduction with the LV-miR-4505 construct. (H) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-4507 after transduction with the LV-miR-4507 construct. (I) qRT-PCR data for GSC 20 demonstrating a significant increase in miR-4530 after transduction with the LV-miR-4530 construct.

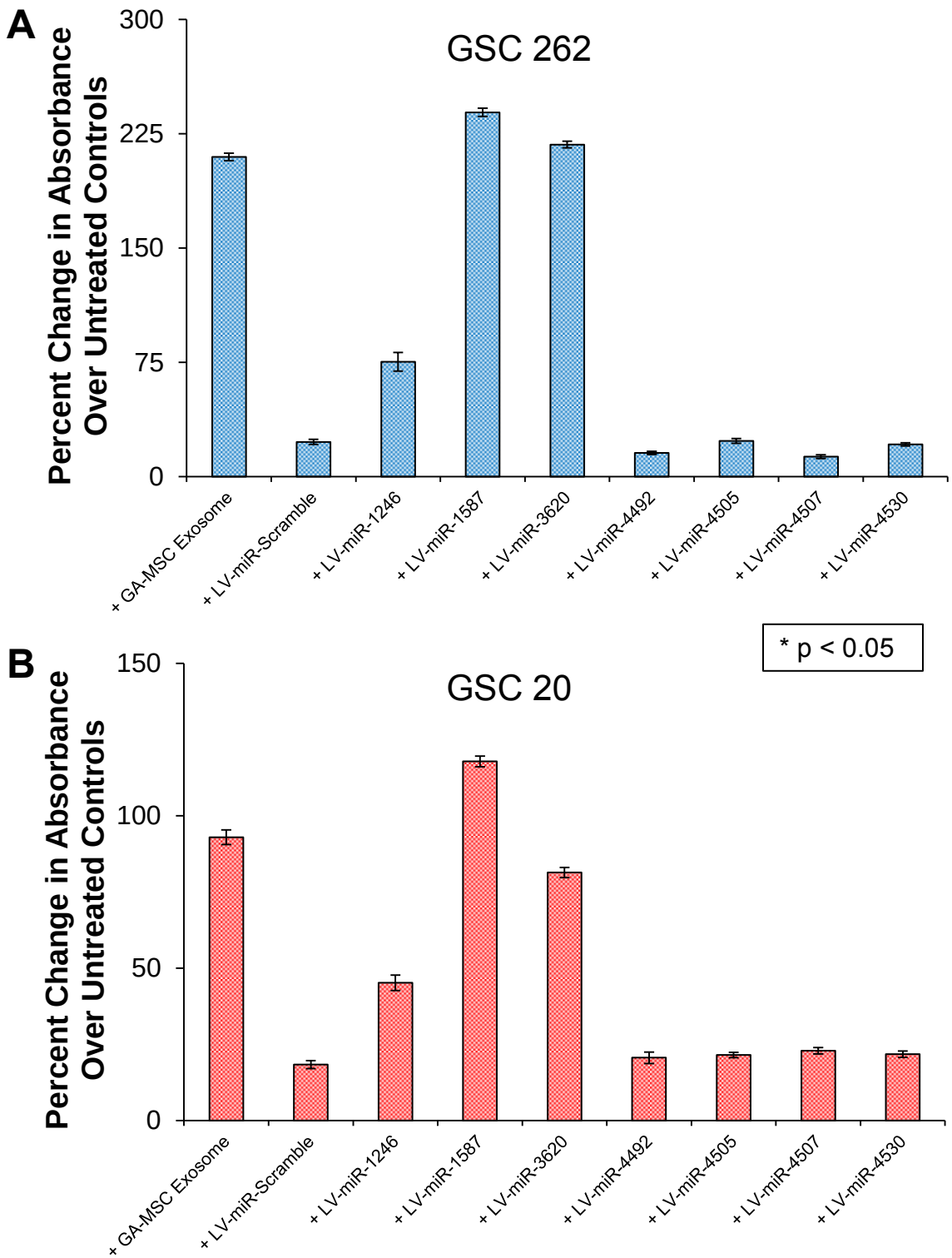

Figure 36. Over-expression of specific exosome enriched miRNA increases proliferation in GSCs. (A) Proliferation assay for GSC 262 over-expressing miRNA that are enriched in GA-MSC-derived exosomes, demonstrating a significant increase in GSC viability with the over-expression of miR-1587 and miR-3620-5p. As expected, treatment of GSC 262 with GA-MSC 262 derived exosomes, also significantly increased viability. (B) Proliferation assay for GSC 20 over-expressing miRNA that are enriched in GA-MSC-derived exosomes, demonstrating a significant increase in GSC viability with the over-expression of miR-1587 and miR-3620-5p. As expected, treatment of GSC 20 with GA-MSC 20 derived exosomes, also significantly increased viability.

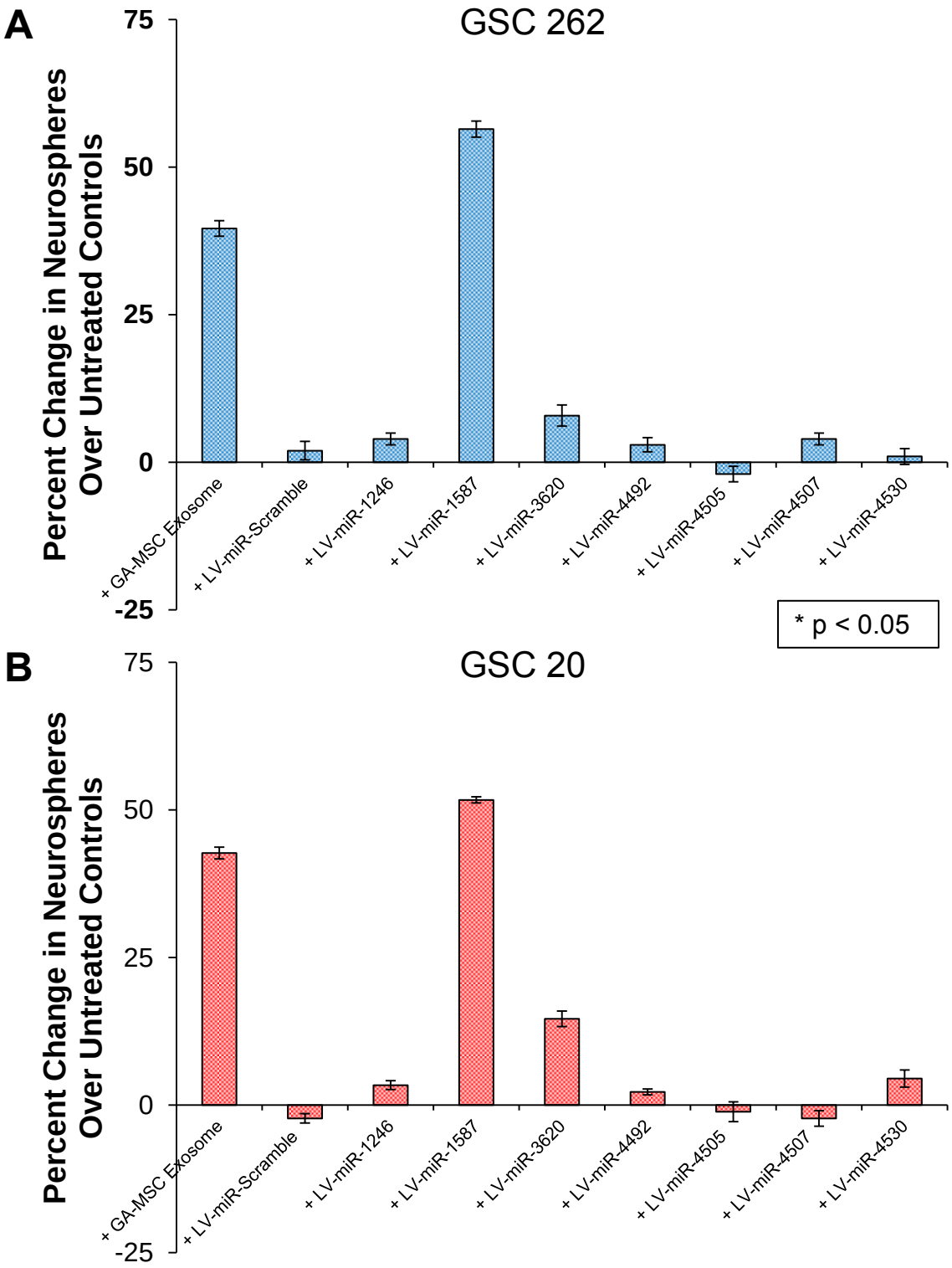

Figure 37. Over-expression of miR-1587 in GSCs increases proliferation. (A) Proliferation assay for GSC 262 over-expressing miR-1587, demonstrating a significant increase in GSC viability similar to that of treatment with GA-MSC 262-derived exosomes. This increase in GSC viability was reversible with the addition of a miR-1587 inhibitor. (B) Proliferation assay for GSC 20 over-expressing miR-1587, demonstrating a significant increase in GSC viability similar to that of treatment with GA-MSC 20-derived exosomes. This increase in GSC viability was reversible with the addition of a miR-1587 inhibitor. .

MicroRNA-1587 in GA-MSC-Derived Exosomes Increases GSC Proliferation

To evaluate a causal role of miR-1587 in the proliferation effects produced by GA-MSC-derived exosomes on GSCs, I conducted further proliferation assays aimed at inhibiting miR-1587. Specifically, I treated GSCs with GA-MSC-derived exosomes and anti-miR-1587 by lipofection, and compared results to GSCs treated with GA-MSC-derived exosomes. As expected, the treatment of GSC with GA-MSC-derived exosomes significantly increased proliferation, and was able to be partially reversed with the addition of anti-miRNA-1587 (**Figure 38**). Importantly, GSC proliferation was not affect by treatment with anti-miRNA alone. This indicates that miR-1587 contained within GA-MSC-derived exosomes has a causal role in increasing the proliferation of GSCs.

MicroRNA-1587 in GA-MSC-Derived Exosomes Increases GSC Clonogenicity

To evaluate a causal role of miR-1587 in the self-renewal effects produced by GA-MSC-derived exosomes on GSCs, I conducted further clonogenicity assays aimed at inhibiting miR-1587. Specifically, I treated GSCs with GA-MSC-derived exosomes and anti-miR-1587 by lipofection, and compared results to GSCs treated with GA-MSC-derived exosomes. As expected, the treatment of GSC with GA-MSC-derived exosomes significantly increased clonogenicity, and was able to be partially reversed with the addition of anti-miRNA-1587 (**Figure 39**). Importantly, GSC clonogenicity was not affect by treatment with anti-miRNA alone. This indicates that miR-1587 contained within GA-MSC-derived exosomes has a causal role in increasing the clonogenicity of GSCs.

Verification of miR-1587 Functionality in GSCs

Given that miR-1587 most significantly increased proliferation and clonogenicity in GSCs when compared to the other six highly expressed and highly enriched exosomal miRNAs, I assessed its functionality by directly analyzing one of its gene targets. Specifically, I focused on a major predicted target of miR-1587, the nuclear hormone receptor co-repressor-1 (NCOR1), which had a predicted targeting score of 90 (miRTarget 2.0 database).[198] I evaluated the expression of NCOR1 in

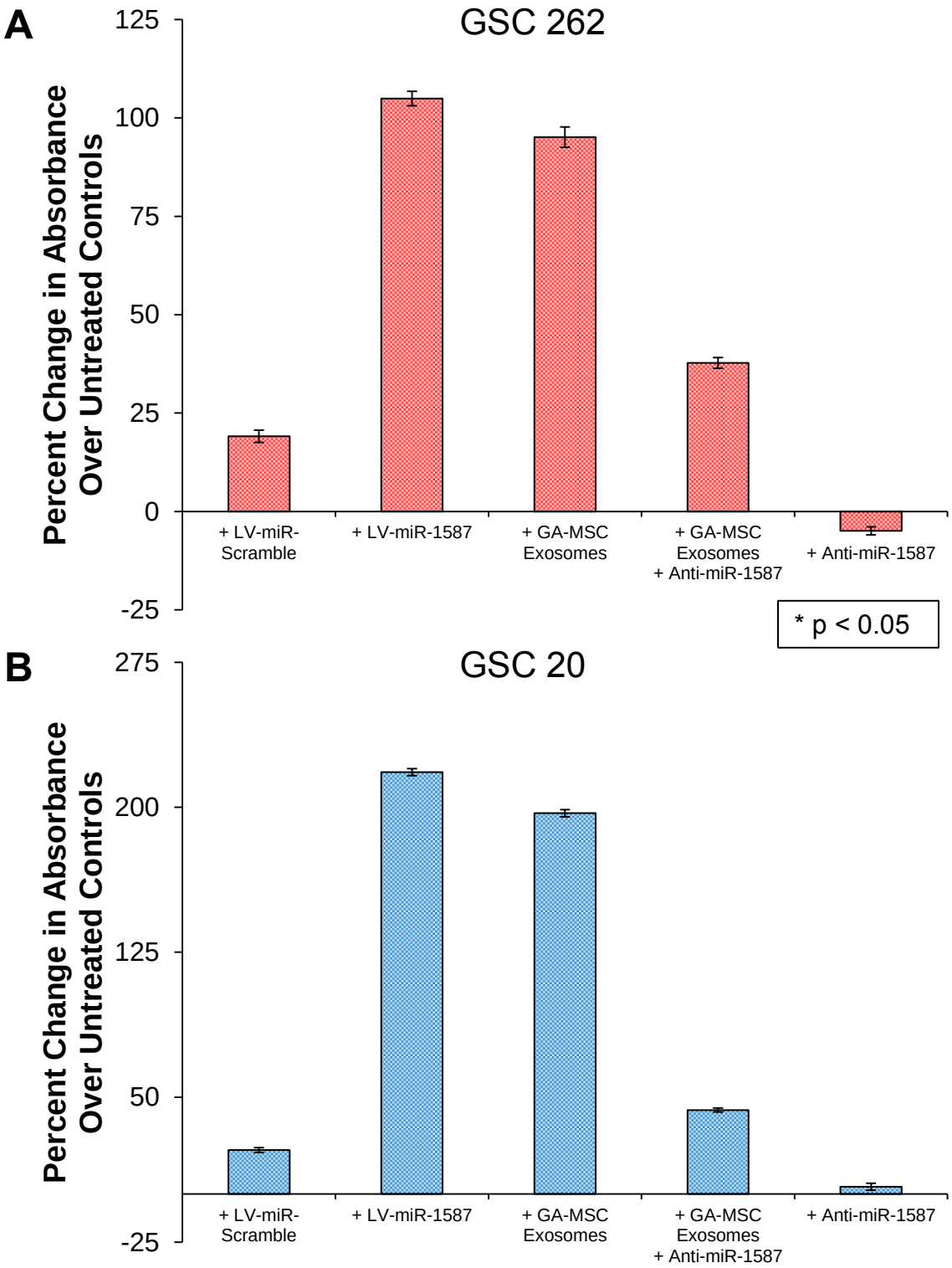

Figure 38. Over-expression of specific exosome enriched miRNA in GSCs increases clonogenicity. (A) Clonogenic assay for GSC 262 over-expressing miRNA that are enriched in GA-MSC-derived exosomes, demonstrating a significant increase in GSC clonogenicity with the over-expression of miR-1587. As expected, treatment of GSC 262 with GA-MSC 262 derived exosomes, also significantly increased clonogenicity. (B) Clonogenic assay for GSC 20 over-expressing miRNA that are enriched in GA-MSC-derived exosomes, demonstrating a significant increase in GSC clonogenicity with the over-expression of miR-1587. As expected, treatment of GSC 20 with GA-MSC 20 derived exosomes, also significantly increased clonogenicity.

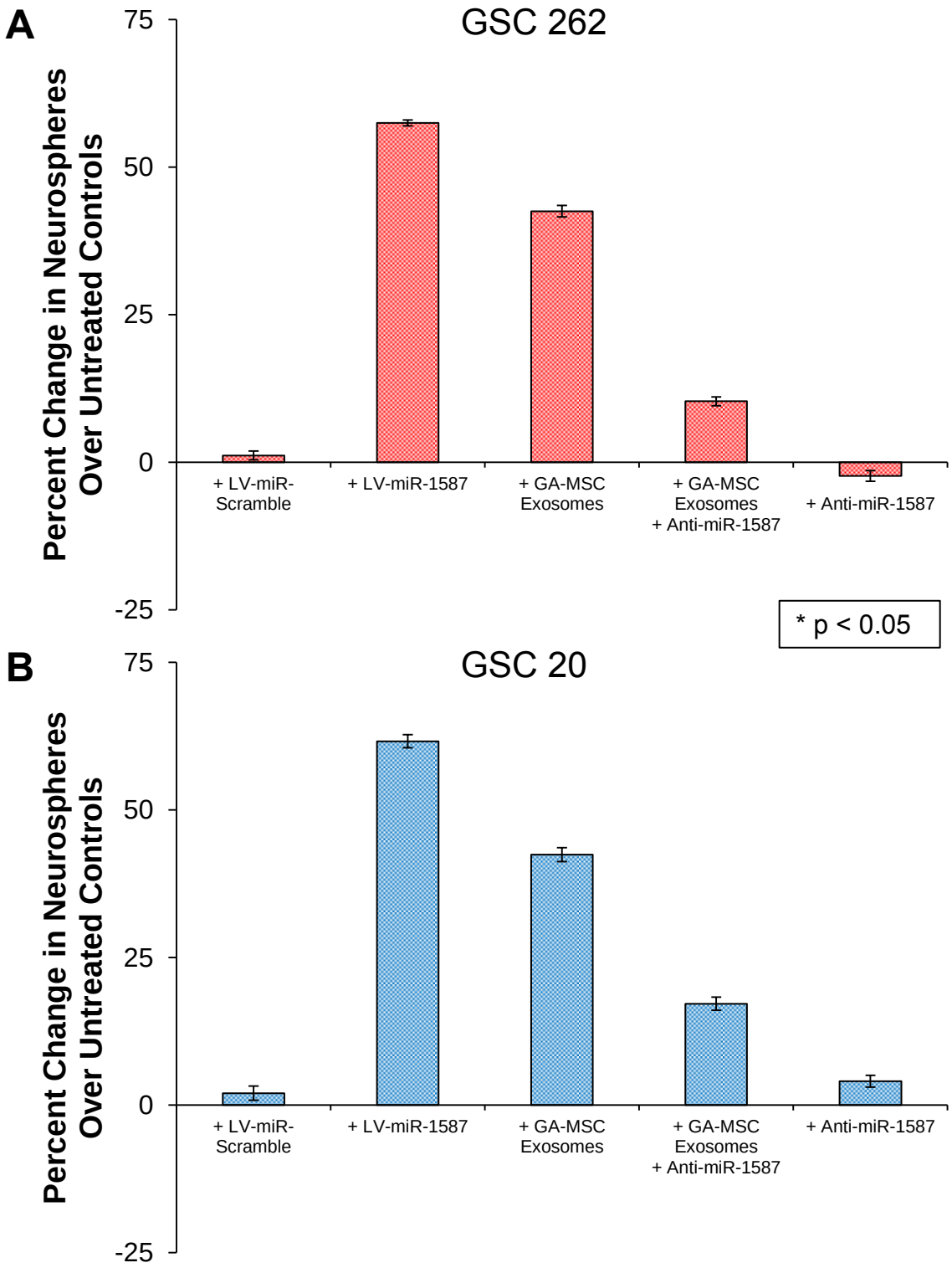

Figure 39. Over-expression of miR-1587 in GSCs increases clonogenicity. (A) Clonogenic assay for GSC 262 over-expressing miR-1587, demonstrating a significant increase in GSC clonogenicity similar to that of treatment with GA-MSC 262-derived exosomes. This increase in GSC clonogenicity was reversible with the addition of a miR-1587 inhibitor. (B) Clonogenic assay for GSC 20 over-expressing miR-1587, demonstrating a significant increase in GSC clonogenicity similar to that of treatment with GA-MSC 20-derived exosomes. This increase in GSC clonogenicity was reversible with the addition of a miR-1587 inhibitor. .

untreated GSC controls, GSCs over-expressing GFP-miR-1587, GSCs treated with GA-MSC-derived exosomes, and GSCs treated with GA-MSC-derived exosomes plus anti-miR-1587. As expected, over-expression of miR-1587 in GSCs, and treatment of GSCs with GA-MSC-derived exosomes, decreased the expression of NCOR1, when compared to untreated GSC controls (**Figure 40**). Most importantly, treatment of GSCs with GA-MSC-derived exosomes plus anti-mir-1587 inhibitor partially reversed the negative regulation of NCOR1. These results indicate that regulation of NCOR1 in GSCs by miR-1587 in GA-MSC-derived exosomes, plays a crucial role in increasing the proliferation and clonogenicity in GSCs.

MicroRNA in GA-MSC-Derived Exosomes Increase GSC Tumorigenicity

In order to evaluate whether miR-1587 could significantly increase the tumorigenicity of GSCs, I conducted *in vivo* experiments using GSC 20 over-expressing miR-1587. These experiments are currently in progress, and mice have been injected with GSC 20 in 6 groups (n=15/group): untreated GSC 20 controls, GSC 20 treated with GA-MSC-derived exosomes, GSC 20 treated with GA-MSC-derived exosomes plus anti-miR-1587 inhibitor, GSC 20 treated with anti-miR-1587 inhibitor alone, GSC 20 over-expressing miR-1587, and GSC 20 over-expressing miR-scramble. Of the 15 mice, 9 will be followed for survival and then euthanized, and 6 will be sacrificed for time point comparison. I expect similar results as those described from my *in vitro* experiments. GSC 20 over-expressing miR-1587 should increase tumor burden and decrease median survival when compared to untreated GSC 20 controls. Additionally, the increase in tumor burden and decrease in median survival should be similar to that of GSC 20 treated with GA-MSC-derived exosomes. Moreover, there should be a significant decrease in tumor burden and increase in median survival between GSC 20 treated with GA-MSC-derived exosomes plus anti-miR-1587. Lastly, there should not be a significant difference in tumor burden or median survival when comparing untreated GSC 20 controls to GSC 20 treated with anti-miR-1587, and to GSC 20 over-expressing miR-scramble. Demonstration of these results would indicate that miR-1587 in GA-MSC-derived exosomes has a causal role in increasing the tumorigenicity of GSCs.

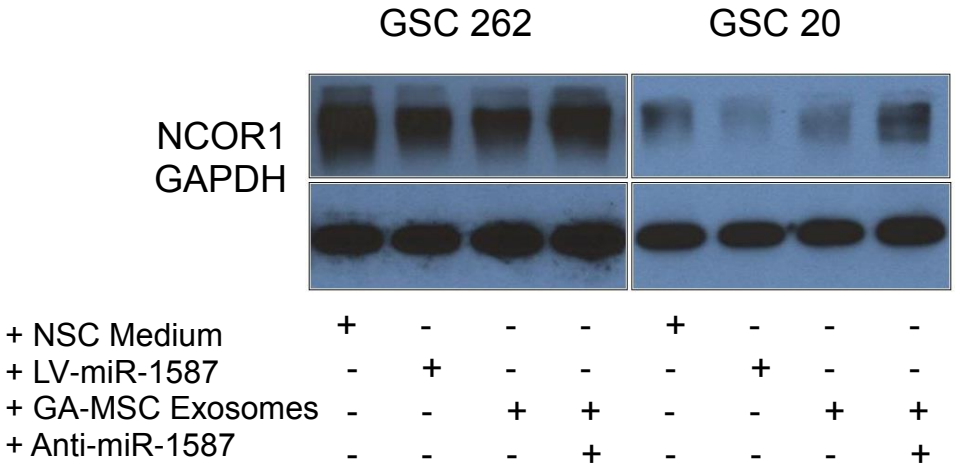

Figure 40. NCOR1 is down-regulated in GSCs over-expressing miR-1587. Western blot analysis of the functionality of miR-1587 in GSC, demonstrating a baseline expression of the predicted miR-1587 target, the nuclear receptor co-repressor-1 (NCOR1) gene, in GSC controls. Expression of NCOR1 is down-regulated with GSCs over-expressing miR-1587, which is similar to that of treatment with GA-MSC-derived exosomes. This down-regulation of NCOR1 expression is reversible with the addition of a miR-1587 inhibitor.

4.3 Discussion

In this chapter I show for the first time that GA-MSC-derived exosomes contain highly expressed and highly enriched miRNA, which share conserved nucleotide sequences. Furthermore, global analysis of the data from gene expression profiling showed that the expression of predicted gene targets of these miRNA was down-regulated in GSCs after treatment with GA-MSC-derived exosomes. Of the highly expressed and highly enriched exosomal miRNA, only miR-1587 was able to increase both GSC proliferation and clonogenicity in *in vitro* experiments. Importantly, increased levels of miR-1587 in GSC correlated with the down-regulation of NCOR1, a major predicted gene target of miR-1587.

The packaging of specific RNA species in exosomes is well established, and is now recognized as an ordered, non-random, process. With respect to MSCs, the miRNA profiles of secreted exosomes are known to be significantly different from that of the parental cell.[199-200] This difference includes certain miRNA that are either enriched or depleted in MSC-derived exosomes.[201] These studies suggest a normal physiologic role for the process of miRNA trafficking and packaging into exosomes. With regards to cancer, RNA that are preferentially packaged and enriched in exosomes are being examined as diagnostic modalites. Exosomes derived from cancer cells, termed *oncosomes*, are viewed as an extension of the tumor itself, and the specific RNA profiles are being investigated in order to screen for and diagnose many different types of cancer, including prostate, bladder, kidney, liver, colorectal, ovarian, lung, breast, leukemia, melanoma and even brain cancers.[81,202-210] Together, these MSC and cancer studies suggest that the enrichment of RNA in exosomes is a process that not only occurs under normal physiologic conditions, but also in the pathologic processes of cancer.

The recognition of specific nucleotide sequences in normal physiologic cell biology is well described. For instance, in the process of transcribing a gene, transcription factors recognize and bind to certain core promoter regions on the DNA strand upstream of the gene, such as the TATA-box sequence of 4-nucleotides.[211] This recognition and binding is essential for subsequent binding of RNA polymerase

and initiation of gene transcription.[211] Likewise, specific 3-nucleotide-long sequences in mRNA transcripts (codons) are then recognized and bound by complementary sequences of tRNA carrying specific amino acids.[212] This recognition is a key step in the ribosomal translation of mRNA into a protein gene product.[212] Thus, gene expression is reliant upon effective recognition of specific nucleotide sequences. Therefore, it is reasonable that nucleotide sequences on miRNA are recognized and bound by proteins responsible for the trafficking of RNA into exosomes.

The process by which RNA is packaged within exosomes is not well established, however there is evidence demonstrating that specific nucleotide sequences may provide recognition sites for directing specific RNA species to exosomes. Specifically, Batagov, et al., in the study of fibroblast-derived exosomes, was the first to demonstrate that the presence of three different 8-nucleotide sequences (ACCAGCCU, CAGUGAGC, and UAAUCCCA), present in mRNA and pre-miRNA, directed enrichment of these RNA transcripts into exosomes.[213] These nucleotide motifs were discovered by computational analysis utilizing cluster algorithms to identify nucleotide commonalities. Since this initial study, Columba-Cabezas and colleagues demonstrated that the Gag p17 matrix protein of HIV-1 contained a 117-nucleotide region that was essential for packing of unspliced HIV-1 mRNA into exosomes derived from infected cells.[214] Without this nucleotide motif, HIV-1 mRNA was not incorporated into exosomes, and instead was diverted into viral partcles.[214] This study highlights the role of a long nucleotide sequence that can be recognized as a region for directing mRNA into exosomes. With respect to gliomas, Bolukbasi and colleagues, recently demonstrated that a 25-nucleotide sequence, with a smaller CTGCC core sequence, that was present in mRNA that were highly enriched in exosomes derived from 2 glioblastoma cell lines.[71] Fusion of this nucleotide motif to non-exosomal mRNA resulted in preferential packaging into exosomes. Interestingly, enriched mRNA also contained a binding site for miR-1289 in their 3'-UTR, which served as a co-enrichment factor once bound.[71] This study further supports the theory of nucleotide pattern recognition for trafficking of RNA species into exosomes. With regard to exosomal packaging proteins, Villarroya-Beltri, et al., demonstrated the binding of SUMO (small ubiquitin-like modifier) -

heterogeneous nuclear ribonucleoprotein A2B1 (SUMO-hnRNPA2B1) to specific nucleotide motifs on miRNAs.[25] Specifically, SUMO-hnRNPA2B1 recognized and bound the 4-nucleotide sequence of GGAG on miRNA. Although, SUMOlyation is ubiquitin-like, it does not result in protein degradation. Instead SUMO-hnRNPA2B1 is targeted to exosomes, and consequently enriches miRNA that is bound to it.[25] This study is the only one to describe the protein-mediated trafficking of miRNA into exosomes. Interestingly, the GGAG miRNA motif is similar to one of the 4-nucleotide sequences identified in my analysis of highly expressed and highly enriched miRNAs in MSC-derived exosomes (GGAC). Therefore it is reasonable that specific nucleotide sequences of miRNA, combined with GC:UA enrichment, can direct the preferential packaging of miRNA into MSC-derived exosomes.

The capability of exosomal miRNA to regulate gene expression in recipient cells has been established in various tissues. With regards to the brain, this process has been described in both physiologic and pathologic conditions. Specifically, Morel, et al., demonstrated that exosomes derived from neurons can affect the expression of amino acid transporters in astrocytes.[215] The delivery of miR-124a via neuron-derived exosomes, increased the expression of glutamate transporter-1 (GLT1) in recipient astrocytes. Furthermore, the disruption of this communication pathway is linked to key changes that occur during the progression of amyotrophic lateral sclerosis (ALS).[215] More relevant to my work, Li at colleagues, demonstrated that exosomes derived from the U251 glioblastoma cell line are able to alter the transcriptome of recipient human brain microvascular endothelial cells (hBMVECs).[72] The communication of exosomal miRNA significantly down-regulated the expression of 19 genes involved in the maintenance of the blood-brain barrier.[72] Together, these two studies indicate that cells can utilize exosome-mediated regulation of gene expression to modify the surrounding microenvironment.

Similar to the designation of genes that inhibits malignant growth as a tumor-suppressors, there are miRNAs that also inhibit malignant growth and are termed tumor-suppressor-miRNAs.[216] Specifically, the group of miRNA that were highly expressed and highly enriched in GA-MSCs, and thus depleted from GA-MSC-

derived exosomes, consists of miRNAs that have been shown to have tumor-suppressor capabilities (**Table 7**). For example, Tan, et al., demonstrated that miR-9 inhibits the proliferation of gliomas cells through the down-regulation of the pro-proliferative cyclic-AMP element binding protein (CREB).[217] Additionally, Moncini and colleagues, demonstrate that miR-103a has a negative effect on cell growth and migration through the down-regulation of cyclin dependent kinas-5R1 (CDK5R1).[218] Similarly, Chen, et al., described the glioma-suppressing role of miR-107, which was linked to the down-regulation of cyclin dependent kinase-2 (CDK2) and neurogenic locus notch homolog protein-2 (NOTCH2).[219] Furthermore, Gu and colleagues demonstrated that miR-199a functions as a tumor-suppressor in gliomas by down-regulating the expression of musculoaponeurotic fibrosarcoma oncogene homolog-B (MAFB).[220] Lastly, Skalsky, et al., demonstrated that miR-376c is highly expressed in normal brain tissue and functions to down-regulate the activin receptor-like kinase-7 (ALK7) in glioblastoma.[221] Each of these studies highlight the relative low expression of these tumor-suppressor miRNAs in gliomas. These reports suggest GA-MSCs have a repertoire of miRNA that can inhibit glioma growth and invasion, yet do not package these tumor-suppressive miRNA into exosomes. Therefore, it is also reasonable that oncogenic miRNAs are instead enriched in GA-MSC-derived exosomes. The group of enriched miRNA, however have only recently been discovered and consequently are not as well characterized as the depleted miRNAs.

Similar to the designation of genes that promote malignant growth as oncogenes, there are miRNAs that also promote malignant growth and are termed *onco-miRNAs*. However, unlike the group of depleted miRNA from MSC-derived exosomes that are tumor-suppressive, the group of highly expressed and highly enriched miRNA in MSC-derived exosomes consists of newly identified miRNA that have not yet been study, and consequently have no known function. The potential for these unstudied miRNA to be onco-miRNAs is suggested by my experimental results in which MSC-derived exosomes increased the tumorigenicity of GSCs. Given the wide range of genes that one miRNA can target, and the similarly wide range of miRNA that can target one gene, the effects of GA-MSC-derived exosomal miRNA on GSCs are most likely not attributable to a single miRNA. However, my

results indicate that miR-1587 is not only highly expressed and highly enriched in MSC-derived exosomes, but is at least one exosomal miRNA that appears to mediate the increase in proliferation and clonogenicity of GSCs. My study is also the first to describe the targeting and down-regulation of the nuclear receptor co-repressor-1 (NCOR1) in gliomas by miR-1587. NCOR1 was first identified and characterized by Horlein and colleagues in 1995.[222] In this study, researchers discovered a transcriptional co-regulatory protein that could repress expression of gene targets for thyroid hormone receptors and retinoic acid receptors, independent of ligand binding. Since the discovery of NCOR1, several other NCORs have been identified, and have been shown to function in both normal physiologic and pathologic conditions, including cancer.[223-225]

The association of NCOR1 with thyroid hormone receptors and retinoic acid receptors suggested a role in both thyroid cancer and retinoblastoma.[226-227] Initially in 2007, Furuya, et al., demonstrated that a loss of function, or lower cellular expression, of NCOR1 resulted in increased proliferation and motility of thyroid tumor cells.[226] These effects resulted from decreased NCOR1-mediated repression of PI3K/AKT signaling, leading to a more aggressive follicular thyroid carcinoma phenotype.[226] This was the first report linking NCOR1 function to thyroid cancer, and it's designation as a tumor-suppressor gene in this type of hormone-related cancer. Recently in 2013, Nazha and colleagues, demonstrated that low expression of NCOR1 in the nucleus of retinoblastoma cells increased their proliferation and promoted the maintenance of an undifferentiated state.[227] This expression pattern was also demonstrated in retina-progenitor cells, which supports the role of NCOR1 as a regulator of cellular differentiation. This study was the first to describe a tumor-suppressor role for NCOR1 in retinoblastoma, and revealed the cellular localization of NCOR1 to be key to its function.[227]

In gliomas, NCOR1 was originally thought to be an oncogene, but recent studies have suggested a tumor suppressor role. A study published by Park and colleagues in 2007, initially established an oncogenic function for NCOR.[228] Outcomes from this study highlighted the tumor-promoting effects resulting from high

expression levels of NCOR in gliomas. These effects were linked to the phosphorylation and subsequent shuttling of NCOR out of the nucleus and into the cytoplasm. Thus, elevated nuclear expression of NCOR was associated with maintenance of an undifferentiated state, and deemed oncogenic in gliomas.[228] However, these researchers were evaluating the NCOR family in the U87 glioblastoma cell line, and did not distinguish between effects caused by NCOR1 and NCOR2. Although both co-repressors associate with the same hormone receptors, their relative expression levels in the cytoplasm and nucleus are quite different, as demonstrated by Campos, et al., in 2010.[229] Results from this study demonstrated a low expression of NCOR1 in both cytoplasmic and nuclear fractions, and high nuclear expression of NCOR2, in tumor cells extracted from 283 patients with varying grades of astrocytic gliomas. This study suggests that NCOR2 may have an oncogenic function, while NCOR1 has a tumor-suppressor function.[229] Consistent with this concept, in 2013, Heldring, et al., also demonstrated the tumor-suppressor function of NCOR1 in gliomas, by showing that the knockdown of NCOR1 resulted in glioma growth and invasiveness both *in vitro* and *in vivo*.[230] Furthermore, decreased expression of NCOR1 correlated with the epithelial-to-mesenchymal transition in glioblastoma.[230] Together, these studies indicate that down-regulation of the tumor-suppressor NCOR1 in gliomas, results in the promotion of growth and evolution of the malignancy. Therefore, my experimental result demonstrating an increase in GSC proliferation and clonogenicity caused by miR-1587-mediated down-regulation of NCOR1 is consistent with the literature.

My future experiments include linking NCOR1, and thus miR-1587, to the increases in GSC tumorigenicity demonstrated in my results. This would involve the use of siRNA to knockdown expression of the NCOR1 gene in GSCs, simulating the effect of miR-1587 in GA-MSC-derived exosomes. Silencing of NCOR1 with shRNA eliminates the non-specific effects that miR-1587 could induce by the down-regulation of other genes in addition to NCOR1. Given the results from Heldring, et al., I expect to find an increase in GSC tumorigenicity when NCOR1 expression is suppressed. This result would mirror that of miR-1587 over-expression in GSCs, and

GSCs treated with GA-MSC-derived exosomes, thus defining a final biological pathway by which GA-MSC-derived exosomes promote gliomas.

One of the weaknesses of my study was the further evaluation of only one miRNA based on the results from my *in vitro* experiments. Specifically, I did not perform western blot analysis or *in* vivo studies of miR-3620-5p, which significantly increased the proliferation of GSCs, but did not have a significant effect on GSC clonogenicity. Additionally, I also did not perform further analysis on miR-1246 which increased both GSC proliferation and clonogenicity, but not to a significant degree. Further investigation of the predicted gene targets of these two miRNA, combined with *in vivo* studies, may indicate a synergistic tumor-promoting function with miR-1587. Another weakness in this study was the evaluation of only one predicted gene target of miR-1587. Although identification of putative miR-1587 targets has not yet been described, verification of its functionality by demonstrating the down-regulation of another predicted gene target would better support my *in vitro* results, classifying miR-1587 as an onco-miRNA in MSC-derived exosomes.

Conversely, a strength of this study was the utilization of computational analysis of the miRNA profiles of BM-MSC-derived and four GA-MSC-derived exosomes in order to develop a list of potential oncogenic miRNAs. This process was significantly more efficient that a shotgun approach analyzing all miRNA in MSC-derived exosomes to identify those which are tumor-promoting. Another strength of this study was the use of gene expression profiling to demonstrate the down-regulation of predicted gene targets for enriched miRNA, after treatment of GSCs with GA-MSC-derived exosomes. This method ensured that the group of potential oncogenic exosomal miRNA had a significant effect on the GSC transcriptome before conducting *in vitro* and *in vivo* experiments. A final strength of this study was utilization of an anti-miR-1587 inhibitor to reverse the effects of GA-MSC-derived exosomes on GSCs in my *in vitro* experiments. This treatment group helped isolate the effects of miR-1587, and ensured that no other exosomal miRNAs were responsible for the increases seen in GSC proliferation and clonogenicity.

4.4 Conclusions

My experimental results demonstrate that GA-MSC-derived exosomes are enriched with a specific group of miRNA, as compared to GA-MSCs. Interestingly, these enriched miRNA contained specific nucleotide motifs that were common among the group, and absent from depleted miRNA. Furthermore, the expression of predicted gene targets for the enriched miRNAs, were significantly decreased in GSCs after treatment with GA-MSC-derived exosomes. Importantly, the over-expression of one of the enriched miRNAs (miR-1587) significantly increased both proliferation and clonogenicity in GSCs. Finally, a predicted gene targets for this specifically enriched miRNA (NCOR1), was decreased after miR-1587 over-expression in GSCs and after treatment of GSCs with GA-MSC-derived exosomes. These results indicate that the mechanism by which GA-MSC-derived exosomes promote glioma growth is through the delivery of specific oncogenic miRNA to recipient GSCs.

Chapter V

Research Summary

Research Summary

For my thesis work, I hypothesized that GA-MSC-derived exosomes enhance the growth and evolution of gliomas. My initial experiments studied the production system of GA-MSC-derived exosomes and subsequently characterized their proteomic and genomic contents. I then investigated the interaction between GA-MSC-derived exosomes and GSCs, and showed that GA-MSC-derived exosomes increases in the tumorigenicity of GSCs. Finally, I discovered a link between a specific miRNA (miR-1587), delivered in GA-MSC-derived exosomes to GSCs, that was responsible for the glioma-promoting properties of this intercellular communication pathway. This system culminated in the down-regulation of a tumor-suppressor protein in gliomas (NCOR1), and revealed the onco-potentiating ability of GA-MSC-derived exosomes. These results validate my hypothesis and show for the first time that stroma-derived exosomes in the glioma microenvironment can influence growth and evolution of the malignancy via the delivery of specific miRNA to recipient GSCs.

5.1 Experimental Conclusions

These experimental results are the first to demonstrate the production of characteristic exosomes by GA-MSCs. Moreover, the production of GA-MSC-derived exosomes varies with exposure to different levels of cellular stressors. Additionally, GA-MSC-derived exosomes do not contain major proteins or cytokines. However, GA-MSC-derived exosomes do contain numerous miRNA with distinct profiles compared to parental MSCs. Indeed, a specific group of miRNAs are highly expressed in GA-MSC-derived exosomes. These results prove that GA-MSCs are capable of producing exosomes whose content have the potential to impact the biology of recipient cells.

By tracking GA-MSC-derived exosomes utilizing a GFP-CD63 fusion protein, I showed that GSCs can internalize GA-MSC-derived exosomes. More importantly, I demonstrate for the first time that GA-MSC-derived exosomes significantly increase the proliferation and clonogenicity of GSCs *in vitro*. Moreover, GA-MSC-derived exosomes can significantly increase the tumorigenicity of GSCs *in vivo*. Furthermore, pre-treatment of GSCs with GA-MSC-derived exosomes increases tumor burden and decrease median survival. These results prove that GA-MSC-derived exosomes can interact with GSCs to influence their biology.

My experimental results demonstrate that GA-MSC-derived exosomes are enriched with specific miRNAs, as compared with parental GA-MSCs. Interestingly, enriched miRNAs contained specific nucleotide motifs that were common among the group. Furthermore, the expression of predicted gene targets for the enriched miRNAs was significantly decreased in GSCs after treatment with GA-MSC-derived exosomes. Importantly, the over-expression of one of the enriched miRNAs (miR-1587) increased both proliferation and clonogenicity in GSCs. Finally, one of the predicted gene targets for this specifically enriched miRNA (NCOR1), was decreased with both miRNA over-expression in GSCs and GSCs treated with GA-MSC-derived exosomes. These results indicate that an enriched miRNA in GA-MSC-derived exosomes alters the expression of a tumor-suppressor in gliomas leading to increased glioma growth.

5.2 Research Significance

The significance of my thesis work is far-reaching, from demonstrating exosomal communication in the tumor microenvironment to describing selective miRNA packaging, to reporting the loss of tumor-suppressor activity in gliomas. These results describe several aspects of cellular biology that have never before been shown in gliomas. Moreover, my results demonstrate the impact of the glioma microenvironment on tumor growth, which can potentially be targeted by adjunct therapy in the future.

The results of my studies elucidated the role of exosomal communication in the tumor microenvironment. My results indicates that exosomes from the microenvironment contribute to maintaining a supportive niche for malignant growth and suggest that this mode of interaction between the tumor stroma and the tumor propagating cells may be consistent throughout all solid tumor cancer types. Therefore one of the major impacts of my work is the necessity to consider the influence of tumor stroma-derived exosomes in glioma growth and future therapeutic strategies to inhibit this tumor-supporting property.

My project also described the packaging of selective miRNA into GA-MSC-derived exosomes. My results suggest that incorporation of certain materials into exosomes is cell-of-origin specific, and may be influenced by interactions with neighboring cells. Additionally, the packing of specific exosomal miRNA suggests that a particular effect can be induced in recipient cells. Therefore, another major impact from my work is the gene-altering capabilities of tumor stroma-derived exosomes which must be taken into account in the process of glioma progression.

Lastly, my research demonstrated the specific down-regulation of a tumor suppressor gene in glioma via delivery of GA-MSC-derived exosomes. Therefore, a final major impact from my work is that the tumor stroma provides a nurturing environment by regulating gene expression in tumor propagating cells, which must be taken into account during the treatment of aggressive gliomas.

5.3 Future Investigations

The intercellular exosomal communication between GA-MSCs and GSCs is only one pathway in a complex network of interactions between cells within the glioma microenvironment. In the future, I plan to expand my investigations of the tumor niche to include evaluating the role of GSC-derived exosomes in modulation of the surrounding stroma. Specifically, I will examine the effects of GSC-derived exosomes on GA-MSCs, brain microvascular endothelial cells (BMVEC), astrocytes and microglia. Given the function of these cells, this communication network may serve to transform the microenvironment to better promote glioma growth. Thus, GSC-derived exosomes may play a role in the recruitment of MSCs from the bone marrow, breakdown of the blood-brain barrier, invasion into brain parenchyma, and immunosuppression within the tumor. As an extension of this study, I would also investigate the diagnostic and prognostic applications of GSC-derived exosomes in serum. Some of these processes have been demonstrated in other cancer types, and their description in gliomas is necessary.

Another direction that I would like to explore is the nucleotide motifs in miRNA that may target them for enrichment into exosomes. Although this study is not necessarily associated with gliomas, or cancer, it has a much broader scope for defining the process of exosomal miRNA packaging. This investigation would involve identifying conserved nucleotide sequences present in enriched miRNA from exosomes from a variety of cell types. Extensive computational and cluster analysis would be necessary for an effective and efficient approach to this experimentation. Once key motifs were identified, artificial mutations in these sequences should result in the decreases expression of previously enriched miRNA in exosomes. Additionally, encoding of these sequences in previously exosome depleted miRNA, should result in their enrichment into exosomes. Positive results of this study would have a broad range of applications, including cancer therapy and diagnostics.

New functions of exosomes are constantly being described, and my results combined with those of future studies will aid in the full understanding of this novel paracrine signaling mechanism.

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Vita

Javier was born in El Paso, Texas where he developed his love for science through his father and teachers. He was very active in sports, orchestra and boy scouting throughout his schooling. Upon graduation from high school, he attended the U.S. Air Force Academy and pursued a degree in Biochemistry, and served as captain of the rugby team. After graduating with distinction in 2002, he pursued a Master's Degree in Biology from the University of Colorado, and worked as a scientist for the Air Force. After a productive military career, he separated from active duty service in 2007 and enrolled as a medical student at the University of Texas Health Science Center at Houston, in the combined M.D./PhD. Program. His research search has been recognized at the national level by the American Association of Neurological Surgeons (AANS) and the Society for Neuro-Oncology (SNO). On July 1, 2014 Javier will begin his 7 year neurosurgery residency training at The Barrow Neurological Institute in Phoenix, Arizona.