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The Role of Extracellular Matrix Proteins on Epithelial to Mesenchymal Transition in Glioblastoma Multiforme

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DEDICATION

This thesis is dedicated to my parents and my girlfriend for their love, endless support and encouragement. Their support was without a doubt crucial in my commitment to overcoming the obstacles I have faced in the pursuit of continuing my education

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common primary brain tumor in humans and is characterized as being highly aggressive and invasive, with the ability to locally invade different areas of the central nervous system (CNS). GBM local invasion undergoes an epithelial to mesenchmal like (EMT) process characterized by the loss of cell-cell adhesion and increased cell mobility. The EMT-like switch in GBM is triggered by a single transcription factor, *Twist1,* and is characterized by the loss of cell clustering, re-organization of the basement membrane, and increased cell migration. GBM invasion depends on the remodeling of the extracellular matrix (ECM) microenvironment, which is induced in part by activated matrix metalloproteinases (MMPs). MMPs have proteolytic activity, acting in the breakdown of the basement membrane (BM), and facilitating cell proliferation, adhesion, migration and angiogenesis. The progression of GBM tumor malignancy is a multistep process that involves cell-cell and cell-ECM adhesion, invasion and migration. In this study, we examined the ability of the neural ECM proteins vitronectin, fibronectin, laminin and collagen IV to trigger an EMT-like response in GBM. We found that, monolayer formation of GBM cells on purified ECM proteins exhibited the mesenchymal phenotype, but this did not lead to the induction of the transcription factor *Twist1,* a marker used to determine GBM invasion. On the contrary, we found that GBM cells grown on collagen IV show heightened levels of *Twist* without the EMT-like switch in morphologies. These findings suggest an important role for collagen IV in the process of GBM local invasion.

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ABBREVIATIONS

Arginine-Glycine-Aspartate sequence **RGD**

Basement membrane **BM**

Basic fibroblast growth factor **bFGF**

Basic helix-loop-helix **bHLH**

Cancer stem cells **CSCs**

Central nervous system **CNS**

Epithelial to mesenchymal transition **EMT**

Extracellular matrix **ECM**

Fibronectin **FN**

Glioblastoma Multiforme **GBM**

Glycosaminoglycan **GAG**

Hyaluronic Acid **HA**

Matrix metalloproteinases **MMP**

Mesenchymal to Epithelial Transition **MET**

Proteoglycan **PG**

Radiation therapy **XRT**

Tissue inhibiting metalloproteinases **TIMPS**

Tumor necrosis factor **TNF**

Vitronectin **VN**

REVIEW OF LITERATURE

I. Glioblastoma Multiforme

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults, and is characterized as highly aggressive and invasive, with the ability to locally invade different areas of the central nervous system (CNS). The invasive properties of GBM make it the most lethal of all primary brain tumor types[1]. Unlike other cancers, GBM does not commonly metastasize to other areas of the body due to the blood brain barrier, but it does invade normal tissue within the brain. Primary CNS tumors known as gliomas are all derived from a common progenitor, the glial cell. Glial cells can consist of ependymal cells, oligodendrocytes, or astrocytes. These subtypes of glial cells give rise to distinct tumor types, but all are referred to as gliomas (Fig.1).

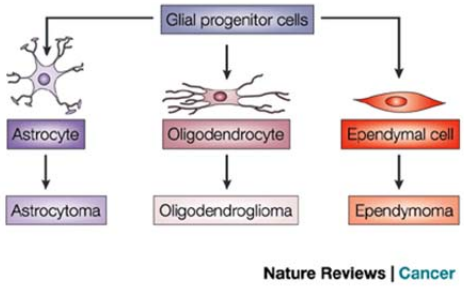


Figure 1. *Cellular classification of gliomas* – The common glial cells of the central nervous system include several cell types, giving rise to distinct tumor types, all of which are classified as gliomas. Within the CNS, neurons act as signaling cells transmitting impulses to and from the CNS, while neuroglial cells act as support cells to the neurons, providing nutrients and protection[2].

GBM is classified as an astrocytoma because it is derived from astrocytes. Astrocytomas are categorized into four grades, higher grades being more deadly than

lower ones; Grade 1-pilocytic Astrocytoma, Grade 2-Low-Grade Astrocytoma, Grade 3-anaplastic Astrocytoma and Grade 4-Glioblastoma (GBM)[1]. Gliomas have been found to grow in the brain stem, optic nerve, spinal cord, and cerebellum[1]. Of all yearly cases of brain cancer, GBM accounts for 40% of diagnoses[3], with a median overall survival rate of 14.6 months and a median progression-free survival rate of 6.9 months [4]. Common clinical therapies for GBM patients include surgery, radiation therapy (XRT), chemotherapy, and drug targeted therapeutics. The highly infiltrating and invasive phenotype of GBM within the brain means that complete surgical excision is difficult, and despite multi-modality treatments, GBM frequently recurs and is hence largely incurable at this time [4]. GBM local invasion is a complex process that involves intricate tumor-host interactions that still remain to be fully elucidated. Through research, scientists aim to interpret these interactions between cancer cells and the adjacent normal brain tissue in hopes of personalizing treatment options for patients, instead of today's broad approach to fighting GBM. The GBM invasive phenotype is characterized by the loss of cell-cell adhesion, adhesion to the extracellular matrix (ECM), and degradation of the basement membrane. This allows cancer stem cells (CSCs) to move outside the tumor's original microenvironment, invading along nearby vasculature and white matter tracts [5].

II. Cancer Stem Cells (CSCs)

Although, there has been large strides in medical therapies and surgical techniques, the outcomes for many GBM patients remains dismal. To blame for this dismal outcome is a small population of tumor cells known as cancer stem cells (CSCs). CSCs are characterized by the ability to self-renew and differentiate into specialized cell types that recapitulate the bulk of the tumor[6]. However, the mechanisms driving CSCs to create this heterogenic cell population is under debate. It is thought that heterogeneity of tumor cells can arise as a response to differences between the external environment and the tumor or by random genetic and epigenetic changes that allow cells to inherit functional and phenotypic changes[6]. Cancers that follow the cancer stem cell model contain a diverse population of cells, some being tumorigenic while others are non-tumorigenic. The non-tumorigenic cells comprise the majority of cells within the tumor and are not linked to tumor progression, while the small fraction of tumorigenic cells leads to tumor progression by having the ability to self-renew and recapitulate the other non-tumorigenic cell types[7] (Fig.2). According to Robert Weinberg the cancer stem cell model does not necessarily involve a normal stem cell as the cell of origin within a tumor. According to his theory, cells that don't have cancer stem cell properties can undergo de-differentiation to become cancer stem cells. He believes that non-CSCs undergo epithelial to mesenchymal transition to induce their de-differentiation into cancer stem cells[8].

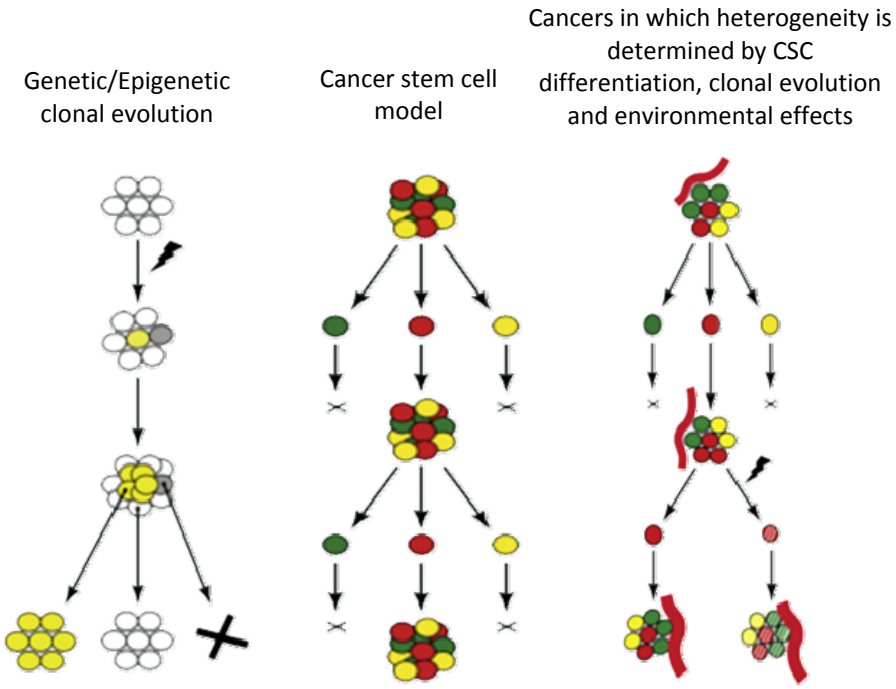


Figure 2. *Cancer stem cell model and clonal evolution* – Heterogeneity within tumors can occur through a variety of factors including clonal evolution and environmental factors. Depicted on the left is a diagram showing clonal evolution where epigenetic changes undergo selection by tumors leading to more aggressive tumors overtime. The middle diagram shows the cancer stem cell model yellow and green cells have non-tumorigenic traits while red cells are tumorigenic and have the capability of reproducing the bulk of the tumor. The depiction on the right shows that clonal evolution and the cancer stem cell model along with environmental factors can all influence tumor growth[7].

Although this concept is widely accepted, there is also evidence suggesting that brain tumor derived stem cells (BTSC) originate from neural stem cells (NSC). Neural stem cells drive neurogenesis within the brain giving rise to neurons, astrocytes and oligodendrocytes[8]. NSCs are found in two germinal regions of the brain the subventricular zone of the lateral ventricle and the dentate gyrus of the hippocampus [8]. Since BTSCs share similar characteristics to NSCs such as self-renewel, multipotency and relative quiescence, it has been proposed that GBM might arise from oncogenic transformation of NSCs and from changes within the local environment [8,9]. The cell of origin is debated with compelling evidence supporting both sides. Since neural stem cells

are highly regulated, it is questioned how these cells could comprise tumors that are not found in proximity to NSC germinal centers of the brain. Further adding to the debate is the presence or absence of specific stem cell markers, and how researchers go about quantifying their abundance or absence. Regardless of these debates, the mechanistic biology behind NSC differentiation should be clarified in order to better our understanding of genetic targets of BTSC differentiation in hopes of finding novel therapies.

III. Epithelial to mesenchymal transition (EMT)

Background

Epithelial to mesenchymal transition (EMT) and its reversion, mesenchymal to epithelial transition (MET), are highly regulated developmental programs that first occur in embryonic progression but are, also known to be key processes during cancer metastasis. EMT is a succession of events that ultimately allows polarized epithelial cells to dissociate from surrounding tissue and undergo a series of biochemical changes to assume the phenotype of mesenchymal cells[5,7]. EMT-MET provides the cellular plasticity between two distinct states: an adherent state (epithelial) and a highly mobile and infiltrative state (mesenchymal). Mesenchymal cells are long spindle shaped ,undifferentiated stem cells that originate from the mesoderm with the ability to later differentiate into distinct tissue types or to induce the differentiation of other nearby cells[8]. These cells are essential building blocks for embryogenesis with the ability to create bone, cartilage and all necessary connective tissue components.[8]

In contrast, their differentiated epithelial cell counterparts do not show great mobility and are organized into tight adherent sheets of polygonal shaped cells held together by adherens junctions[11]. These cell junctions are particularly abundant in epithelial tissues and consist of protein complexes that provide contact between neighboring cells and the extracellular matrix. The major cell-cell adhesion protein, E-cadherin, holds the sheets of epithelial cells together and prevents them from dissociating from their neighbors[5,11].When a switch in these phenotypes is triggered, it allows enhanced cell migration, invasiveness and resistance to apoptosis [5]. The multistep process of EMT is typically orchestrated through activated transcription factors such as Snail,

Slug, Twist, and Zeb1/2[5,10,12]. These transcription factors are expressed in differing combinations in a variety of cancers and have been shown *in vitro* to play important roles in EMT invasion programming[12]. The changes evoked by these transcription factors include the loss of E-cadherin, and the associated cell-cell adhesion, change from the polygonal shape of an epithelial cell to the more elongated fibroblastic morphology of the mesenchymal cell, expression of matrix degrading enzymes, and increased resistance to apoptosis (Fig. 3). These traits are all known to occur during the invasion-metastasis cascade[3–5].

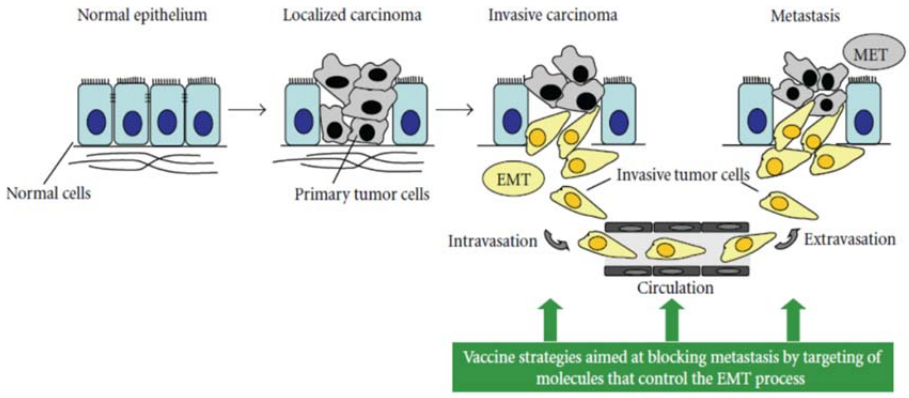


Figure 3. *The mechanism of EMT and its reversion process MET* – The mechanism of EMT is associated with loss of cell-cell adhesion and increased motility allowing cells to intravasate nearby vasculature or tissue seeding new metastasis. Abbreviation: EMT, epithelial to mesenchymal transition; MET, Mesenchymal to epithelial transition[13].

In cancers of epithelial cell origin, EMT is characterized by the loss of E-cadherin and the upregulation of N-cadherin. This cadherin switch is a hallmark of carcinomas, and is a fundamental step in EMT, allowing cells to dissociate from the primary tumor mass to seed new metastases [5,10,12,14]. In contrast with epithelial tissue, E-cadherin is not normally expressed in healthy brain tissue, neural cells, astrocytes or oligodendrocytes[15,16]. Similarly, expression of E-cadherin within the diseased GBM brain

is also rare and appears to be different from carcinomas since its expression is linked to tumorigenesis and poor prognosis[15]. In order to locally invade, GBM cells undergo a different form of EMT that does not involve the canonical cadherin switch that is typically seen in the majority of carcinomas[16,17]. Although the transcription factors Snail, Slug, Twist, and Zeb1/2, have all been shown to coordinate EMT in carcinomas, astrocytomas such as GBM use a single transcription factor, Twist, to initiate EMT [16,17]. Another notable difference is that in carcinoma EMT, mesenchymal cells intravasate blood vessels to metastasize to distant sites of the body. In comparison, the infiltrative path of GBM into the normal brain parenchyma occurs along the fibrous tracts lining the outer walls of the brain vasculature and along myelinated white matter tracts[18].

GBM Transcription Factor Twist

The basic helix-loop-helix (bHLH) protein, *Twist,* has elevated expression in high grade gliomas as well as in developing embryonic brains, but is absent from glial cells of normal brain tissue [17]. Its expression is associated with reduced cell aggregation, cytoskeleton re-organization, enhanced migration and adhesion to ECM protein substrates[16]. These characteristics provide compelling evidence of *Twist's* ability to induce EMT in GBM. *In vivo, Twist* mRNA expression is directly correlated with tumor grade, suggesting that *Twist* plays a possible role in the progression of GBM malignancy and progression[17]. When *Twist* is over-expressed in GBM cell lines, this increased *Twist* expression is associated with increased cell invasiveness and migration, two known properties of high grade gliomas[16,17].

Conversely, knock-down of *Twist* results in the inhibition of glioma invasion [16]. Taken together these findings suggest a therapeutic significance for *Twist* inhibition and its role in GBM progression should be further elucidated.

MMPs in basement membrane degradation

A major component of EMT is the triggering of CSCs to leave their original microenvironments in order to seed metastases elsewhere in the body. Aiding in the degradation of the basement membrane along with the normal extracellular matrix is a family of hydrolytic enzymes known as matrix metalloproteinases (MMPs). MMP's are secreted by glioma cells as well as stromal cells of the tumor to aid in the remodeling of the basement membrane (BM) during EMT[19]. These enzymes are organized into four subclasses based on their substrate specificity[2].There are 25 known MMP genes in humans and many of these have implications in cancer[2]. MMP expression is induced by cytokines, growth factors, tumor promoters, physical stress, oncogenic transformation, and cell-matrix and cell-cell interaction[2]. They are regulated in a variety of ways including gene expression, pro-enzyme activation, and inhibition through specific tissue inhibitors (TIMPs)[20]. MMPs aid in the invasiveness of tumor cells by degrading ECM proteins and releasing the growth factors embedded within the ECM to activate signal transduction cascades that promote migration[21]. In the gelatinase sub-class of MMPs, MMP-2 and MMP-9 predominate in glioblastoma and their mRNA and protein expression levels are known to be higher in GBM patient biopsy tissue[22,23]. Both of these MMPs are implicated in GBM proliferation and migration through the activation of transforming growth factor- α (TGF-α) and β (TGF- β)[2]. In addition, the levels of these

MMPs largely correlate with the histological grade of malignancy[23]. Likewise, MMP-9 and MMP-2 promote GBM invasion *in vitro* and in xenograft models[18,21] and their inhibition dramatically reduces the invasive phenotype[26]. Equally important, MMP2 gene expression is thought to be upregulated by the transcription factor Twist in GBM[16].

IV. Extracellular matrix proteins

The normal brain parenchyma surrounding GBM tumors plays an essential role in invasion progression. Normal brain function relies on the ability of ligands within the ECM to bind cell surface receptors known as integrins. This binding activates biological pathways that regulate the physical properties of the ECM and tissue function such as cell proliferation, differentiation, adhesion and mobility[27]. During EMT progression, MMPs disrupt this normal environment, causing changes in the interaction between ECM ligands and their cell surface receptors. The disruption of the ECM enables ligands embedded within the matrix to freely bind to cell surface receptors, enabling pathways that were once regulated to go unchecked. Furthermore, glioma cells actively secrete fibrous proteins that have greater protein cross-linking, remodeling the ECM from the soft normal brain to a more rigid tumor- like ECM [28]. The increased protein cross-linking facilitates cell migration along with the binding of ligands to integrin receptors leading to the progression of cancer through EMT [29].

The developing fetal brain shows characteristics similar to that of the GBM brain, having elevated levels of fibrous ECM proteins. This is in contrast to the mature adult brain, which shows significantly lower expression of the same ECM proteins[30]. The fetal brain ECM expresses elevated levels of the fibrous proteins, fibronectin, laminin, vitronectin and collagen[31]. The most abundant brain protein hyaluronan (HA), has also been shown to be elevated in the matrices of gliomas, similar to what is seen for the embryonic brain matrix[32].

Normal Brain ECM

The composition and organization of the ECM within the normal brain is significantly different from the ECM outside of the CNS. The ECM outside of the CNS is well defined in composition and organization, consisting mainly of collagens, glycosaminoglycans (GAG) or proteoglycans (PG) that exhibit unique variations and amounts of ECM molecules. This accounts for the great diversity amongst differing organ systems. In contrast, the ECM within the CNS is not as well organized and has yet to be fully defined. It was not until the late 1970s with the use of electron microscopy that the ECM of the brain was investigated.

The high degree of variation amongst PGs , GAGs, and ECM proteins allows them to vary in function, acting as cofactors and regulators of growth factors that influence neural cell adhesion, neuron growth, ECM organization and tumor cell invasion[33] .The parenchyma surrounding the brain has been characterized as an amorphous matrix consisting of hyaluronic acid (HA), collagen and fibrous proteins[2,34]. Carbohydrates are also abundant in the brain parenchyma and may be bound to core proteins to form proteoglycans (PG), or unbound as the GAG hyaluron (HA) [33]. Hyaluron, also known as hyaluronic acid (HA), is the most broadly distributed ECM molecule and is found in both grey and white matter. Its role is to organize the structure of the ECM by eliciting changes in matrix conformation, from extensive chains to condensed rods, and coils[35]. HA also plays an important role in creating the water-rich environment of the brain. Due to its highly anionic nature, it attracts cations, causing an osmotic influx of water[18]. In addition, proteoglycans are present in the ECM of the brain at high concentrations, being formed by the covalent attachment of one or more GAG

side chains to a core protein. Due to variations in the types of side chains, lengths and alternative splicing, GAGs exist in many different isoforms[33]. Fibrous proteins have been detected throughout the brain, mostly residing in the basement membranes surrounding the cerebral vasculature and perivascular space[33,36]. The fibrous proteins associated with these blood vessels are type-IV and type-V-collagens, fibronectin, laminin, and heparan-sulfate proteoglycans[2,36,37].

GBM Brain ECM

Malignant brain tumor fatality is caused by the invasiveness of glioma cells under the influence of the newly synthesized cellular microenvironment. During the progression of GBM malignancy, EMT triggers the invasive behavior of glioma cells, signaling them to degrade the normal ECM and replace it with a rigid fibrous ECM. Degradation of the ECM by MMPs allows for aberrant interactions between GBM cells and the ECM, resulting in the dissemination of single tumor cells into the brain parenchyma by traveling along myelinated white matter tracts and blood vessels[33] . In addition, glioma cells begin synthesizing and secreting new ECM molecules with greater amounts of cross-linking, making them more rigid when compared to the normal brain[28].

Rigidity directly coincides to glioma grade, with higher grades demonstrating more rigidity than lower ones [31]. As an example vitronectin (VN), a fibrous protein detected in GBM, is reported to be undetectable within the normal brain and is unassociated in glial cells, neuronal cells, or cerebral white matter[36]
VN expression is detectable in late stage GBM, but is absent in normal and early stage GBM brains [34,35].

Table 1: Differences in the ECM of normal brain tissue and GBM diseased brains

Molecules	Abundance		Function
	Normal Brain	Glioma	
Hyaluronic Acid (HA) **Proteoglycan (PG)**	↑ ↑	↑↑↑ ↑↑↑	HA: space filling molecule, regulates cell proliferation, adhesion and motility. PG: HA binding proteins
Fibrous Proteins ▪ **Vitronectin** ▪ **Fibronectin** ▪ **Laminin** ▪ **Collagen IV**	Found in trace amounts along brain vasculature and basement membrane. Vitronectin exists at extremely low levels.	↑↑↑ ↑↑↑ ↑↑↑ ↑↑↑	Fibrous Proteins: structural elements of connective tissue in the basement membrane of blood vessels of the normal brain and gliomas; in high grade gliomas they are also expressed and secreted by glioma cells.
Matrix metalloproteinases (MMPs)	↑	↑↑↑	MMPs: Molecules that aid in the breakdown of the basement membrane during ECM remodeling; MMP-2, MMP-9

Vitronectin is thought to be one of the major factors in human serum that promotes the migration of glioma cells and the differentiation of cancer stem cells[39]. When CSCs are cultured in VN-containing serum, they lose expression of stem cell maintenance genes and have an induced cytokeratin profile that is typically seen in mesenchymal conversion[40]. Vitronectin has been localized to the tumor-brain interface in GBM, primarily displayed at the plasma membrane and in the cytoplasm of the primary tumor[41]. Because of its localization to the tumor mass and not the vasculature, it has been suggested that VN is tumor cell derived[41].

Likewise, hyaluronan (HA) expression is elevated approximately four-fold in the matrices of gliomas relative to normal ECM[42]. The protein fibronectin is similar in biochemical structure to VN and is also expressed by GBM cells *in vitro* and along blood vessel migratory tracts surrounding tumors *in vivo*[2]. Ulrich et al. revealed that GBM cells

grown on fibronectin coated plates show an altered morphology and cytoskeletal organization similar to that of the mesenchymal phenotype. They discovered that multiple GBM cell lines show this altered morphology when plated on rigid substrates and that plating on softer substrates results in decreased cell spreading, stress fibers and focal adhesions. The protein laminin is detectable in and along blood vessels and is found to associate with the foot processes of neurons in the basal lamina of the brain[43]. Interestingly, laminin was found to take on multiple isoforms and these were associated with different grades of gliomas[44]. Laminin along with collagen type IV are the major components making up the basement membrane of the brain and are commonly associated with the neural vasculature[2,23,45].

V. Cellular communication

Role of Integrins

EMT- induced GBM tumor cell invasion is influenced by a variety of growth factors and extracellular matrix (ECM) signals within the brain microenvironment[45]. Neuronal cells communicate with protein components within the ECM via a family of transmembrane surface receptors known as integrins[46]. Integrins are comprised of α and β subunits and their heterodimeric pairing confers specificity of binding to one or more substrates. The five members of the αv integrin subfamily αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 are expressed in neural and vascular cells of the brain and bind to arginine-glycine-aspartic acid (RGD) peptide motifs present in many shared ECM ligands[47]. In contrast with other cell surface receptors integrins are able to communicate through "inside-out" and "outside-in" signaling, signals from the cell's interior travel out of the cell to the external environment and signals from the external environment are transferable to a cells interior[48]. Integrin signaling is important in many cellular processes such as adhesion, migration, polarity, cell survival, apoptosis and proliferation[49].

Integrin expression within GBM

Of all the integrins associated with GBM, none has been more documented than αvβ3. The αvβ3 integrin is documented to colocalize with MMP2 at the leading edge of invading tumor cells[48] and within endothelial cells along newly formed vasculature[41]. The integrins αvβ3 and αvβ5 have been implicated in tumor-induced angiogenesis within gliomas were they up-regulate basic fibroblast growth factor (bFGF) along with tumor necrosis factor (TNF- α)[48]. A driver of αvβ3 signaling is the ECM ligand VN[41].

Expression of αvβ3 is associated with the increased expression of the ECM protein VN at the leading edge of invasive glioma tumors[41]. Given these points, it is fair to suggest that the interaction between the αvβ3 integrin and VN contributes to the invasive behavior of GBM. If in fact it does, this could lead to better use of anti-glioma drugs like the RGD inhibitor cilengitide, while giving researchers more insight into personalized treatment options for patients.

Cilengitide in GBM therapy

Because integrin signaling leads to tumor cell migration, proliferation, and invasion, integrin-inhibiting drugs have been a recent development in the fight against GBM. Of these αv integrin antagonists, the antitumor drug Cilengitide has made headway in the reduction of GBM progression. Cilengitide is a cyclic RGD peptide inhibitor for the αvβ3 and αvβ5 integrins and is currently being tested in a phase III clinical trial[50,51] . In glioblastoma the integrin αvβ3 has been found to localize with the protein vitronectin and the matrix metalloproteinase MMP2 at the tumor brain interface[41]. Early clinical trials of Cilengitide have demonstrated anti-tumor activity, increased patient remission and even increased patient survival for a small populations of GBM patients[50]. The early results from these phase trials show the efficiency of integrin inhibition and it is now being determined if Cilengitide in conjunction with other agents might enhance the effectiveness of these therapies[50,51].Going forward, the future success of developing therapeutic integrin inhibitors for GBM patients depends on the understanding of integrin expression amongst differing cell types, in particular those in neural microenvironments.

MANUSCRIPT

I. Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in humans[1] and is characterized as being highly aggressive and invasive, with the ability to locally invade different areas of the central nervous system (CNS). GBM tumors are known to contain sub-populations of cells referred to as cancer stem cells (CSCs). CSCs are important for producing the cells that compose the bulk of the tumor, initiating secondary tumors through local invasion and contributing to the development of drug resistance. CSCs display the innate characteristics of stem cells in their ability to self-renew and recapitulate the multiple cell types that compose the bulk heterogeneous tumor[9,10,47 .]

A key event in cancer metastasis is the, epithelial to mesenchymal transition (EMT) a biological process characterized by the loss of cell-cell adhesion and increased cell mobility similar to that occurring in embryonic developement[5,10,12]. Unlike the metastasis of carcinomas, GBM does not undergo EMT via a canonical cadherin switch. The mesenchymal conversion in GBM is induced by the transcription factor *Twist*, as *Twist* expression correlates with less cell clustering, re-organization of the basement membrane, and increased cell migration[16,17]. GBM invasion depends on the remodeling of the extracellular matrix (ECM) microenvironment, which is induced in part by activated matrix metalloproteinases (MMPs). MMP expression has been implicated in several cancers and its overexpression correlates with progression of disease. MMPs have proteolytic activity, acting in the breakdown of the basement membrane (BM), and facilitating cell proliferation, adhesion, migration and angiogenesis[22].

The progression to tumor malignancy is a multistep process that involves cell-cell and cell-ECM adhesion, invasion, migration, and angiogenesis. . It has been shown in numerous studies that GBM tumor cells interact with the ligands laminin, fibronectin collagen IV and vitronectin and respond by altering morphology, invasiveness, proliferation, and enabling differentiation [23,31,36,38,40,41,51]. These fibrous ECM proteins are elevated in high grade gliomas, thus providing a more rigid framework for cell migration along white matter tracts and blood vessels the predominant highways for GBM invasion. Although, it is widely believed that this cell-substrate interaction in GBM is EMT-like, it has not been proven. In this study, we aim to address whether one or more of these fibrous ECM proteins of the brain vasculature initiates EMT in gliomas through the up regulation of *Twist*, triggering recruitment of MMPs to facilitate subsequent invasion. We seek to elucidate this by assaying for the up regulation of the mesenchymal marker Twist1in response to the various ECM proteins. By studying this step in EMT transition we will be able to determine which of these specific ECM proteins initiates tumor metastasis. We also hypothesize that if Twist is induced by one or more of these substrates, then *MMP-2* expression will increase with heightened Twist levels. By assaying for *MMP*-2 expression, we will be able to provide more evidence proving that an EMT-like switch is associated or initiated by the proteins of the brains ECM. Furthermore, it is our belief that potential therapeutic advances in GBM should target this EMT-MET switch to disable the invasive machinery of GBM. By targeting this switch we could potentially increase life expectancy for these patients and help reduce the dismal outcomes many GBM patients and their families face.

II. Preliminary studies

Henry Ford Hospital (Ana DeCarvalo) has recently developed an *in vitro* cell culturing system that mimics the EMT-MET process. We have adopted this *in vitro* cell culture model in order to study the events of the EMT switch that occurs in GBM. When HF2587 glioma cells are grown in serum free neurosphere media (NMGF) (Table 2), they form groups of clusters or 3-dimensional spheres. The cells take on an epithelial-like phenotype with cell-cell adherence and increased proliferation. Upon neurosphere dissociation and transfer to media containing 10% fetal bovine serum (FBS, Table 3) these same cells exhibit a mesenchymal morphology with focal adhesions, cell-substrate interactions, increased migration and slower proliferation rates. Transferring cells from serum-free neurosphere media to media containing 10% fetal bovine serum induces an EMT-like switch that is reversible (mesenchymal to epithelial transition, MET) by transferring cells out of serum-containing media into serum-free neurosphere media. The plasticity between EMT and MET is implicated in cancer metastasis and our system accurately models these switches.

Table 2: Formulation of neurosphere media with growth factors (NMGF)

Material :	**Composition:**	**Concentration:**
Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) media (Gibco 11330)	L-glutamine, D-glucose, Sodium Pyruvate, HEPES	
N-2 Supplement (Gibco 17502-048)	Insulin, human transferrin, progesterone, putrescine, selenite	50mg/ml
Bovine serum albumin (BSA) (Sigma A4919)	Albumin	50mg/ml
Gentamicin Reagent (Gibco 15750-060)	Aminoglycoside antibiotic	25 µg/ml
Epidermal growth factor (EGF) (Peprotech 100-15)	Recombinant Human EGF	50µg/ml
Fibroblast growth factor-B (FGF)(Peprotech 100-18B)	Recombinant Human FGF-2 (1 of 23)	50µg/ml

Table 3: Formulation of media supplemented with 10 % fetal bovine serum (FBS)		
Material:	**Composition:**	**Concentration:**
Dulbecco's Modified Eagle Medium (DMEM) (Gibco 11995-065)	Glucose, L-Glutamine, Sodium Pyruvate	
Fetal bovine serum (FBS)	Blood serum from fetal calf	10%

In order to validate the EMT-MET cell culture system, our first experiment was to look at morphological changes of HF2587 cells grown in FBS, NMGF, and NMGF supplemented with the fibrous protein vitronectin. The glioblastoma cell line HF2587 was plated in two separate culture conditions, serum free and FBS. Cells grown in serum free media supplemented with N2 (insulin), bovine serum albumin (BSA), epidermal growth factor (EGF), basic fibroblast growth factor-2 (FGF2), maintained an epithelial-like phenotype with robust cell-to-cell adhesion and well- formed neurospheres. Under these conditions HF2587 cells exhibit a high proliferation rate, strong intercellular adhesion to form spherical clusters, and an epithelial like morphology. Cells grown in media containing 10% FBS shifted to a fibroblast-like morphology with slower proliferation and cell-substrate (Fig. 4.A) (ECM) adherence vs. cell-cell adhesion (Fig. 4.B). Most noticeably, the cells grown in FBS grew as a monolayer rather than in spherical clusters. Our next step was to determine whether the protein vitronectin, a major component in serum, induces the same switch in phenotype from spheres to a monolayer with increased cell-substrate adherence. Cells cultured in serum-free media supplemented with purified vitronectin alone underwent a morphological change similar to that induced by serum (Fig. 4.C). This demonstrates that vitronectin, a major component of serum, may play a key role in mesenchymal conversion in this GBM model system and suggests a pivotal role for the extracellular matrix in GBM migration.

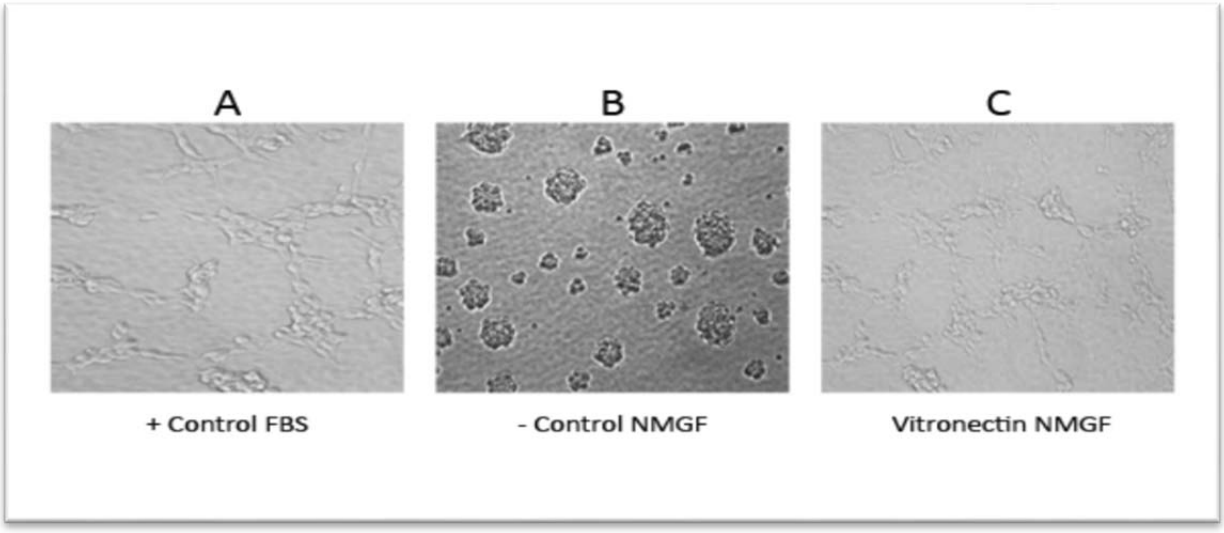


Figure 4. *In vitro HF2587 cell culturing system* – HF2587 cultured cells showing differing morphologies. A.) HF2587 cells are grown in serum containing media and form adherent monolayer. B.) HF2587 cells grown in serum free NMGF media exhibit the epithelial phenotype. C.) HF2587 cells are grown in serum free NMGF media supplemented with VN (3µg/µl). These cells were allowed to grow for 5 days at 37°C.

To confirm the accuracy of our EMT-MET cell culturing system at the transcriptional level, we sought to detect gene expression of the mesenchymal marker *Twist1*. HF2587 GBM cells were grown as neurospheres or as adherent cells for two weeks and harvested. A portion of the adherent cells weren't harvested and were allowed to continue growing as adherent cells while the remainder underwent reversion by placing them in serum-free media. After 2 weeks of reversion, RNA was extracted from all the groups of the harvested cells and qPCR for *TWIST1* expression was performed. The results from the first harvest showed that the cells grown in serum had a 5 fold induction of *TWIST1,* suggesting that a Twist-mediated mesenchymal conversion was occurring (Fig. 5.A). The same qPCR of *Twist1* in the cells reverted by removal of serum was associated with the loss of the mesenchynal marker *Twist1* (Fig. 5.B).

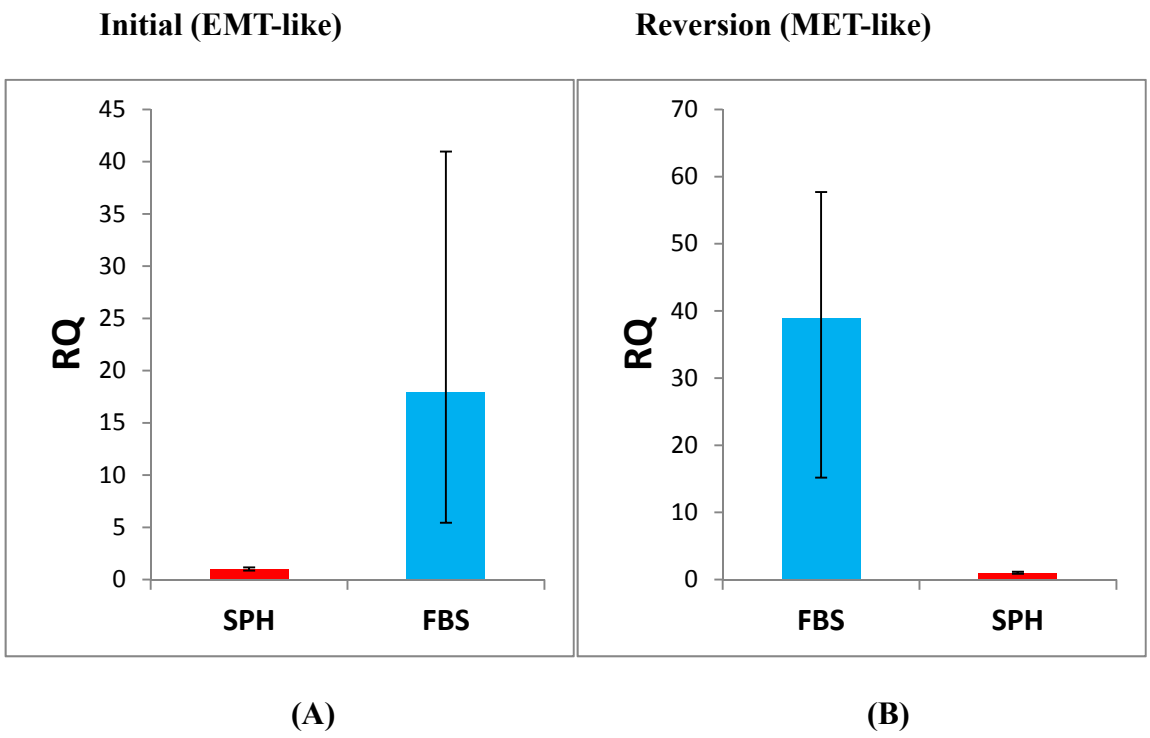


Figure 5. *Gene expression differences of Twist1 from neurospheres to adherent cells, and the reversion of adherent cells back to neurospheres – TWIST* gene expression levels for HF2587 cells maintain for 4 weeks in FBS and NMGF. Bars are represented by minimum and maximum values. A.) Gene expression levels for *TWIST* show that cells grown in serum containing media exhibit a 5 fold induction of *TWIST* compared to cells grown in serum free media. This induction of *TWIST* has been shown to trigger EMT in GBM. B.) To show the reversion of this process (MET) a portion of the cells grown in FBS from figure A were split into two cultures FBS and NMGF. The cells maintained in FBS continued to express *TWIST* but the cells grown in NMGF reverted back to the normal levels of *TWIST* seen in figure A.

The results from the qPCR data verify the accuracy of our *in vitro* EMT cell culturing system. To support the qPCR data a Western blot for TWIST1 protein expression (Fig. 6) was carried out for cells grown in serum and cells grown in neurosphere media. The figure demonstrates that TWIST1 was expressed in HF2587 cells grown in FBS but the level of TWIST was undetectable in neurospheres. T98G is a GBM cell line that was used as a positive control for its high expression of TWIST1 even when grown in neurosphere media.

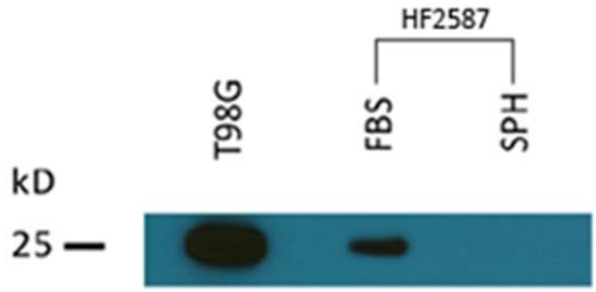


Figure 6. *Protein analysis of Twist in adherent cells compared to neurospheres* – TWIST1 (MW: 26) protein expression via Western blotting. T98G (+ control) is a human glioma cell line that over expresses TWIST and was grown in NMGF media. HF2587 cells where grown in both FBS and NMGF media, the results support our qPCR data showing that TWIST expression is higher in cells grown in serum containing media.

To access the invasive capabilities of our cell line, immunodeficient mice were orthotopically injected with HF2587 cells. The cells were grown in NMGF neurosphere media prior to being injected into the mouse cranium. After 7 weeks of growth, the mice were sacrificed and the brain tissue stained for human MHC class1. As depicted in figure 7, the cells underwent an EMT-like phenomenon, spreading out from the original injection site.

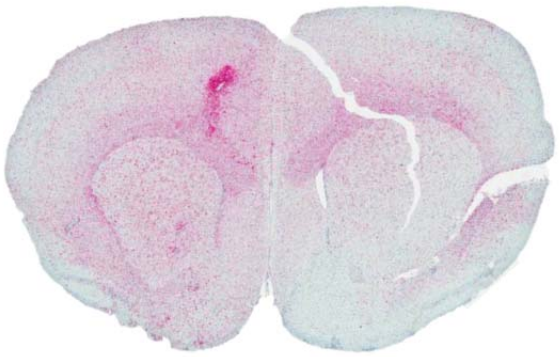


Figure 7.*Invasive nature of HF2587 in mice given orthotopic injections* – Human MHC class 1 stain (red/pink) after 7 weeks of growth. The dark accumulation of stain is the injection site. This demonstrates the highly invasive and aggressive nature of GBM. Work done by David Monsma and Dawna Dylewski,

III. Methods

Materials

Purified human extracellular matrix proteins where purchased as follows: Human vitronectin and fibronectin from Invitrogen (Grand Island, NY). Collagen IV and laminin from Santa Cruz Biotechnology (Dallas, TX). qPCR Taqman probes for both MMP2 and TWIST where purchased from Applied Biosystems (Grand Island, NY).). The HF2587 cell line was kindly provided by Ana Decarvalo of Henry Ford Hospital (Detroit Michigan). All experiments used HF2587 cells that were under passage 21.

Cell Culture

HF2587 cells were routinely grown as neurospheres at 37°C with 10% CO_2 in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 with glucose (DMEM/F12, Gibco 11330) supplemented with N2, EGF, FGF2, and BSA (table 1). The cells were grown for a period of three weeks, undergoing nine passages prior to being seeded in specific ECM- coated wells

ECM coating

Flat bottom 12-well plates (Corning) were coated overnight at 37°C with purified ECM proteins suspended in PBS (VN, FN, LAM) or 0.05N HCl (collagen IV). Human vitronectin, laminin, and collagen IV were coated at a concentration of 4.5µg/cm². Fibronectin coated wells were found to mediate attachment at a higher concentration of 10µg/cm². After overnight incubation, wells were blocked with BSA (1% w/v in PBS) for 1 hour at room temperature.

Wells coated with BSA (10% w/v in PBS) served as controls for nonspecific adhesion. HF2587 cells were plated at a density of 6 x 10^4 cells/ ml in NMGF media.

Cell Harvesting

Media was aspirated from the wells and the adherent cells were washed with PBS. Adherent cells were then collected after addition of 0.25% trypsin EDTA (Gibco 15050-065) for 1 minute at 37°C. Trypsin was inactivated by addition of twice the volume of FBS cell media. Cells were collected and washed once in PBS. The cell pellets were then flash frozen on dry ice prior to being stored at -80°C. HF2587 cells grown in suspension were collected, washed once in PBS and the cell pellets were flash frozen on dry ice prior to being stored at -80°C.

RNA Isolation

RNA was isolated from HF2587 cells, using the protocols from the RNeasy mini kit and QIAshedder from Qiagen (Maryland). Extracted RNA was quantitated using the NanoDrop spectrophotometer and then stored at -80°C.

cDNA Synthesis

Complementary DNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol.

Quantitative RT-PCR & Analysis

Quantitative RT-PCR was done on an Applied Biosystems Step One using Taqman© master mix and one of the following Taqman© assays (Applied Biosystems): MMP2, Twist1. Three biological replicates and three technical replicates were used for each target analyzed. All threshold cycle (Ct) values were normalized to the endogenous control ubiquitin c (UBC) and relative quantification was calculated from the median Ct value.

Protein extraction

Nuclear and cytoplasmic proteins where extracted from HF2587 cells, using protocol from the Nuclear Extraction Kit from Millipore (2900). Extracted nuclear proteins where then quantified using the BCA assay.

Western Analysis

Nuclear and cytoplasmic proteins were separated by SDS-PAGE gel electrophoresis and transferred to PVDF for Western blotting. The PVDF membrane was blocked in 5% milk in Tris-buffered saline-0.05% Tween 20 (TBST) for 1 hour. The primary antibody mouse monoclonal IgG 1:250 (Santa Cruz – 81417) was added and incubated overnight on a rocking platform at 4°C. Following overnight incubation, the membrane was rinsed 3 times in TBST for 10 minutes each. An anti-mouse IgG-HRP secondary (GE Healthcare- NA931V) was then applied 1:5000 in 5% milk TBST. The proteins were detected with ECL prime (GE Healthcare-RPN2232), with exposure complete after 90 seconds.

IV. Results

The HF2587 GBM cell line was originally derived from a patient GBM tumor biopsy sample. Our preliminary findings with the in vitro EMT culturing system indicated that after a two week time point, cells grown in FBS media expressed 5-fold or greater expression of the transcription factor Twist when compared to cells grown in non-serum containing neurosphere media. When the plates were coated with vitronectin we saw a similar change in phenotype from 3-D spheres to single cell as seen with FBS media. Because of this we believed that a possible role of ECM proteins could be to initiate metastasis through the formation of monolayers and the induction of Twist and MMP2.

Time-dependent effects of ECM proteins on monolayer formation

Based on our preliminary work we knew that HF2587 cells would grow as a monolayer on VN coated plates even in the presence of serum-free media. To test if other ECM proteins induced the same behavior we coated Corning 12-well plates with either fibronectin, collagen IV, vitronectin, or laminin. Serum- containing media (FBS) served as a positive control, while cells grown in neurosphere media acted as a negative control. To test for non-specific adherance10 % (w/v) BSA coated plates where used, since BSA is an inert protein with regard to cell adhesion. Pictures were taken at 4, 12, 24, 48 and 72 hours intervals respectively.

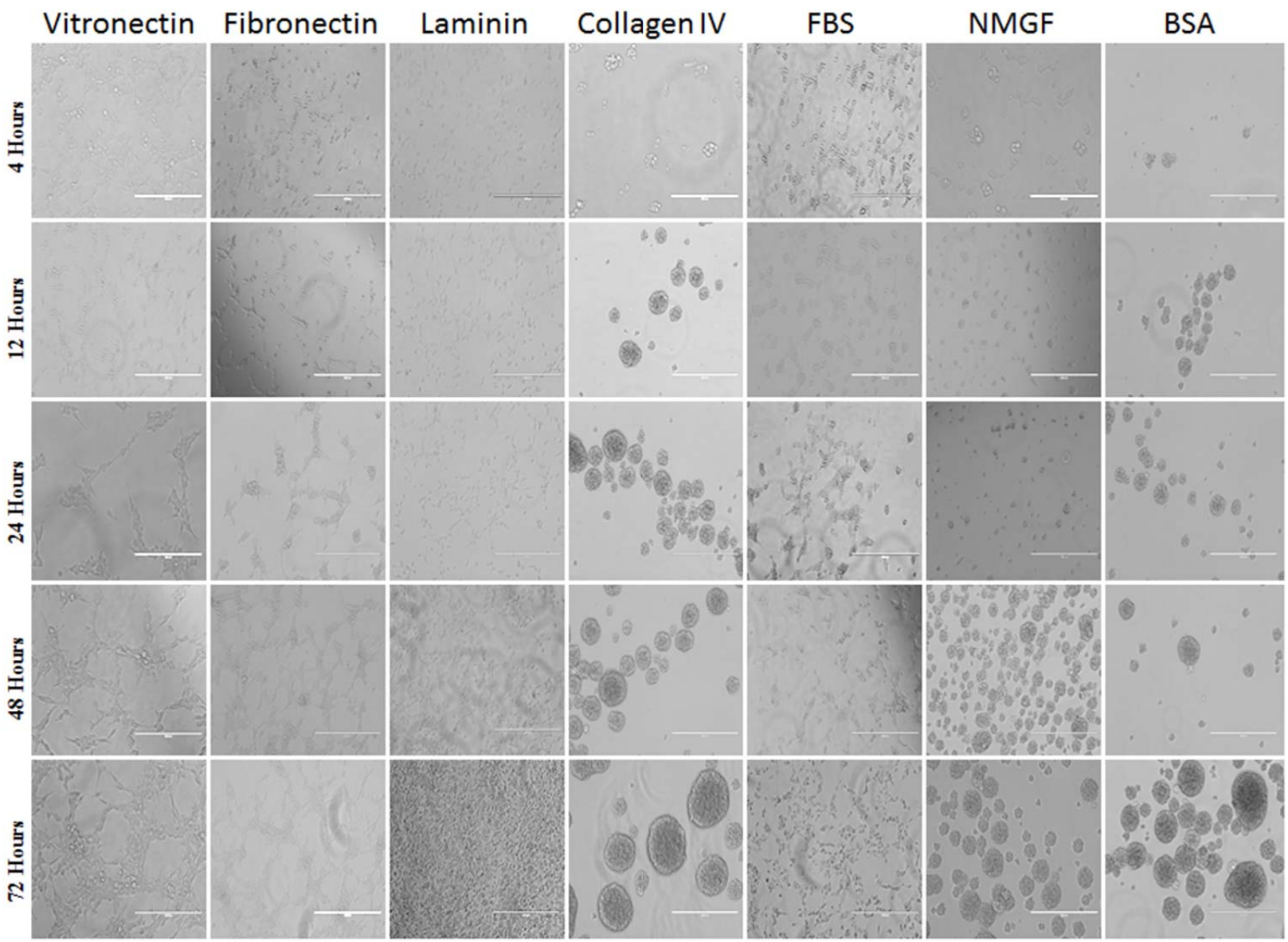


Figure 8. *Time lapse of HF2587 monolayer formation on ECM substrates* – Ability of HF2587 cells to form monolayers upon interaction with specific proteins of the neural ECM. HF2587 grew as monolayers on the proteins vitronectin, fibronectin, laminin and the positive control FBS. Collagen IV appeared to have no affect on HF2587 neurospheres since they retained their spherical phenotype

ECM protein effect on cellular adhesion

Four hours following cell seeding on various ECM substrates, monolayers or adherence occurred in the presence of fibronectin, vitronectin, and laminin. In contrast cells grown on collagen IV started to sphere and there was no apparent adherence.

By 24 hours it was clear that collagen IV had no effect on the ability of HF2587 cells to form monolayers, but it was interesting to note that sphere size in these cultures was, notably larger than with NMGF or BSA. Cells grown on vitronectin,fibronectin and laminin retained their adherence and focal adhesions were present, similar to what is seen with FBS. It should be noted, however that the monolayer morphology amongst these three substrates was different. The adherence to fibronectin was unusual in that there seemed to be some cell-cell adherence amongst the cells in the monolayer. In contrast, laminin was completely in single cell monolayer with no cell-cell adherence, and the cells adhered to the plate in fibroblast-like shapes. Vitronectin adherence was morphologically similar to what is seen with fetal bovine serum. These cells were not in fibroblast shape but they retained single cell adherence.

At 72 hours the monolayers remained intact and collagen IV still caused HF2587 to sphere. The unexpected observance was that the cells grown on collagen were not rapidly dividing but rather sticking to one another forming increasingly large spheres. When compared to cells grown in NMGF or on BSA it was evident that cell division had slowed down dramatically on collagen. This observation was further clarified at 7 days with a cell count during, the 7-day harvest. The wells containing cells grown on collagen IV had the lowest number of cells per well. The morphology of the adherent cells had

not changed from 24-72 hours; fibronectin still showed adherent clumping, and cells grown on vitronectin were morphologically similar to the cells grown in FBS. Cells grown on laminin continued to adhere in fibroblast-like shape. What was most alarming at this time point was that cells grown on laminin had reach 80-90% confluence and needed passaging. Our previous data from our EMT-MET *in vitro* cell culturing system had shown that when cells formed monolayers, the rate of cell proliferation slowed down, similiar to the characteristics of EMT. Because these cells were dividing so fast, it was a strong indication that this was not EMT- like. Throughout the remainder of the 2 week period the above observances did not change and remained static, although due to human error the 13 day cells grown on fibronectin did not survive.

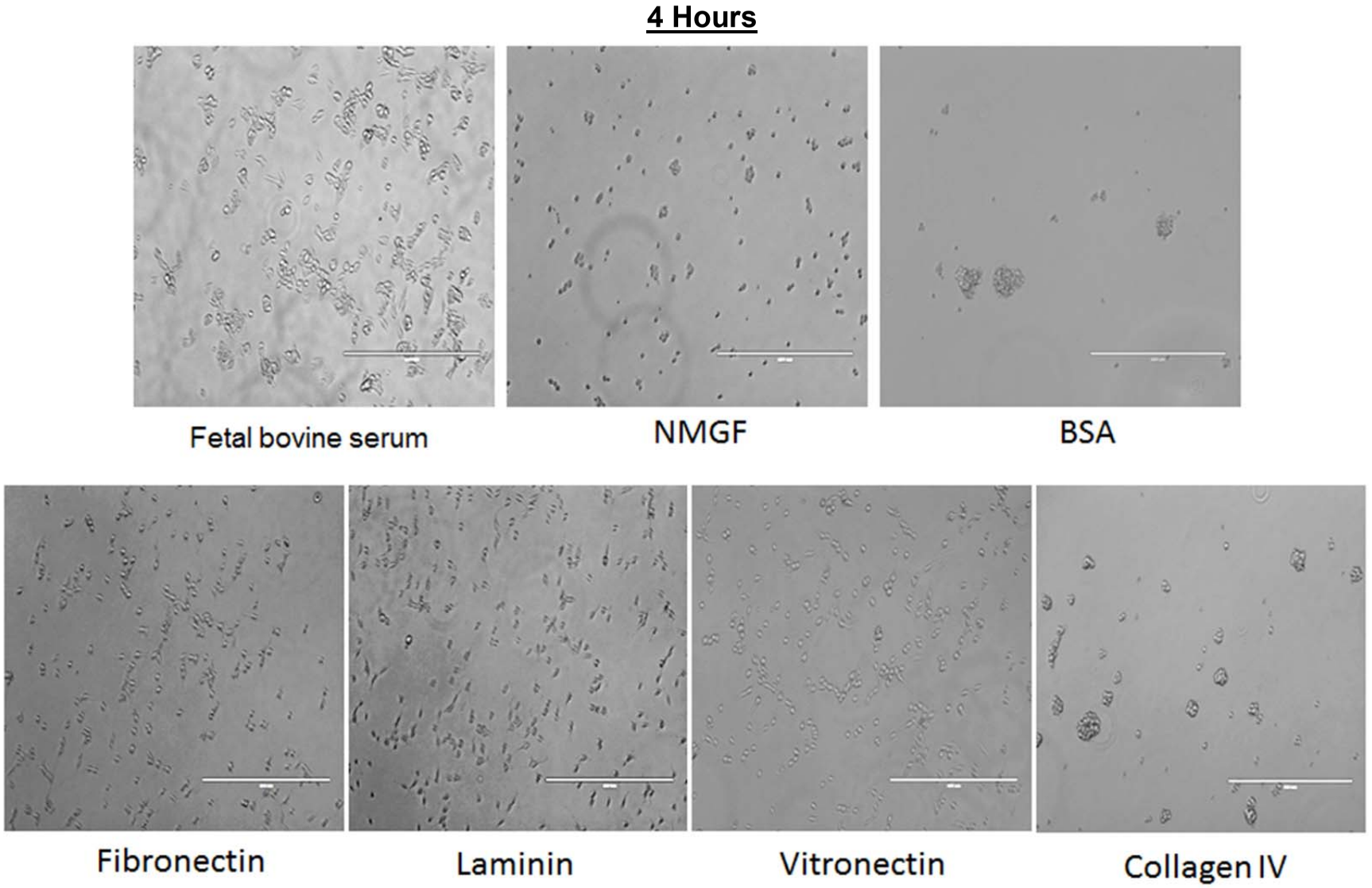


Figure 9. *HF2587 cells at 4 hours following plating on specific ECM proteins* – HF2587 cells at 4 hours following plating on specific ECM proteins. Cells on fibronectin, laminin and vitronectin maintain single cell phenotype similar to the positive control (FBS). Collagen IV caused cells to revert back to their cell-cell adherence and formed spheres.

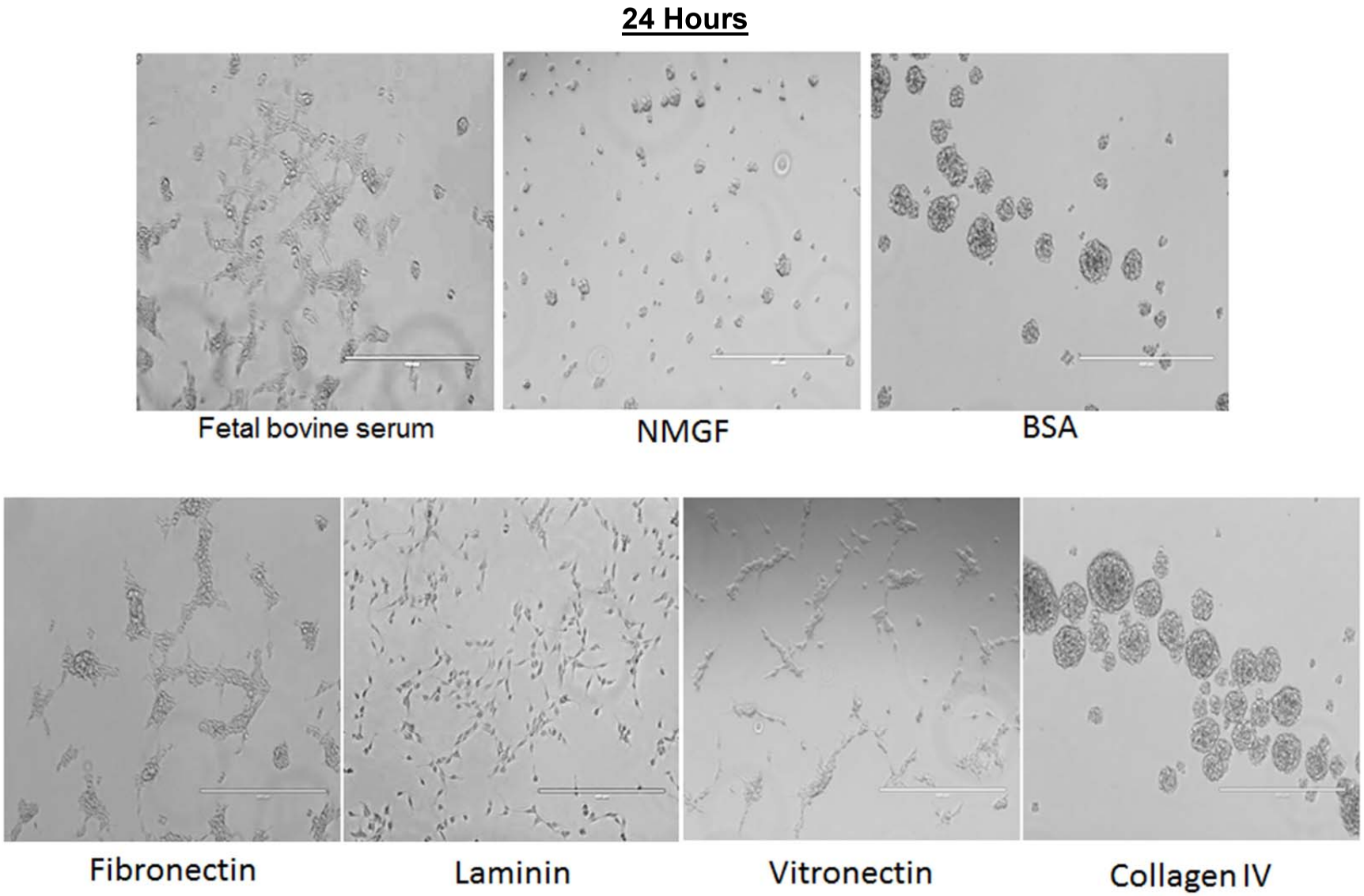


Figure 10. *HF2587 cells at 24 hours following plating on specific ECM proteins* – HF2587 cells at 24 hours following plating on specific ECM proteins. Cells on fibronectin, laminin and vitronectin maintain single cell phenotype similar to the positive control (FBS). But these 3 substrates had differing morphologies amongst the monolayers. Collagen IV continued to cause cells to maintain cell-cell adherence and sphere size was greatly increased from that of cells grown in NMGF.

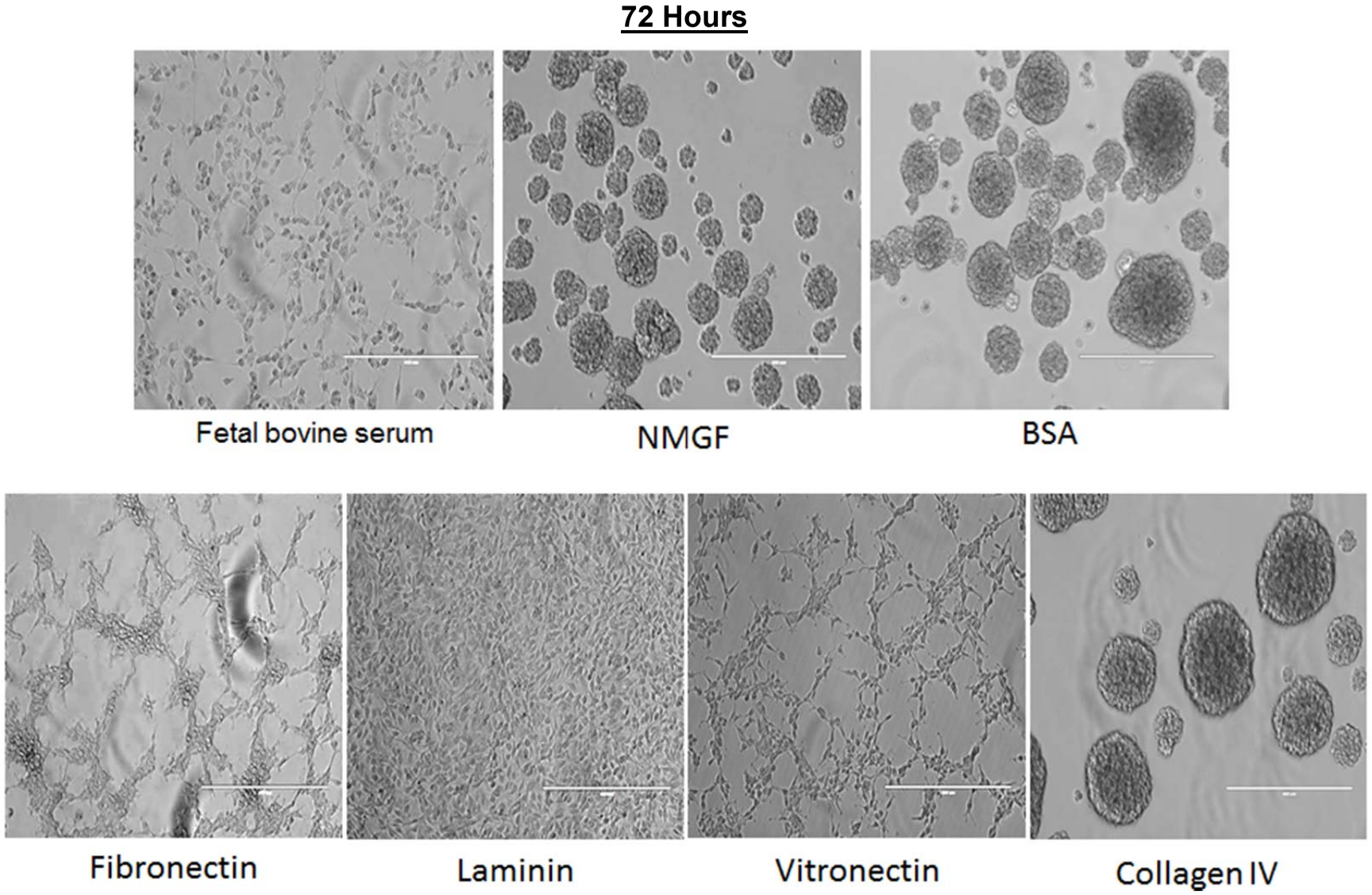


Figure 11. *HF2587 cells at 72 hours following plating on specific ECM proteins* – HF2587 cells did not vary in morphology adherent cells and spherical cells remainied the same as they were at 24 and 48 hours.

Ability of ECM proteins to upregulate *Twist1*

We believed neural ECM substrates would cause HF2587 to form monolayers, and *Twist1* expression would increase as a result of this, since this was true in our *in vitro* culturing system. It is known that ECM proteins cause glioma cells to become more migratory and invasive. To test whether this invasiveness is associated with the induction of EMT we performed gene expression analysis on the transcription factor *Twist1*. *Twist1* has been found to initiate EMT in GBM, and is up-regulated in high grade gliomas. However, real-time quantitative PCR (qPCR) revealed that mRNA expression of *Twist1* was minimally affected by the formation of monolayers on specific ECM substrates. We found that over a 7 day period the ECM proteins fibronectin, vitronectin, and laminin actually had a decrease in *Twist* expression when compared to cells grown as neurospheres. These results strongly disagree with our hypothesis that monolayer formation on ECM substrates triggers increased *Twist* expression. Also, contradicting our hypothesis, cells grown on the sphere forming collagen IV revealed a relatively insignificant 1.46 fold increase in *Twist* when compared to NMGF. Consistent with previous findings FBS had a 2.33 fold increase from NMGF *Twist* expression (Fig. 12).

Twist expression is time dependent; this has been seen in our preliminary experiments where *Twist* was shown to increase 40 fold in a 4 week period. When the current experiment was extended to 13 days, FBS expression of *Twist* increased from 2.33 to 5.60 fold. Laminin and vitronectin continued to show insignificant changes in Twist at day 13. Similarly, collagen continued to increase expression of Twist1 from 1.46-fold on day 7 to 3.74-fold by day 13.

BSA coated plates were used as a control in our experiment for non-specific binding. Once again data is unavailable for the fibronectin 13 day time points (Fig. 13).

Ability of ECM proteins to upregulate *MMP2*

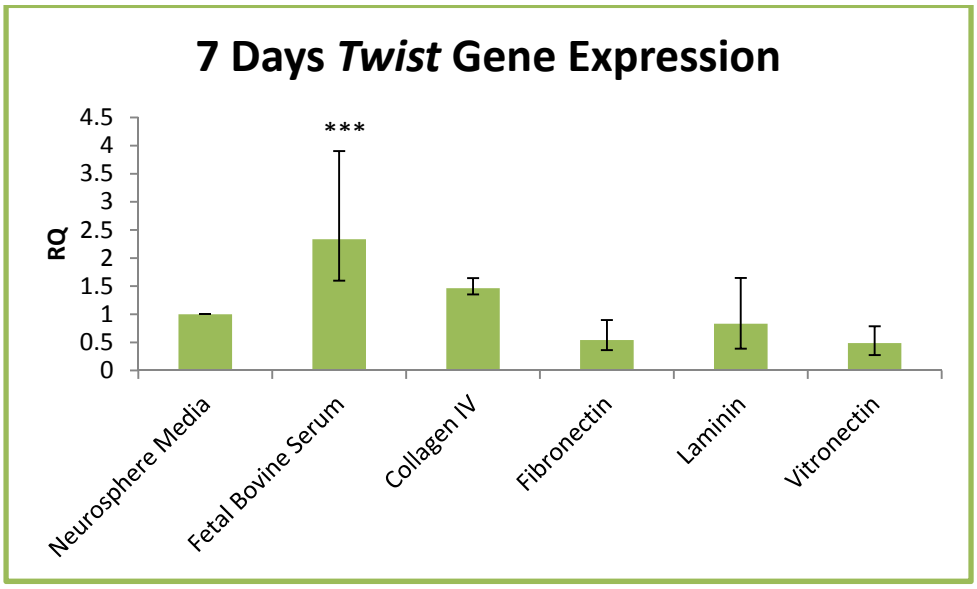
MMP2 is also a mesenchymal marker of EMT, enabling glioma cells to travel outside the original tumor environment to seed secondary tumors, and has been linked to high grade gliomas. If there is a true EMT-MET switch in our *in vitro* cell culturing system then *MMP2* expression should increase with increased expression levels of the transcription factor *Twist1*. The results from our 7 day harvest concluded that MMP2 is up-regulated 2.54 times more in FBS containing media in comparison to NMGF. This result supports our belief that our *In vitro* system does in fact mimic an EMT-MET transition. The ECM proteins vitronectin, fibronectin and collagen IV had minimal effects on gene expression of *MMP2,* and expression levels were in fact lower than in control cells grown in NMGF. Laminin increased expression of the MMP gene about 1.44fold relative to the control cell population.

As with Twist1, increasing the growth time on the ECM substrates to 13 days allowed continued increases in MMP2 expression across all samples, confirming this gene up-regulation is a time-dependent event. Surprisingly vitronectin had the largest increase in MMP2 from the previous RQ values at 7 days, approximately 4 times as high as control cells, but increased over 5 times in expression between 7 and 13 days. For the 13 day harvest, the highest RQ value was recorded by FBS, coming in at 10 times higher than control cells. Laminin was found to have heightened expression of MMP2 about 4.4 fold that of control cells; there was no 13 day recording for fibronectin.

Ratio of Twist*/MMP2 expression*

We initially predicted that *MMP2* expression correlated with increased gene expression of Twist; this hypothesis was confirmed in our experiment. When HF2587 cells were grown on the substrates fibronectin, laminin, vitronectin, collagen IV or FBS, heightened *MMP2* gene expression correlated with increased expression of Twist. Interestingly, the ratio of *MMP2* expression to *Twist* expression for the cells that formed monolayers on FBS, vitronectin, fibronectin and laminin was in favor of MMP2. As for collagen IV, *Twist* expression was greater than MMP2 at both 7 and 13 days (Fig. 16).

A.

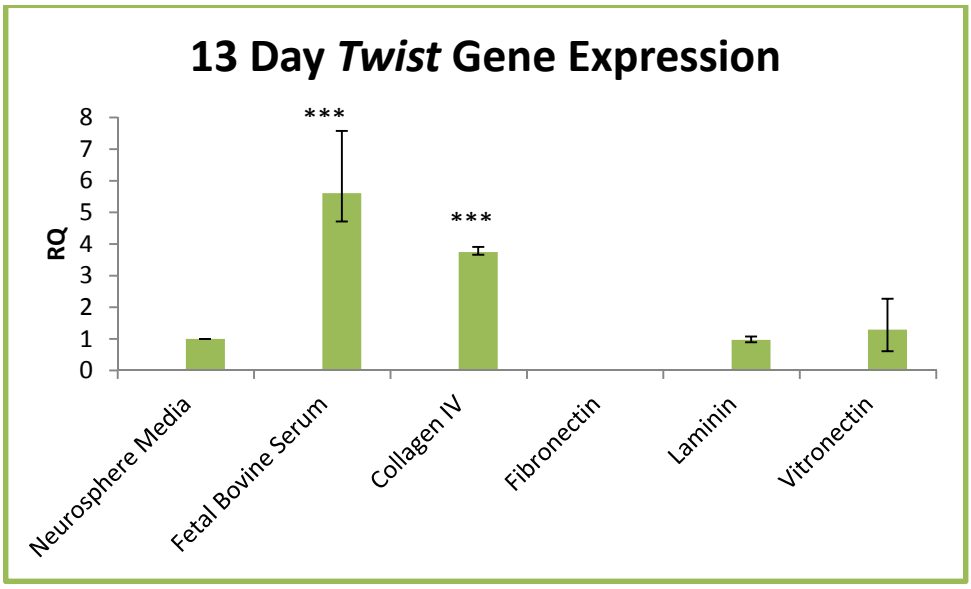


B.

Sample	RQ	Maximum	Minimum
Neurosphere Media	1	1	1
Fetal Bovine Serum	2.34	3.90	1.60
Collagen IV	1.46	1.64	1.35
Fibronectin	0.542	0.90	0.36
Laminin	0.833	1.65	.39
Vitronectin	.487	.79	.28

Figure 12. *HF2587 cell Twist gene expression following 7 day harvest* – A.) Graphical depiction of RQ values from 7 day cell harvest. Error bars represent maximum and minimum values. B.) Table of exact RQ, maximum and minimum values. RQ values where calculated using the comparative Ct method. $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference $\Delta Ct = Ct$ gene of interest – Ct endogenous control. UBC was used as an endogenous control and our values where normalized to the 7 day HF2587 cells grown in NMGF.

A.

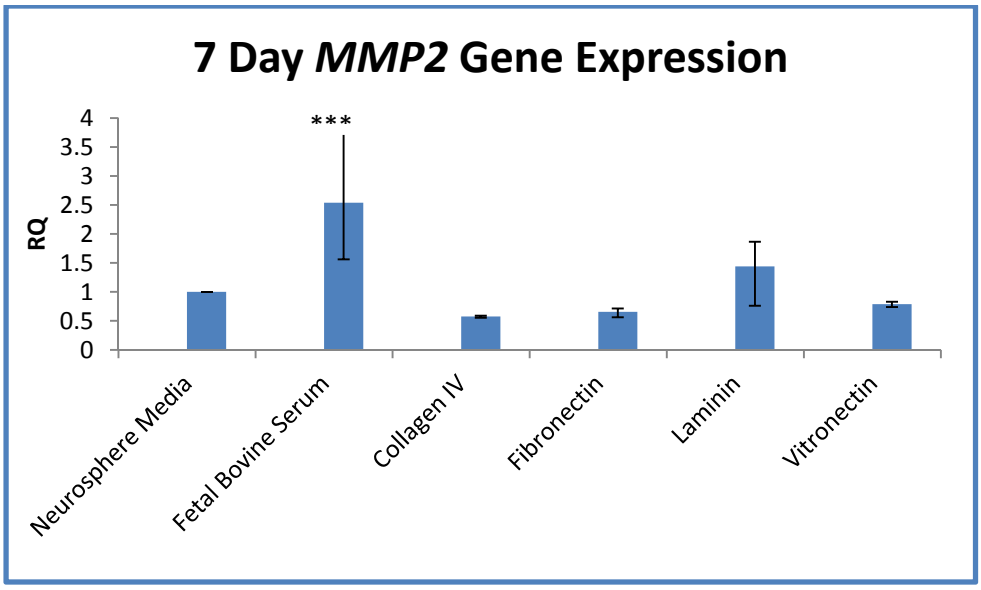


B.

Sample	RQ	Maximum	Minimum
Neurosphere Media	1	1	1
Fetal Bovine Serum	5.61	7.58	4.71
Collagen IV	3.75	3.91	3.66
Fibronectin	0	0	0
Laminin	.971	1.08	.89
Vitronectin	1.30	2.27	.61

Figure 13. *HF2587 cell Twist gene expression following 13 day harvest* – A.) Graphical depiction of RQ values from 13 day cell harvest. Error bars represent maximum and minimum values. B.) Table of exact RQ, maximum and minimum values. RQ values where calculated using the comparative Ct method. $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference $\Delta Ct = Ct$ gene of interest – Ct endogenous control. UBC was used as an endogenous control and our values where normalized to the 13 day HF2587 cells grown in NMGF.

A.

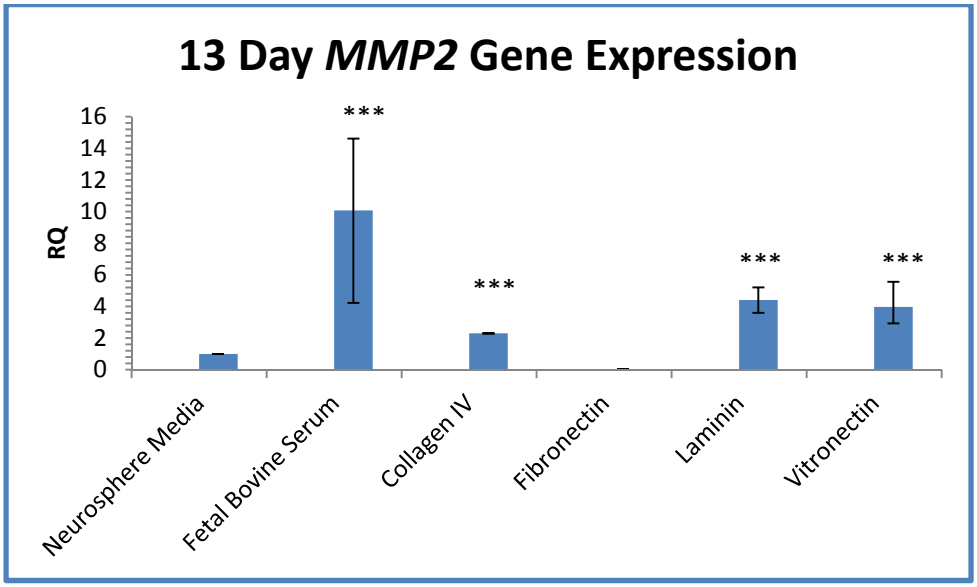


B

Sample	RQ	Maximum	Minimum
Neurosphere Media	1	1	1
Collagen IV	0.575	.591	.0554
Fetal Bovine Serum	2.54	3.794	1.561
Fibronectin	0.657	0.715	0.562
Laminin	1.44	1.867	0.761
Vitronectin	0.789	0.831	0.738

Figure 14. *HF2587 cell MMP2 gene expression following 7 day harvest* – A.) Graphical depiction of *MMP2* RQ values from 7 day cell harvest. Error bars represent maximum and minimum values. B.) Table of exact RQ, maximum and minimum values. RQ values where calculated using the comparative Ct method. $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference $\Delta Ct = Ct$ gene of interest – Ct endogenous control. UBC was used as an endogenous control and our values where normalized to the 7 day HF2587 cells grown in NMGF.

A.

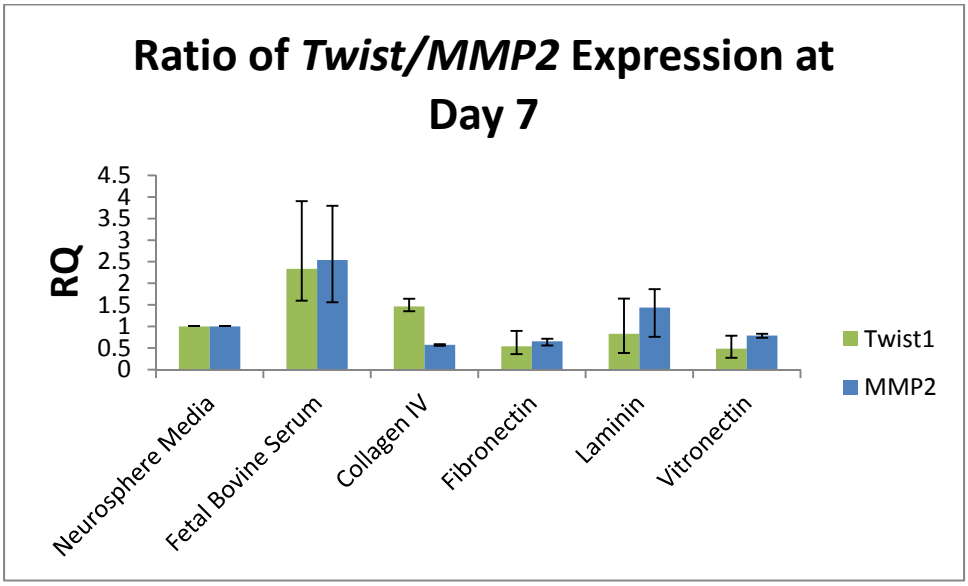


B.

Sample	RQ	Maximum	Minimum
Neurosphere Media	1	1	1
Fetal Bovine Serum	10.07	14.616	4.226
Collagen IV	2.30	2.319	2.267
Fibronectin	0	0	0
Laminin	4.41	5.201	3.593
Vitronectin	3.97	5.558	2.926

Figure 15. *HF2587 cell MMP2 gene expression following 13 day harvest* – A.) Graphical depiction of *MMP2* RQ values from 13 day cell harvest. Error bars represent maximum and minimum values. B.) Table of exact RQ, maximum and minimum values. RQ values where calculated using the comparative Ct method. ΔΔCt = ΔCt sample - ΔCt reference ΔCt = Ct gene of interest – Ct endogenous control. UBC was used as an endogenous control and our values where normalized to the 13 day HF2587 cells grown in NMGF.

A.



B.

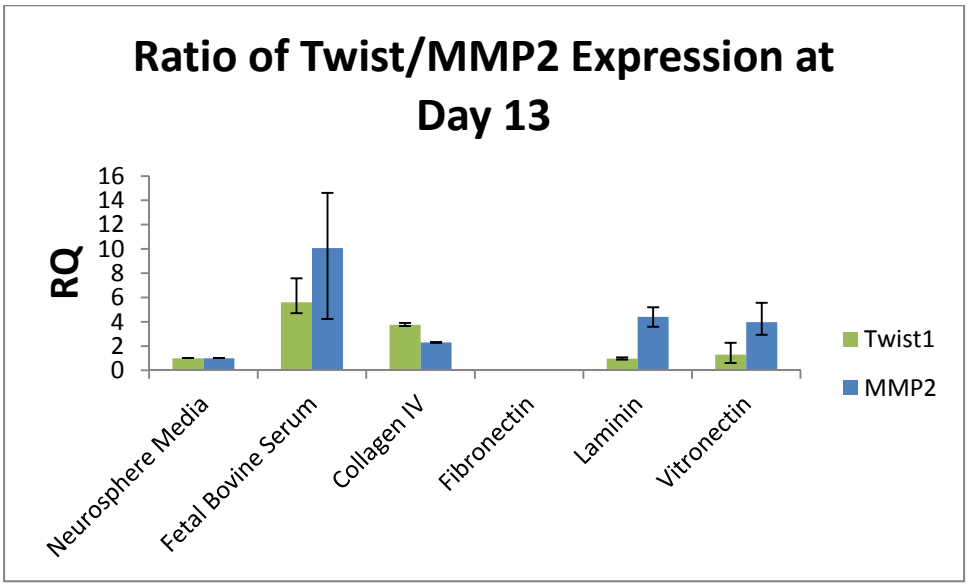


Figure 16. *Ratio of Twist/MMP2 gene expression following 7 and 13 days* – A.) Side by side comparison of *Twist* and *MMP2* expression following 7 day harvest. It appears as though *MMP2* expression is dependent of *Twist* expression. B.) Coexpression at 13 day harvest, once again is appears as though *MMP2* expression is dependent of *Twist* expression, but it's interesting to note that MMP2 is increased the most in cells that formed monolayers, linking it to migration as other researchers have.

V. Discussion

In this study the effects of neural ECM proteins on GBM invasive properties were investigated by observing growth morphology and by measuring Twist1 and MMP2 gene expression to reflect an EMT-like transition. We predicted that monolayer formation caused by ECM protein plate coating would increase Twist expression and MMP2 expression would also increase as a downstream response to Twist. Our results, however, suggest that while several ECM proteins induce monolayer formation, this growth property is not uniformly linked to Twist1 expression as seen with whole serum induction. Similarly, increases in Twist1 expression do not appear to directly induce MMP2 expression, since some ECM proteins were able to induce MMP2 expression in the absence of Twist1.

It has been previously reported that GBM undergoes an EMT-like transition, although this EMT-like response does not involve the canonical cadherin switch and is triggered but a sole transcription factor *Twist* [16,17]. Our *In vitro* cell culturing system mimics the findings of these earlier reports in that we have found TWIST to be up-regulated in the presence of serum, and heightened *Twist* expression is associated with an increase in *MMP2* expression. Our results from day 7 to day 13 show significant increases in both EMT markers *Twist* and *MMP2* which verifies our cell culture system as an accurate means of modeling glioma invasion. It has been widely accepted that environmental factors of the neurological ECM have considerable influence on the ability of glioma cells to migrate and invade[18,36,39,41,52,53]. However, the role of these proteins in EMT glioma invasion has yet to be elucidated.

We have made headway into this task by examining, the ability of the fibrous proteins vitronectin, fibronectin, laminin and collagen IV in the initiation of EMT through the upregulation of *Twist* and *MMP2*. Our findings provide new insight into the formation of monolayers due to these ECM ligands and the connection of monolayer formation with *Twist* induction. Our results confirmed that the fibrous ECM proteins fibronectin, laminin and vitronectin formed monolayers to various degrees. Comparatively, these monolayers where not similar in morphology, which shows that cell-ECM binding occurs, are different affinities. This is in correspondence to others who determined that monolayer formation of various ECM substrates is cell line specific[32,53].

HF2587 cells grown on fibronectin, laminin, and vitronectin coated plates had altered monolayer phenotypes. When these cells were grown on fibronectin the monolayers exemplified slight cell-cell adherence and loose binding to the substrate. This same finding has been seen with the GBM cell lines U-251 and SF-767, which show no specific attachment to fibronectin at normal concentrations but slight attachment at higher dose concentrations[53]. While harvesting the seeded HF2587 cells from these coated plates the attachment was so loose that, pipetting the media back and forth lifted the cells form the bottom of the wells diminishing the need to use trypsin. On the contrary, HF2587 cells grown on laminin coated wells showed pronounced adherence, within the first four hours of seeding and continued to do so throughout the experiment. Laminin coated plates reached 90% confluence within the first 72 hours of seeding, and cell counts from the first harvest revealed that cell proliferation was unaffected by monolayer formation. In fact, cell counts from the 7 day harvest, confirmed that cells grown on laminin coated wells had the highest proliferation rate amongst the ECM proteins. Since no proliferation

assay was undertaken, these results are more qualitative then quantitative. Results from other studies have confirmed that laminin is the optimal adhesion substrate for GBM cells, but there is conflicting evidence in regard to proliferation rates[52,53].

The extracellular matrix protein vitronectin has been extensively studied and our observations include increased cell-substrate adherence and formation of monolayers as observed by others[52,53]. Like laminin, our cell counts during cell harvesting indicated higher than expected proliferation rates. Our most intriguing ECM protein was collagen IV, when cells where grown on collagen IV, monolayers did not form, while intact spherical clusters of cells gathered to form massive cellular sheets. Cell counts from collagen IV coated wells were substantially lower than all other ECM coated wells. This finding is unlike others who have demonstrated the ability of collagen IV to form monolayers and increase migration [52,53].

To determine the effectiveness of ECM proteins on the induction of EMT in GBM, gene expression analysis for *Twist* and *MMP2* was performed to conclude if ECM proteins elicit expressional changes. This led us to several significant discoveries, the first being that monolayer formation on vitronectin, laminin, and fibronectin had little to no effect on the transcriptional activity of *Twist*. Comparatively, these same proteins showed little effect on *MMP2* expression at day 7 but, by day 13 the expression level of *MMP2* was significantly altered showing that there is a direct correlation to monolayer formation and *MMP2* expression. More importantly this time course also proves that transcriptional up-regulation is a time dependent event. *Twist* expression was not significantly altered from day 7 to 13, which opens up to discussion whether *MMP2* is a

direct downstream marker of *Twist*. Our most compelling evidence in the ECMs ability to trigger *Twist* expression comes from the protein collagen IV.

Unlike, the proteins that elicited monolayer formation of HF2587 cells, cells grown on collagen IV remained in spherical clusters and exhibited high *Twist* expression and low *MMP2* expression. In fact, MMP2 expression was much greater in vitronectin and laminin when compared to collagen IV. This finding that collagen IV causes GBM CSCs to sphere and up-regulate *Twist* is an exception to the findings done by others who have demonstrated that Twists up-regulation is associated with increased migration and cell-substrate binding rather than cell-cell adherance[16,17].

These findings suggest that monolayer cellular adherence does not necessarily mean an EMT-like conversion and the data presented here supports this idea. In an attempt to make a logical explanation of the findings of this experiment, the possible role of collagen IV could be to up-regulate *Twist* in GBM. Cells grown on collagen IV were EMT-like in their slow growth rates and their induction of *Twist*, but did not show the change in phenotypes from an epithelial-like state to a mesenchymal state. During the progression of GBM EMT, the enlarging tumor mass comes into close contact with the vasculature of the brain. This vasculature contains high concentrations of collagen IV and laminin. When tumor cells associate with collagen IV this could initiate the up-regulation of *Twist,1* while laminin acts as a permissive substrate for migration. The fact that MMP2 was entirely up- regulated in monolayers further shows its role in aiding in invasiveness.

We can conclude that the monolayers formed on laminin, vitronectin and fibronectin were not EMT –like due to high amounts of proliferation and low

transcription of Twist. On the contrary, collagen IV was similar to an EMT-like phenomenon in that proliferation slowed and transcription of *Twist1* was heightened. But further experiments must be carried out to clarify these results.

Areas of improvement for this study would include the use of a migration assay to determine the effects of these proteins on cellular migration. Along with this a cell-titer glow experiment should be done in order to determine the proliferation rates of HF2587 cells on the various ECM proteins. Equally important it would be interesting to find out if other GBM cell lines exhibit similar *Twist* induction on collagen IV.

To conclude, our preliminary experiments show that collagen IV induces *Twist* expression in the presence of serum free media which is a novel finding in the realm of current GBM research. To confirm these preliminary results this experiment should be carried out once again to confirm this, and it is now more than evident that our *in vitro* culturing system is an accurate means to further conduct this research.

VI. References

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