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RIVERSIDE

Identification of Potential Molecular Targets in Tumor-Associated Endothelial Cell in
Glioblastoma Multiforme

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of the requirements for the degree of

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in

Genetics, Genomics, and Bioinformatics

by

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December 2020

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“Do not follow where the path may lead... go instead where there is no path and leave the trail...” That was the best phrase to describe my PhD journey. It was the hardest trip and the longest night where many times I think I should give up....

Here comes out I am writing the page of acknowledgements.

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“We must believe in our souls that we are somebody, that we are significant, that we are worthwhile, and we must walk the streets of life every day with this sense of dignity, the sense of somebody-ness.” -- Colson Whitehead, *The Nickel Boys*

Wish the next success come because I learned the great adventure from this journey.

ABSTRACT OF THE DISSERTATION

Identification of Potential Molecular Targets in Tumor-Associated Endothelial Cells in
Glioblastoma Multiforme

by

Jui-Yu Liao

Doctor of Philosophy, Graduate Program in Genetics, Genomics and Bioinformatics
University of California, Riverside, December 2020
Dr. Raphael Zidovetzki, Chairperson

Glioblastoma multiforme (GBM) is an aggressive, primary brain tumor characterized by extensive vascularization and a high degree of invasion. The median survival time of the patient is only 12-15 months with the high recurring rate despite the earlier diagnosis and conventional therapy. In this thesis, we mainly considered the endothelial cell as the key role in tumor microenvironment with the clinical significance by applying the cell model from isolated human specimen. Brain endothelial cells originally play as the critical role to tightly attach as the inner layer of blood vessel and closely connect with the nearby cells. During the tumor development, the difference of cell morphology and genetics variances were observed between healthy brain endothelial cells (BEC) and tumor-associated endothelial cells (TuBEC). Isolated TuBEC presented relatively larger, veil-like, irregular cell shape compared to the BEC which had smaller and rod shape with tight junctions. We then hypothesized that the cellular process endothelial-to-mesenchymal transition (EndMT) occurs in the brain endothelial barrier in GBM with the properties to support the angiogenesis and invasion. Our *in silico* gene enrichment results from microarray indicated

that EndMT was one of the top significant variances with the significant enrichment score. Several candidate targets/ pathways were summarized and investigated to trigger the EndMT from gene expression data. The main blockades of Nicotinamide (NAM) metabolism-- salvage pathway and clearance pathway, were linked with EndMT in GBM. By using the inhibitor FK228 to block the clearance pathway, we further successfully confirmed that one of NAM blockade impaired the EndMT. Other two novel candidates, inducing the periostin (POSTN) and metallotheionin (MT), were investigated in our model with EndMT. To sum, the significance of this thesis is to clarify the potential molecular mechanism of the specialized BEC transition in GBM and further identify an approach for terminating the mechanism in primary brain tumors.

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CONTRIBUTION

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Jui-Yu Liao wrote the manuscript. Don Armstrong, Jui-Yu Liao, and Raphael Zidovetzki analyzed the microarray and summarized the table of candidates.

CHAPTER 1

Jui-Yu Liao wrote the manuscript. Florence Hofman and Raphael Zidovetzki conceived the project. Jui-Yu Liao performed the experiments and analyzed the data. Nagore Isabel Marin Ramos, Don Armstrong, and Raphael Zidovetzki revised the manuscript

CHAPTER 2

Jui-Yu Liao wrote the manuscript. Florence Hofman and Raphael Zidovetzki conceived the project. Jui-Yu Liao performed the validated experiments and analyzed the data.

INTRODUCTION

Role of Brain Endothelial Cells during the Tumor Progression and its Therapeutic Opportunities in GBM

ABSTRACT

Glioblastoma multiforme (GBM) is notorious for patients who have the extremely short survival time- around 14.6 months, in average. The healthy brain endothelial cells (BEC), which form the normal brain blood vasculature, exhibit tight junctions and are relatively quiescent. When GBM occurs, glioma tumor has the distinguishing features of the highly vascularity where the blood-brain barrier (BBB) is quickly controlled by the tumor cell in brain microenvironment. Anti-angiogenic therapies provide the possible solution for GBM treatment. To reveal the novel solution for GBM, the major player, the tumor-associated brain endothelial cells (TuBEC), is aiming to discuss with its morphology, molecular mechanism, and genetic variance in this chapter. TuBEC presented decreased tight junction proteins resulting the extensive permeability in vasculature, as well as high levels of endothelial cell proliferation, migration, and invasion of normal brain tissue [1]. Isolated TuBEC from human specimen have large, flat, and veil-like appearance and exhibited mesenchymal properties. Based on cell morphology, gene expression and *in silico* evidence, we proposed that endothelial-to-mesenchymal transition (EndMT) is the key mechanism to mediate the transdifferentiation in endothelial cell. Two major questions are further explored: (1) Which gene(s) are responsible for this EndMT? (2) What pathways are involved in the transition from BEC to TuBEC? The significance in this chapter is to integrate the current understandings of TuBEC in GBM in order to further discussing the potential target/ strategy of anti-angiogenic therapy in GBM.

I.1. Background

Brain blood vessels support the brain circuits for the sensation, memory and motion [2, 3]. The adequate blood supply, oxygen delivery and energy metabolite supply are instrumental to regulate the proper structural and normal brain functioning [4]. This highly regulatory activity in brain was maintained in the neurovascular unit (NVU) which define as the area cooperation with multiple cells, including endothelial cells, pericytes, smooth muscle cells, glia, and neurons [5]. In pathological situation, NVC compromise the regulation of blood supply and integrity of the unique feature in brain, the blood-brain barrier (BBB), to adapt. Abnormal brain vasculature provides uncoordinated cell growth and rapid cell proliferation to change the function of normal vasculature.

Glioblastoma (GBM) is the most aggressive and common primary brain tumor in adults, accounted for 47.7% of primary malignant brain tumors and defined as the highest grade of gliomas [6, 7]. The median survival life for patients is approximately only 14.6 months in average from time of initial diagnosis despite the current treatments with standard surgery, radiation and alkylating chemotherapy [8]. GBM is notorious based on the characteristics of high proliferation index, complexity of microvasculature among all the solid tumors [9]. This extremely high vascular proliferation presents the highly abnormal and dysfunctional vasculature system in morphology, functionality and molecular characteristics. In clinical, the malignancy level of glioma is directly associated with abnormal endothelial cells in BBB where GBM belongs to the highest grade (grade IV) of glioma [10].

With the complex feature of blood vessels in GBM, neovascularization is crucial for the glioma growth and survival. The rapid growth of tumor is mainly facilitated by the interaction between tumor cells and blood vessels [9, 11]. During the glioma development, the abnormal BBB, mainly formed by tumor-associated brain endothelial cells (TuBEC) within the NVC, undergoes the rapid formation with the properties of vessel leakage and vessel permeability [12]. The corrupted vasculature allows the movement of extensive growth factors and cell migration from glioma tumor, especially the glioma stem cells (GSC), to pass through the blood flow [11]. GSC regulate and promote to the tumor vasculature for trans-differentiation in endothelial cells while blood vessels also support the glioma cells [11]. By passing through and migration in vasculature, glioma tumor cells are able to occupy the healthy tissue, induce the formation of new abnormal vessels, and extend the tumor growth in the NVU. Glioma cell stimulate the rapid blood supply in order to provide the high nutrient and oxygen demand. For example, the mechanism of hypoxia is the typical and well known driver during the abnormal neovascularization. Brain cells in hypoxic exposure induced the expression of vascular endothelial growth factor (VEGF) and cause the vascular leakage [13]. Further evidence indicated that non-hypoxia-driven mechanisms still exist and require to be explored.

Anti-angiogenic therapy can consider as a critical strategy to effectively eliminate the tumor growth because most tumors become dormant without well-developed neovascularization [14]. The anti-angiogenic inhibitor bevacizumab, a humanized monoclonal anti-VEGF-A antibody by neutralizing VEGFs, has been in clinical trial application for over 15 years [15]. However, the bevacizumab did not provide the longer

survival rate for patients in GBM. [16]. Even when in some of cancers, such as ovarian cancer, it provides positive response, the resistance and recurrence of tumor is still observed in GBM patients [16, 17]. These results illustrated that the loss of VEGF is not the only role in controlling the formation of tumor vasculature. It is still unclear what the real mechanism is, and it requires to be investigated in neovascularization. Antiangiogenic therapies are the valuable strategy which not only inhibit the tumor growth but also normalize the vasculature to improve the rate of drug and oxygen delivery. More solutions and detail mechanisms should be clarified with the combination of other radiotherapy and chemotherapy in the future.

In vasculature, BEC has been confirmed as the primary targets in the neovascularization for optimal tumor progression [9]. Normal BEC belongs to the inner layer which tightly covered the blood vessel. The plasticity of BEC is demonstrated to potentially modify their phenotype and differentiate into mesenchymal type of cell undergo the endothelial-to-mesenchymal transition (EndMT). The transformed BEC, known as tumor-associated brain endothelial cells (TuBEC), were also considered as one critical source for the cancer-associated fibroblasts (CAF), the most abundant stromal cells for invasion, migration and expansion in tumor microenvironment (Figure I.2) (Section I.6). The diagnostic criteria for GBM is corresponded to the morphologically atypical shape and results the dysfunctional vessel during this abnormal processes [18]. Thus, the objective of this chapter is to provide the comprehensive understanding in the BEC during tumor development. The main contribution was coming via, BEC and its further therapeutic application, will be mainly discussed and summarized in this chapter.

I.2. Brain vasculature: cellular components of blood brain barrier

The vasculature plays a crucial role in transport of oxygen and nutrients, removal of cellular waste and regulation of blood flow [11]. A defining feature in brain is the specialized vasculature, particularly the BBB. The specialty of BBB limits the entry of potentially neurotoxic plasma components and separates the blood cells and pathogens from the brains. It functions as a dynamic regulator of ion balance, facilitator of nutrient transport, and barrier to harmful molecules [19]. Over the past two decades, researches have demonstrated that the substantial intercellular communication happened between cells of the vasculature as well as the neurons and glia that connect to the vasculature [20]. These findings illustrate that the BBB does not function alone, but as a module within the greater context, which called neurovascular unit (NVU), and includes surrounding neurons, astrocytes, pericytes, and microglia as well as the blood vessels themselves. Among these cells, BEC belong to the core unit of BBB with the characteristics to tightly attach as the inner layer of blood vessels and contact to other cells [21].

The BEC are surrounded as a thin inner layer in capillaries and larger vessels. This critical endothelium layer allows the entrance of rapid free nutrients and oxygen from blood to brain and tightly regulate the pH and normal brain metabolism. Only the smaller lipophilic molecules and drugs can pass this layer [22]. In capillaries, tightly packed BEC were accompanied by pericytes; in larger vessels, arterioles and arteries, BEC are associated with both pericytes and smooth muscle cells. The basement membrane, secreted

by BEC, is the extracellular matrix for cell stability, structural support and anti-coagulation status [23]. BEC play as the cross-functional role among these cells which are directly associated with astrocytes, pericytes, and smooth muscle cells.

I.3. General characteristic of normal brain endothelial cells (BEC)

The normal BEC in blood vessels are the primary cells in angiogenesis. They are unique among the cells in NVU with the cell morphology of rod, spindle-shaped and small height [24]. They express the adhesion molecules for the adherens junction (AJ), such as cadherins and PECAM-1, and express specific proteins for tight junction (TJ), and membrane-associated guanylate kinases (ZOs) [5, 24]. As the physical barrier, the structure of TJ in brain has been found to be the most complex among the vasculature of the entire body [25]. As the distinguished feature of BEC, TJ is composed of the combinations of integral membrane proteins including occludins, claudins and cytoplasmic proteins that couple these transmembrane proteins to the actin cytoskeleton [20].

In addition to a physical barrier, BEC form a selective transport interface between the blood and the brain which contains polarized expression of transporters, metabolite-degrading enzymes, receptors, ion channels, and ion transporters [26]. The selective transport also ensures the delivery of nutrients such as glucose, amino acids, nucleosides, and electrolytes from the blood to the brain and that solutes, nutrients, and metabolite waste products are transported from the brain to the blood [26]. The specialization of BECs reflects the unique requirements of an organ in a homeostatic ionic environment and

protection from neuroactive blood-borne solutes [21]. In the health/ normal situation in brain, BEC are usually quiescent with only 0.01% cell dividing in the normal vasculature [27, 28]. The balance between pre-angiogenic factors and anti-angiogenic factors, such as vascular endothelial growth factor (VEGF) and thrombospondin (TSP-1), were supported by this quiescence from normal endothelial cell [29].

I.4. Brain vasculature: the interaction when GBM occurs

The abundant aberrant vasculature with high permeability is the hallmark characteristic in GBM diagnosis. When the GBM occurs, glioma tumor cells usually localize in the perivascular spaces of the NVU. They can later remodel, destroy, and vascularize abnormal, dysfunctional NVU structure in order to ensure adequate oxygen and metabolic support through these newly induced vessels. In order to achieving the tumor invasion, tumor cells near blood vessel employ and loosen the extracellular matrix (ECM) proteins, such as collagens, fibronectins and vitronectin by secreting the growth factor proteins [30]. While supporting the survival and proliferation of the new tumor cells, glioma cells activate the signaling pathways of TGF- β , cytokine, notch, sonic hedgehog, and further strengthen the tumor's resistance [18, 31-34].

The BBB did not show the apparent disruption during early stage of GBM development while the tumor still supported the normal vessel and not yet destroyed the pre-existing vasculature [18]. When glioma progresses, the BEC start to migrate and transform in several new angiogenic spots. Secreted soluble molecules and extracellular

vesicles which are released from tumor cells contact with the basement membrane, destroy the TJs between BECs and cause the morphology changing of microvasculature [10]. During the process, BEC start to transform into TuBEC with not only morphology differ but also the genetic instability [1].

The certain spots from existing normal vessel formed the tumor blood vessel, defined as angiogenesis, by inducing the abnormality of BEC [10]. The phenomenon occurs and causes the irregular, permeability abnormal vessels by the stimulation of secreted soluble factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor, and angiopoietin from glioma tumor cells and cancer associated fibroblasts (CAF)[18]. The CAF, as the most abundant stromal cells in the tumor microenvironment, were considered to originate from different cells, including normal fibroblast, mesenchymal stromal cells, epithelial cells as well as the endothelial cells by the process of EndMT [35, 36]. Hypoxia response and accumulated reactive oxygen species (ROS) have been confirmed to increase the permeability of TJs in BBB and support the inflammation, apoptotic resistance, metabolic reprogramming, and EMT in order to facilitate the tumor progression [13, 20]. These molecular mechanism are critical because the molecular crosstalk and signaling networks among brain vascular components and tumor cells have been shown to predict patient survival, disease progression, and further extend to therapy in GBM [37, 38].

I.5. Differential characteristics of tumor-associated brain endothelial cells

TuBEC are considered to have the morphological difference and genetic variance compared to the BEC. The isolated TuBEC from human specimen presented the relatively flat appearance, with large nuclei, abundant cytoplasm, multiple nucleoli, and veil-like structures [1]. TuBEC do not have the rod- or cobblestone-like appearance as BEC and have relatively wide cytoplasm.

There were several hypotheses to explain the origin of TuBEC. The first hypothesis is that TuBEC directly developed from BEC by receiving the growth factors from tumor. Several reports, including our researches, supported this hypothesis by detecting the expression of endothelial markers and identifying the morphology difference from isolated TuBEC and the *in vitro* co-cultured experiment with GSC (Section I.8) [11, 39-41]. The second critical proposal illustrated that GSC may transform to TuBEC. This statement was confirmed based on the same position of gene alternations and chromosomal aberration between the subpopulation of TuBEC and glioma cell [42-44]. Other hypothesis illustrated that the transdifferentiation into endothelial cells may occur by observing with EC-liked behaviors from other cell type, including the dendritic cells and monocytes [45, 46]. However, it is not confirmed yet with molecular evidence in GBM. These variations cause the diversity of TuBEC.

Regarding the functionality, TuBEC not only involve in oxygen and nutrient supply but also actively promote the tumor progression. Initially, in the tumor microenvironment, the rapid increased expression of VEGF receptors in TuBEC receive the high amounts of VEGF [47]. It promotes the formation of abnormal blood vessel with high permeability

and then trigger the tumor cell to get into blood stream easier. By changing the expression of adhesion molecules in TuBEC, tumor cells are able to invade between TuBECs. Meanwhile, TuBEC secrete a large amounts of inductive factors to stimulate the tumor growth and tumor migration. These factors include the paracrine factors, such as endothelin-1, bFGF, TGFbeta, interleukin-6 and IL-8, and angiocrine factors, such as IL-6, IL-3 and nitric oxide (related to Chapter 1) (Figure I.1A). For example, in the cross-talk, TuBEC can produce Jag1 and DLL4 to recognize the Notch receptors on GSCs [48]. Self-renewal from GSC was activated to promote tumor growth. The paracrine growth factor IL-8 which was secreted by TuBEC stimulates migration of GSC *in vivo* [49]. The angiocrine factor Ang1 produced by EC binds to Tie2 on GSC and mediates adhesion, invasion and chemoresistance [50]. TuBEC can also secrete factors that maintain GSC self-renewal and survival through activation of the mTOR pathway [51]. These cross-talks by different factors from the side of TuBEC to glioma cells keep maintaining the tumorigenesis and promoting the tumor angiogenesis.

I.6. Role of endothelial-to-mesenchymal transition (EndMT) in tumorigenesis

Glioma tumor cells can overcome the BBB. There were at least three strategies for tumor cell transmigration through the monolayer of BEC, including 1) tumor cell migration through the endothelial cell body, 2) induction of apoptosis in endothelial cell, 3) tumor cell migration through the cell-to-cell junction [52]. From previous morphology founding in our team, the isolated TuBEC from human specimen presented relatively flat, veil-like

appearance [1]. In order to validate the potential strategies, some of gene marker which belongs to the typical mesenchymal and endothelial functions was investigated and published in 2019 in our lab [40]. Given these observations in our previous researches, we set out to investigate whether the difference between TuBEC and BEC correlated with the endothelial-to-mesenchymal transition (EndMT). To give the overview picture, Gene set enrichment analysis (GSEA) is used to determine the significant biological process. We then collected the gene expression data of endothelial cells from public references and database for enrichment analysis. The results showed that the cellular process, epithelial-to-mesenchymal transition (EMT), has the significant enrichment score (p -value <0.05) in biological process (Figure I.3) (Materials and methods were shared with Don Armstrong and was described in the legend of Figure I.3).

EMT is integral mechanism in development and pathological processes in wound healing, fibrosis and cancer progression. Epithelial cells lose their cell-to-cell junction and apical-basal polarity and undergo a change of signaling network which increase the cell motility and enable the invasive phenotype. Similar to the EMT, endothelial-to-mesenchymal transition (EndMT) is one of the pathological mechanisms that happened in endothelial cells. EndMT take place during tumor progression to achieve neovascularization and to open the blood-brain barrier (BBB) [18, 53, 54]. The properties of BBB are determined by junctional protein complexes between the brain endothelial cells (BEC) [55]. In order to break through the BBB during the tumor progression, EndMT induces the trans-differentiation of mature BEC into mesenchymal cells with large, flat, and veil-like appearance within the tumor [56], This morphological appearance of this type

of cell is similar to what we observed in TuBEC [42, 57, 58]. During EndMT, TuBEC lose cell-to-cell junctions and acquire a mesenchymal-like phenotype, as well as invasive and migratory properties [35].

Based on the data in morphological founding [1], molecular evidence [40], and statistical difference (Figure I.3), we propose EndMT as the main strategy for tumor cell to overcome BBB. EndMT is the transdifferentiation of mature EC into mesenchymal cells induced by locally-secreted cytokines and growth factors in the tumor microenvironment [59]. This cellular process originally belongs to a fundamental cellular process which has been first described in embryogenesis and cardiac valve disease [60]. EndMT has been known to be associated with the formation of carcinoma-associated fibroblasts and the promotion of cancer metastasis [35, 54]. The process of EndMT is initiated by autocrine and/or paracrine inflammatory signals from the surrounding cells [54]. During EndMT, these specialized EC show degradation from their vascular basement membrane, and loss of the cell-to-cell junction [10]. The EC furthermore present the mesenchymal stem cell-like phenotype and acquire the invasive and migratory properties [61, 62]. By detecting the expression level from the well-known endothelial or mesenchymal markers (Figure I.1C), investigators will be able to distinguish two different cell types during the process of EndMT [63].

I.7. Main Signaling Pathways of EndMT in GBM tumorigenesis

Several well-known signaling pathways in EndMT, are identified and associated with the BBB function. The five well-known signaling pathways, including transforming growth factor beta (TGF- β), bone morphogenetic proteins (BMPs), Notch, Wnt, and Hedgehog (Hh), all exert their effectors in the endothelial cell (Figure I.1B). For example, in the Smad-dependent pathway, TGF- β can induce the EndMT by the expression of the Snail family members [64, 65]. The studies have further shown that TGF- β stimulation in the mouse BEC can change the BBB permeability [66, 67]. For the BMP signaling, the loss of the *CCM1* gene leads to an up-regulation of BMP-6 [68]. This upregulation can, in turn, activate both TGF- β and BMP signaling pathways and then lead to the induction of EndMT. The BMP2 treatment *in vitro* can also activate endocardial EndMT through an ALK2-dependent manner and attenuate the Notch1 signaling with the expression of the *hey1* and *hey2* transcription factors [69].

The Notch signaling cascade also contributes to the EndMT as well as the integrity of the BBB. Loss of the *Jag1*, which is one of the five Notch ligands, disrupts EndMT during endocardial cushion formation [70]. The nonsense mutation of Notch3 in the brain of zebra fish has enhanced BBB permeability and led to a reduced number of pericytes [71]. In the canonical Wnt/ β -catenin pathway, the vascular ECs exhibited the reduced cell-cell adhesion and increased the permeability in the β -catenin-deficient mice [72]. However, it needs to be noted that many studies of EndMT were performed on the embryo development or the non-brain-derived ECs. Recently, studies started to connect the EndMT with the various brain diseases. Maddaluno et al., 2013, demonstrated that the disruption of any of

the three CCM genes can trigger the EndMT in the microvascular BEC [68]. The most recent study also indicated that primary cultures of rat BEC can undergo the EndMT with the stimulation of TGF- β from other cancer metastasis [73]. They further confirm that the metastatic tumor cells from other organs can gain access into the brain by crossing the BBB under the mesenchymal phenotypic cell. Hence, it is more convincing that EndMT may be required for breaking the BBB under the invasion of the primary brain tumors.

In this thesis, we aim to identify the non-typical candidates in EndMT in GBM. Table I.1 was the candidates we selected. These candidates were selected based on significant p-value in GSEA. They represent different five pathways/ biological process, including Nicotinate and nicotinamide metabolism, Wnt/beta-catenin, Purine Metabolism Metallothioneins and EMT, were going to discuss later in Chapter 1 and Chapter 2.

I.8. Current model in lab to study the transition of brain endothelial cells

In order to understand if the certain mechanism/genes can impair the EndMT, the *in vitro* model with cell culture are required to assist us for exploring the biological meaning from our candidates. GSC play a major role in transforming the healthy BEC into TuBEC [74]. Previous studies from our laboratory using an *in vitro* co-culture system with GSC and patient-derived BEC, successfully established that GSC induce EndMT in BEC [40]. This model was applied in the following experiment in thesis (Figure I.4).

In the standard process from our previous research, GSC seed on the plate with gelatin-coating first. After 24 hrs, medium removed and another cell, BEC, seed with

specific EBM medium. After 72-120 hrs of co-culture with GSC, the population of BEC started to display mesenchymal features, as evidenced by high expression of the mesenchymal marker alpha smooth muscle actin (α -SMA) (Figure I.3) compared to the BEC line alone. We expected the *in vitro* model to present mesenchymal feature of BEC by applying the immunostaining. The typical markers in Figure I.1C can be examined for the progress of EndMT. The standard results represented that the BEC with mesenchymal feature transitioned into TuBEC during the co-culturing with GSC. In the following works, we used this conducting EndMT model *in vitro* to determine whether inhibiting the NNMT can terminate the EndMT in BECs.

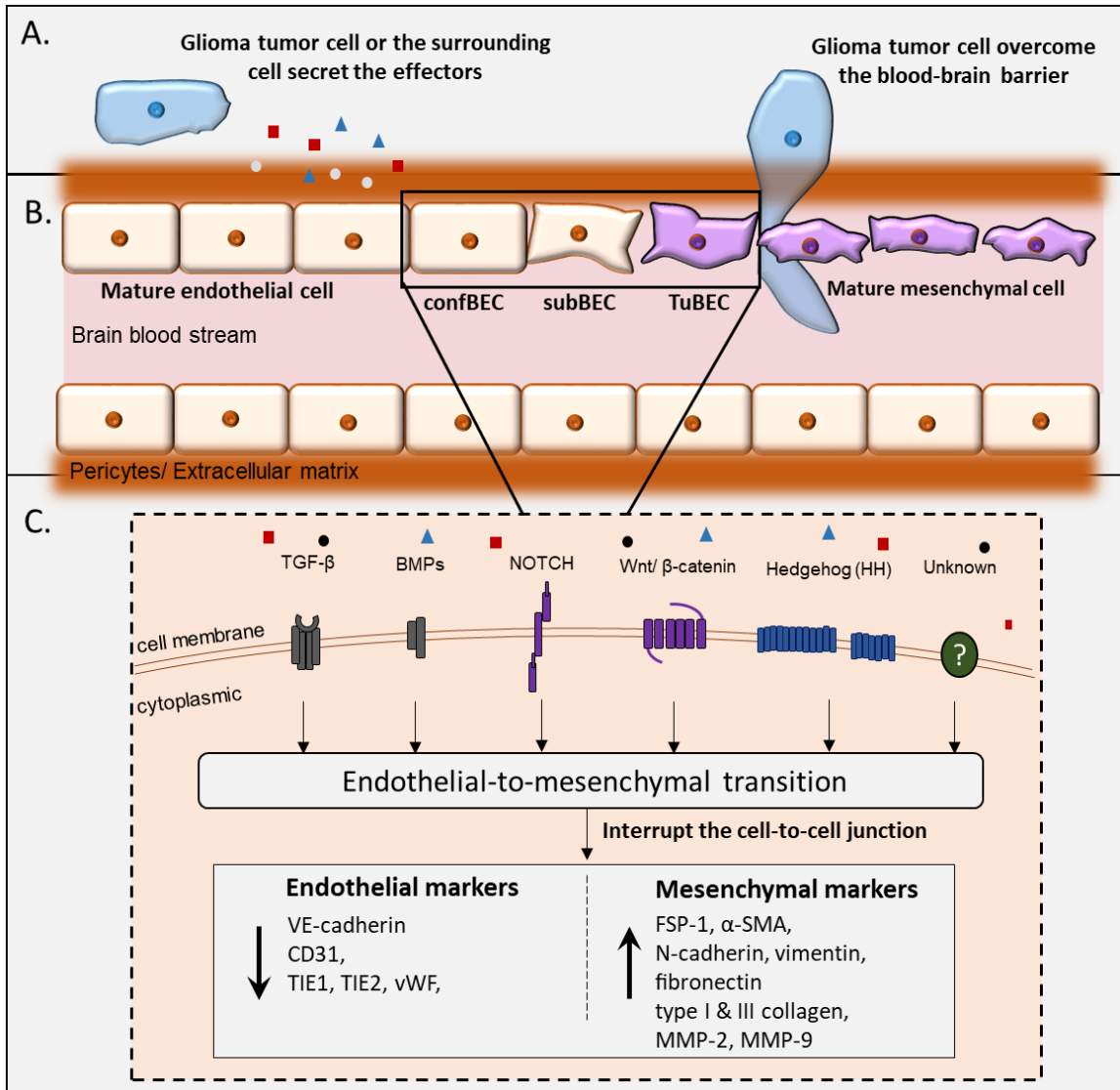


Figure I. 1 Overview of the hypothesis in brain tumor microenvironment.

(Figure description continue to the next page.)

(Figure I.1., description)

(A) Glioma tumor cells (blue) can secrete the effectors which are received by the tightest mature brain endothelial cell (BEC). After disrupting this blood-brain barrier (BBB), glioma tumor cell would be able to further invade into the blood vessel [75].

(B) The proposed model is highlighted in the brain blood vessel. After receiving the effectors from the surrounding tumor environment, BEC is proposed to undergo the transition into the mesenchymal cell. The confluent BEC (confBEC) represent the “natural” healthy cell. The sub-confluent BEC (subconf BEC) is the cell type which already partially activated by GBM cells. And the tumor-associated BEC (TuBEC) represented the cell transformed into tumor supporters. Three type of BECs in this potential transition process will be used as our experimental materials [58].

(C) The pathway in GBM microenvironment is still unclear. At least five potential pathways have been described in endothelial-to-mesenchymal transition (EndMT) in the embryo development or other disease. Six arrows contain lots of kinases, regulators, and transcription factors which is simplified here. After triggering the EndMT, the expression of endothelial markers is decreased. The mesenchymal markers are attenuated and the cell-to-cell junctions are loosen between those transformed-endothelial cells [63, 76].

This figure is combined the summary from these review articles [58, 63, 75, 76].

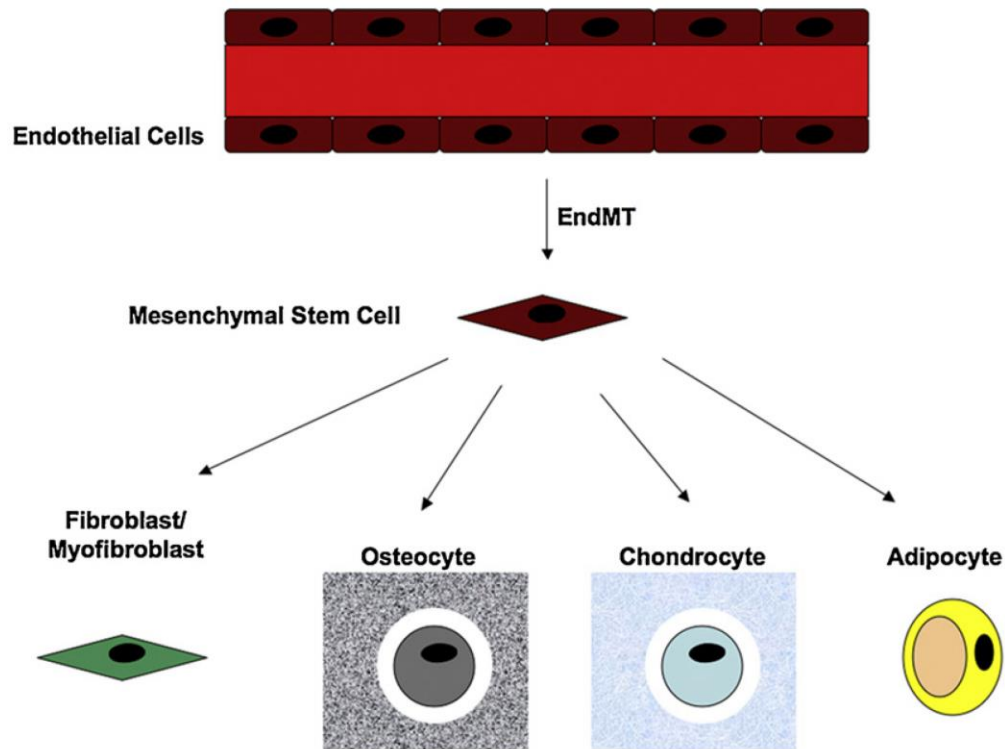


Figure I. 2 Basic cellular process of endothelial-to-mesenchymal transition (EndMT) in the embryo development.

Figure was made by Medici, D., and Kalluri, R. in 2012 [63]. Originally, EndMT is the basic cellular process in embryo development. Picture were showed that the endothelial cell undergoes the EndMT and acquired the stem-cell-liked ability to transform firstly into mesenchymal-type-cell. They further transdifferentiated into several different type of cell in the cellular process [63]. When it occurs in the pathological condition, such as GBM, the potential process was presented in Figure I.1.

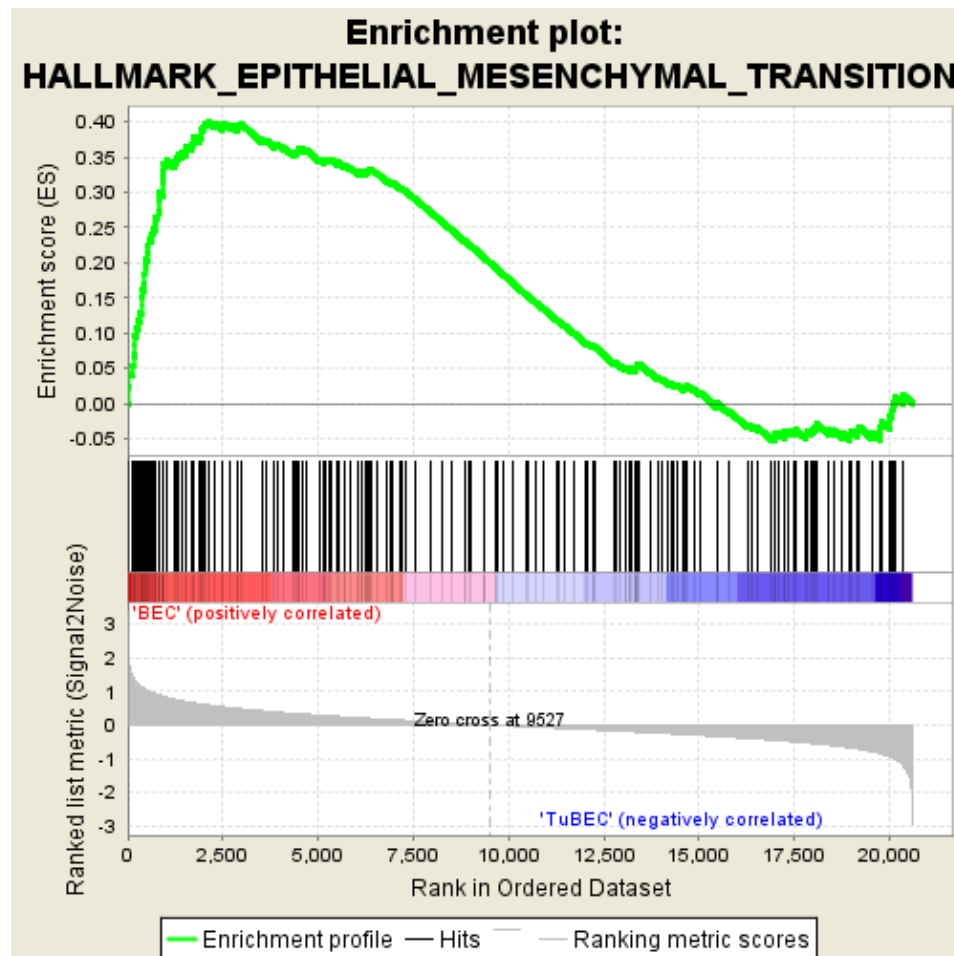


Figure I. 3 Gene set enrichment analysis (GSEA) of the regulated genes in relation to EndMT in TuBEC compared to BEC.

Shown is the enrichment plot for the epithelial-to-mesenchymal transition gene set correlated with the healthy BEC versus TuBEC from human specimen in GBM. The enrichment score (ES) is indicated. The evaluated p -value is 0.042 which is <0.05 .

(Materials and methods were made together with Dr. Don Armstrong and Dr. Raphael Zidovetzki. Detail descriptions are in next page)

Supplementary of materials and methods in Figure I.3:

Gene Microarray Analysis

Cells were isolated as the description in Chapter 1. Both BEC and TuBEC in microarray analysis were seeded at a density of 5×10^5 per 10-cm dish and grown for 48 hours. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen Inc., Valencia, California) according to the manufacturer's instructions. Total RNA quantity and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, California). The RNA duplicate with the highest quality was reverse-transcribed and hybridized with an Affymetrix HU133 Plus 2.0 (Affymetrix Inc., Santa Clara, California) microarray system. Probe pair intensities were extracted using the statistical software package R (R Foundation for Statistical Computing, Vienna, Austria), and normalized using the MvA kernel density method. P-values were calculated using the Student t test modified with a Bayesian statistic. The q-value procedure, an estimate of the false discovery rate (FDR), was used to correct for multiple testing. To avoid the biases but with enough tolerant, the $FDR < 0.1$ was considered statistically significant. Total 93 genes were pre-selected.

Gene Set Enrichment Analysis (GSEA)

The total 93 genes were considered as the input of GSEA. The package was applied in python 3.0 on the standard respiratory clone from the cloud server Github (<https://github.com/juiyuliao/GSEA>). The database for matching were downloaded from the original source of GSEA website (<https://www.gsea-msigdb.org/gsea/index.jsp>), the

MSigDB database. GSEA calculates the enrichment score (ES) by walking down the ranked-ordered list of genes, increasing a running-sum statistic when a gene is arranged based on its p -value in the gene set without consider the fold change [77, 78]. The generated plot (Figure I.3) contains in the middle a rank-ordered list of 93 genes identified as the most variable targets. The top of this list (red color, Figure I.3) contains genes upregulated in BEC-downregulated samples. The bottom of the list (blue color, Figure I.3) contains downregulated genes in BEC-upregulated samples. Anytime a gene from the gene set is found along the list, a vertical black bar is plotted (hit). If most of the hits are at the top of the list, then this gene set is enriched in BEC-downregulated cases. However, if they are distributed homogenously across the rank-ordered list of genes, then that gene set is not enriched in any of the gene expression profiles.

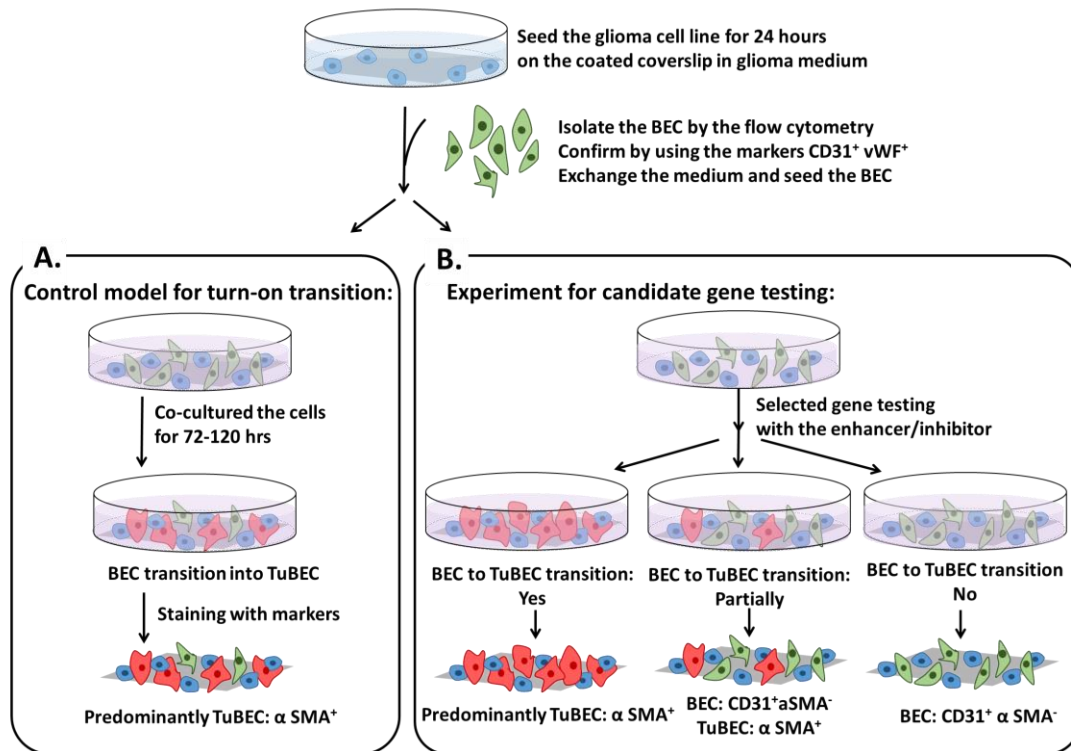


Figure I. 4 Co-culture system of BEC with GSC in vitro.

Co-culture of BEC with GSC (USC02) is the basis of the BEC to TuBEC transition in vitro assay. This assay monitors the transition and the effects of drugs or proteins. In brief, U251 glioma cells are plated on coverslips coated with collagen for 24 hours. Subsequently, BEC are seeded on the plates with the glioma cells with the appropriate activators/inhibitors and experimental controls. The co-cultured cells are then incubated for another 72-120 hrs, Finally, coverslips are fixed and stained for distinguishing markers, Markers for the BEC are CD31 and von Willebrand factor (vWF). The markers alpha smooth muscle actin (αSMA) and CD31 will be used to confirm the TuBEC phenotype. Cells in blue are the glioma cells. Cells in green are the BEC, and can be stained with the CD31-green fluorescent protein (GFP) antibody. Red cells represent TuBEC which express αSMA.

Table I. 1 Five pathways are identified by Gene Set Analysis and 16 genes that are associated with BEC – TuBEC transition.

Nicotinate and nicotinamide metabolism^a	Wnt/beta-catenin^b	Purine Metabolism^c	Metallothioenins^d	EMT^e
QPRT	AKT3	PNP	MT1H	GAS1
PNP	DKK3	AK5	MT1M	NNMT
NMNAT2	DACT1	ENTPD1	MT1X	POSTN
NNMT		PDE5A		CALD1

^a. Previous GBM/cancer studies in the pathway of the Nicotinate and nicotinamide metabolism [79, 80]. ^b. Previous GBM/cancer studies in the Wnt/beta-catenin pathway [81, 82]. ^c. Less reference in the Purine Metabolism directly link with GBM [83]. Some of them mentioned the connection with cancer [84]. ^d. Metallothioenins also have the novelty in glioma [85]. However, its regulation in endothelial cell have been mentioned to participate in the regulation of angiogenesis [86]. E. EMT can be referred as EndMT in the endothelial cell which have been mentioned recently in cancer [87, 88].

CHAPTER 1.

Nicotinamide N-methyltransferase induces an endothelial-to-mesenchymal (EndMT) transition in GBM

ABSTRACT

Tumor-associated brain endothelial cells (TuBEC) cover the inner surface of tumor blood vessels and require an urgent energy metabolism to rapidly supply nutrients to tumor cells. Nicotinamide (NAM) mediates the critical metabolic pathways that connects cellular redox states with high-energetic supply. Here, we found that Nicotinamide N-methyltransferase (NNMT), one of the critical enzymes in NAM metabolism, was upregulated in TuBEC in glioblastoma multiforme (GBM). We had previously demonstrated that glioma stem cells (GSC) have the ability to induce an endothelial-to-mesenchymal transition (EndMT) in healthy normal brain endothelial cells (BEC) in co-culture. In this work, by using siRNA transfection assay and FK228 drug inhibition, we demonstrated that knockdown of NNMT in BEC decreases mesenchymal marker alpha smooth muscle actin (α -SMA) levels while keeping the expression of the endothelial marker CD31 in co-culture with GSC. Specifically, NNMT knockdown reduced the expression levels of Vimentin (VIM) and matrix metalloproteinase 2 (MMP2), blocking the EndMT induction. Furthermore, NNMT-induced EndMT mechanism can be blocked by inhibitor FK228. These data suggest that NNMT could be a novel therapeutic target to block EndMT in GBM.

1.1. Introduction

Metabolic aberration in signal transduction networks has a profound effect on tumorigenesis and it is considered as a hallmark in cancer [89]. The role of these key metabolic enzymes remains largely unexplored in tumorigenic processes [90]. They likely act as key regulators of the activation and inactivation of oncogenes and tumor suppressor genes, respectively. Nicotinamide N-methyltransferase (NNMT), a representative enzyme in Nicotinamide (NAM) metabolism, is considered a high priority potential cancer therapeutic target, not only because it represents a critical route to obtain energy in cancer cells, but also because of its role in the regulation of DNA methylation [91-93]. NAM metabolism involves two major metabolic pathways, known as the reversible salvage pathway and the irreversible clearance pathway [92]. NNMT serves as the key enzyme in the NAM irreversible clearance pathway [94].

NAM metabolism via the reversible salvage pathway is conducted by the key enzyme nicotinamide nucleotide adenylyltransferase (NMNAT). In this pathway, NAM is converted to NAM mononucleotide (NMN). NMN is then further converted into NAM adenine dinucleotide (NAD) by NMNAT which can be reversibly converted back to NAM, known as reversible pathway [92]. When investigating the endogenous NMNAT2 levels which is the most abundant NMNAT isoforms in brain, overexpressing NMNAT2 provides neuroprotection in various neurological disorders [95]. In colorectal cancer, activity of NMNAT2 was correlated with cancer suppressed process with p53 transcription factor [96]. The research of salvage pathway connected with GBM still remains largely unknown and needs to be investigated.

In several studies reporting high-throughput data mining in GBM, NNMT ranked as one of the top genes among the most consistently overexpressed metabolic genes in cancer, compared to normal brain [91, 97]. The role of clearance pathway in GBM is relatively clear and important. In NAM metabolism, the clearance pathway relies on the key essential cytoplasmic enzyme, NNMT. NNMT irreversibly transfers the methyl group from S-adenosylmethionine (SAM) to NAM and produces S-adenosylhomocysteine (SAH) and 1-methylnicotinamide (1-MNA) [91, 98]. By transferring the methyl group, NNMT alters the hypomethylated histones in epigenetic regulation and other cancer-related proteins leading to cancer progression [98, 99]. Recently, it was also suggested that NNMT promotes the EMT by activating TGF β 1 expression in gastric cancer cells [100]. However, no studies showing a direct link between NNMT and EndMT have been performed. Thus the exact role of NNMT in EndMT remains unclear.

The goal of this study is to characterize the specific metabolic pathway involved in the EndMT process in GBM. In two of the major NAM pathways, we found that NNMT was activated in TuBEC compared to BEC where the reversible pathway of NMNAT2 was not upregulated. We hypothesized that the irreversible clearance pathway of NNMT can mediate the induction of EndMT. Immunostaining studies in both mesenchymal marker α smooth muscle actin (α SMA) and the endothelial marker CD31 showed that knockdown of NNMT impairs the EndMT process in GBM. By using the NNMT chemical inhibitor assay, we demonstrated that EndMT can be blocked in the GBM environment through NNMT blockade. These results suggest that the NAM metabolic pathway involved

in the EndMT process in GBM is the irreversible clearance pathway. These findings contribute to the understanding of the EndMT signaling in GBM and provide with potential new target NNMT for GBM therapy. The fact that the clearance pathway in nicotinamide is irreversible could explain why reversing the EndMT is so difficult.

1.2. Materials and Methods

Cell isolation and culture

All human tissue specimens were obtained in accordance with the Institutional Review Board guidelines established at Keck School of Medicine, University of Southern California (Los Angeles, CA). The human primary TuBEC and the glioma stem cells (GSC) were isolated from glioma patients as previously described [101, 102]. The normal BEC were obtained from patients with cerebral epilepsy. The isolation process has been described in Jhaveri, Agasse, Armstrong, Peng, Commins, Wang, Rosenstein-Sisson, Vaikari, Santiago, Santos, Chen, Schonthal, Chen and Hofman [102]. The endothelial cells were grown on 1% gelatin-coated flasks, using ECM medium [40], and cells were used between passages 3-8. GSC were cultured in cancer stem cell (CSC) medium. For co-cultures, GSC were seeded on 1% gelatin-coated coverslips in CSC medium with 0.1% FBS to promote cell attachment. BEC were seeded on top of the CSC monolayer after 24 h and medium was then replaced with EC medium.

RNA isolation and real-time quantitative PCR

Total RNA was isolated by using the RNeasy Plus Mini Kit (Quiagen). cDNA was obtained with iScript Advanced cDNA Synthesis Kit (Bio-Rad) following manufacturer's instructions. The primers were designed by using the software Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) and computed the T_m value with Oligoanalyzer from IDT (<https://www.idtdna.com/pages/tools/oligoanalyzer>). Primer sequences used for qPCR amplification are as following: NNMT (sense 5'-TGGCTTCTGGAGG

AAAGAGA-3', antisense 5'- AATCCCGAGGGTTAAA ATGG-3'); NMNAT2 (sense 5'-TCTTGGGGAAG-GTGGGAGAA-3', antisense 5'-TCACTACCACACAGCAG-CAG-3'). Each value was normalized to human β -actin RNA levels as an internal control: sense 5'- TCCCTGGAGAAG AGCTACGA-3', antisense 5'- AGCACTGTGTT-GGCGTACAG-3'. The gene expression levels were assessed by using the SYBR Green Real Time PCR Detection System (Applied Biosystems). Amplifications were run in a StepOnePlus cycler (Applied Biosystems), with an initial denaturation step of 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec using an ABI PRISM 7500 Fast Real-Time PCR System. At least three independent experiments were performed in triplicate. The results were calculated using $2^{-\Delta\Delta C_t}$ method (Applied Biosystems).

Transfection of siRNA

Cells were seeded at a density of 2×10^4 cells per well in a 12 well plate in EC medium and allowed to grow for 24 h. Then, cells were transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's reverse transfection protocol. Six hours after transfection, the effective transfection reagent-DNA medium mixture was replaced with fresh medium. The siRNA templates were SASI_Hs01_00209920, named as siR20; SASI_Hs01_00209921, named as siR21; and SASI_Hs01_00209922, named as siR22 (Sigma-Aldrich). MISSION® siRNA Universal Negative Control #1 (Sigma-Aldrich) was used as the scramble control.

Treatment with inhibitor

FK228, also known as romidepsin or depsipeptide, is a bicyclic tetrapeptide which was originally isolated from a bacteria strain of *Chromobacterium* [103]. FK228, acting as an inhibitor of histone deacetylase (HDAC), has confirmed to effectively decrease the activity of NNMT in human papillary thyroid cancer cells [104, 105]. The drug was purchased from APExBio technology (Taiwan), dissolved in DMSO and immediately stored at -20 °C.

Cell death ELISA

The optimal concentration of FK228 in BEC alone and in co-culture with GSC was determined using the cell death ELISA assay (Roche). Cells were seeded at a density of 5×10^3 cells per well in 96-well plates and allowed to adhere overnight. Then, cells were treated with increasing concentrations of FK228 (0, 0.5, 1, 1.5, 2, 2.5, and 5 ng/mL) for 48 hr. Equivalent volumes of DMSO were used as vehicle control. In all cases, the final concentration of DMSO remained lower than 0.5%. Attached cells were collected and analyzed for apoptosis using commercially available ELISA kit per the manufacturers' instructions (Cell Death Detection ELISAPLUS, Roche Applied Science). No differences were observed between the untreated controls and the DMSO-treated controls.

Western blot analysis

Cells were washed with PBS and lysed with ice-cold RIPA lysis buffer (Thermo Fisher Scientific). The detailed protocol for western blot and protein detection was performed as previously described [106]. Protein concentration of cell lysates was

determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories). Protein bands were visualized using enhanced chemiluminescence detection reagents (GE Healthcare, Amersham) in an ImageQuant LAS4000 (GE Healthcare), and quantified using ImageJ (NIH). Data from three independent experiments performed in triplicate were presented as mean \pm SEM.

Primary antibodies used with the dilution and manufacturer were: rabbit polyclonal anti-NNMT antibody (1: 1000; Proteintech), mouse monoclonal anti-NMNAT2 antibody (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-Vimentin antibody (1:1000; Abgent), mouse monoclonal anti-MMP2 antibody (1:1000; Abgent), and mouse monoclonal anti-beta-tubulin antibody (1:1000; Proteintech) used according to manufacturer's recommendations. Secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG HRP conjugates (1:5000; Santa Cruz Biotechnology), accordingly.

Immunostaining

Immunostaining was performed as previously described [107, 108]. Primary antibodies were: rabbit polyclonal anti-NNMT antibody (1: 200; Proteintech), mouse monoclonal anti-NMNAT2 antibody (1:200; Santa Cruz Biotechnology), and mouse monoclonal anti-alpha-SMA antibody (1:150; DAKO), followed the manufactures' manuals. Secondary antibodies were biotinylated anti-mouse and biotinylated anti-rabbit (1:200; Vector Laboratories). Pictures were taken in an Olympus BH-2 for biotinylated antibodies. Images are representative of three independent experiments performed in triplicates.

Statistical Analysis

The data represent at least three independent experiments. Statistical analyses were performed using GraphPad Prism 5.0. $P < 0.05$ was considered significant using paired two-tailed t -test or 2-way ANOVA followed by Dunnet's multiple comparison test.

1.3. Results

TuBEC have higher expression of NNMT in both RNA and protein level.

Previous studies using data mining analysis indicated that NNMT, as the critical enzyme of the clearance pathway in NAM metabolism, is one of the top genes whose expression highly increased in glioma tumor cells [91, 97]. It was confirmed that targeting on NNMT expression reduced the cellular proliferation, self-renew, and in vivo tumor growth in glioma stem cells [91]. NNMT also promotes EMT in certain types of carcinomas, whereas the EndMT in endothelial cell has not been investigated [100]. We hypothesized that NNMT may contribute to the transition of BEC during their interaction with GSC. To test the role of NNT in EndMT, we compared the patient-isolated TuBEC and BEC populations.

In cell culture, the morphology of TuBEC is clearly recognizable due to the larger cellular and nuclear size, with the cell appearance of flat and veil-like structure, compared to normal BEC, which are smaller and plump in appearance [39]. Both TuBEC and BEC were seeded in EC medium. Expression of NNMT was analyzed in TuBEC and compared to control BEC. As shown in Figure 1A, mRNA levels of NNMT were significantly higher in patient-derived TuBEC compared to control patient-derived BEC. These results were confirmed at the protein level, as evidenced by western blot studies Showing a 4-fold higher NNMT expression in TuBEC, compared to control BEC. Thus, NNMT basal expression mRNA and protein levels are upregulated in TuBEC, relative to healthy BEC.

BEC have higher expression of NMNAT2 in both RNA and protein level

NMNAT belongs to the key-regulated enzymes, which catalyzed the biosynthesis of Nicotinamide (NAM) from nicotinamide mononucleotide (NMN) and ATP, in reversible salvage pathway [92]. High levels of NMNAT2 transcripts are detected predominately among the isoforms of NMNAT in the mammalian brain [109]. Limited information of NMNAT2 is available in GBM. In our research, the expression levels of NMNAT2 was first investigated in brain endothelial cell in GBM. Our quantitative real time-PCR results showed that the levels of NMNAT2 were approximately 60 times higher in healthy BEC compared to TuBEC (Figure 1.2A). This significant difference was further confirmed at the protein levels, by performing western blot studies of three independent patient-derived samples. NMNAT2 protein expression in BEC was significantly higher than in TuBEC (Figure 1.2B). Overall, we confirmed that NMNAT2 had increased expression levels in BEC compared to TuBEC, showing an opposite effect to the results obtained when studying with NNMT comparative expression (Figure 1.1).

Knock-down of NNMT impairs the EndMT

The *in vitro* co-culture system established in our laboratory [40] was applied to monitor the process of EndMT (Figure 1.3A). Patient-derived GSC (USC02) [1] and BEC were co-cultured for 72 hr, and then the expression of the mesenchymal marker α -SMA was analyzed for by immunostaining. After 72 hr, the higher expression levels of α -SMA in the subpopulation of BEC co-cultured with GSC reflected their EndMT towards a TuBEC phenotype (Figure 1.3A).

To further confirm the role of NNMT in EndMT, the siRNA transient transfection is required to establish. In order to test what region of NNMT would cause stronger effects when knockdown in brain endothelial cells, several siRNAs targeting three different regions within the exons of the NNMT gene, known as siR20, siR21, and siR22, were used alone and in combination in TuBEC (Supplementary Figure 1.1A). After transfection and cell collection on TuBEC, western blot analysis of NNMT suggested that siR20 has relatively lower knockdown expression of NNMT compared to another two siRNA (Supplementary Figure 1.2A). Multiple knockdown also did not improve the efficiency (Supplementary Figure 1.2B). The relatively effective knockdown region by using the siR20 was applied in the following experiments.

In order to monitor the EndMT process, co-cultured USC02 and BEC were treated with siR20 or scramble siRNA as the negative control to obtain an NNMT knockdown for 4-6 hr. The results showed that cells with siR20 transfection exhibited a weaker signal of the mesenchymal marker α -SMA, compared to cells transfected with scrambled siRNA (Figure 1.3B). Conversely, the expression of the endothelial marker CD31 was increased in siR20-transfected cells, compared to the control siRNA-treated cells (Figure 1.3C). These data show that direct knockdown of NNMT blocks GSC-induced EndMT in BEC in the co-culture system. Since the α -SMA staining has relatively clear staining than CD31 and other EndMT markers for evaluation, the following up experiments were stained by using the α -SMA as the representative.

Inhibitor analysis for NNMT impairing

For potential therapeutic application in GBM, we also explored the available chemicals which undergo the mechanism of NNMT in EndMT. The inhibitor FK228, which belongs as an approved drug for treating lymphoma in clinical, has confirmed to effectively decrease the activity of NNMT in human papillary thyroid cancer cells [104, 105, 110]. The mechanism of action of FK228 was described as a class I histone deacetylase (HDAC) inhibitor which directly contribute to the NNMT (Furumai, Matsuyama, Kobashi, Lee, Nishiyama, Nakajima, Tanaka, Komatsu, Nishino, Yoshida and Horinouchi [111]). FK228 is also known to decrease angiogenesis without thrombosis or hemorrhage in mouse models [112]. Therefore, with the rationale of boarder application, FK228 was applied as the inhibitor of NNMT in GBM in our work.

We first determined the optimal dose of FK228 in BECs cultured alone and in co-culture with USC02, by performing a cytotoxicity assay with increasing concentrations of the drug. Based on *in vitro* co-culture model, three cell groups were examined for cytotoxicity, including a group of healthy BEC, a group of both BEC and USC02, and a group of glioma stem cells USC02. FK228 was added with fresh medium and incubated for 48 hr. Cell survival rates were measured at 72 hr post treatment. When co-cultures of BEC and USC02 were treated with FK228, the concentrations under 2 ng/mL did not have significant difference compared to the vehicle control (DMSO) (Figure 1.4A). Treating with BEC (Figure 4B), the concentration under 2 ng/mL did not cause the significant difference relative to vehicle-treated cells. However, BEC cell lines presented the sensitivity with the relatively obvious error bar when the concentration was 1 ng/mL

(Figure 1.4B). Additionally, if we treated the glioma stem cells USC02 alone, USC02 started to be induced significant cell death at 1 ng/mL of the drug concentration (Supplementary Figure 1.3).

The concentration under 2 ng/mL, including 0.5, 1.0 and 2 ng/mL, was chosen to further examine the effects of FK228 on EndMT. BEC in co-culture with GSC treated with vehicle (negative control) presented strong positive staining of mesenchymal marker α -SMA, compared to BEC cultured alone (Figure 1.5A). Compared to the negative control, treatment of co-cultures with the NNMT inhibitor FK228 decreased the expression of α -SMA in three individual patient-derived BEC, suggesting EndMT was being impaired. At the concentration of 0.5 ng/mL, the EndMT was already significantly impaired from the results of immunostaining by using mesenchymal marker α -SMA (Figure 1.5A). To confirm whether NNMT inhibited by FK228 in TuBEC, we examined the protein levels of NNMT in TuBEC at 72 hr post treatment with FK228. The results showed that protein expression level of NNMT indeed decreased with the drug treatment in TuBEC (Figure 5B). In summary, FK228 can impair EndMT in our patient-derived co-culture system at subcytotoxic doses as low as 0.5 ng/mL. To consider the toxicity effect (more than 1ng/mL has the cell death in USC02 alone), the lowest concentration of 0.5 ng/mL was applied in the following experiments.

Downregulation of NNMT in BEC alone is enough to block the EndMT

To understand whether the EndMT blockade was due to biological changes directly occurring in the BEC, we pre-treated the BEC alone with the chemical inhibitor

FK228 for 48 hr or performed a knock down assay using siRNA (siR20) for 4 hr. After pre-treatments (to avoid toxicity, for FK228, pretreatment was 48 hr for transfection with siRNA, 24 hr was applied), pre-treated BEC were re-seeded on the 12 well plates with GSC. The cells were stained with α -SMA marker. Immunostaining analysis showed that, when pre-treated with siRNA, knocking down the expression in BEC alone also presented less α -SMA signals as compared to the scramble control after co-culturing with GSC. The α -SMA expression was also weaker in BECs (Figure 1.6, lower panels). Similarly, the BEC pre-treated with FK228 also exhibited lower expression levels of the mesenchymal marker than the vehicle-treated cells after co-culturing with non-treated GSCs (Figure 1.7, lower panels). These immunostaining results confirmed that loss function of NNMT impaired the progression of EndMT in BEC (Figure 1.5 and 1.6). By knocking down the gene NNMT only in BEC, the EndMT was impaired when co-culturing with USC02, confirming that the NNMT induced-regulation came from those BEC.

Marker genes in EndMT participates in NNMT-dependent activation

We further investigated the possible molecular routes of EndMT corresponding to the potential mechanism of NNMT-induced EndMT in TuBEC. The mesenchymal structural and cell-to-cell junction proteins, including vimentin, cadherin, etc, were examined in the validation of activating EndMT. Also, from previous study, the proposed model of NNMT overexpression also described that NNMT controlled the downstream of AKT regulated genes MMP2-induced signaling mechanism in renal cancer cells [113]. These data lead us to hypothesize a potential linkage between NNMT and MMP2 signaling in our model.

To test the hypothesis, we conducted the siRNA transfection assay using knockdown NNMT directly in TuBEC. The TuBEC were seeded first and then treated with siRNA reagent. The cells were collected for examination after 48 hr post treatment. In Figure 8, the mesenchymal structural protein vimentin was confirmed to decrease when knockdown the expression of NNMT. Also, the expression of MMP2 in TuBEC decreased upon knocking down the NNMT. The matrix metalloproteinases (MMPs), which are usually considered as the downstream in AKT signaling with the functions of degrading the basement membranes and allowing for endothelial cell invasion, were also tested together in our assay based on the mechanism described in Tang et al., (2011)[113]. MMP2 pathway has been demonstrated to be induced by NNMT in other cancer cells but not yet in GBM [114, 115]. The results showed that the expression of MMP2 significantly decreased upon knocking down the NNMT in TuBEC (Figure 1.8). To summarize, the results, it implies that NNMT expression is involved in turning off the MMP2 signaling in TuBEC as well as changing the structure protein in order to leading the transition happened.

1.4. Discussion

EndMT is a critical process in carcinogenesis, metastasis, and angiogenesis in GBM [11, 40]. During the EndMT process, BEC lose their features and obtain mesenchymal-like characteristics, allowing tumor cells to invade, break through the capillary vessel, and control the nutrient supply [54]. Previously, NNMT was reported to lead the morphological changes in epithelial cells in cancer [100]. NNMT has also been described to play a role in the maintenance of the metabolism balance and to be preferentially expressed in mesenchymal GSC [91]. Here, we studied the regulating role of NNMT in NAM metabolic pathways and its effect on EndMT of GBM progression. We hypothesized that NNMT is involved in EndMT in TuBEC. The results using the *in vitro* co-culture model showed that EndMT can be impaired when NNMT expression is decreased (Figure 1.3 and 1.6), suggesting that NNMT induces the EndMT from BEC in GBM.

NAM metabolism has been recently described as a novel potential target in cancer therapeutics, due to its essential roles in regulating cancer proliferation, invasion, and DNA repair [92]. NAM metabolism is mainly divided into two individual routes: clearance pathway and salvage pathway (Figure 1.9)[92, 94] NNMT, which is involved in the clearance pathway, is not only overexpressed in glioma cells and different types of cancers but also promotes the invasion and migration during carcinogenesis [91]. The role of NNMT-induced EndMT was first evaluated in GBM in this study. We demonstrated here that the basal levels of NNMT are higher in TuBEC as compared to the normal BEC (Figure 1.1). Conversely, the basal levels of NMNAT2 from the salvage pathway are higher in BEC as compared to the TuBEC (Figure 1.2).

These findings were corresponded to the previous studies where NMNAT2 has been confirmed to increase the expression in p53-dependent suppression in colorectal cancer [116, 117]. As the tumor suppressor p53, their results imply that NMNAT2 present the lower expression pattern in tumor cell compared to health cell. This was similar to our results where the healthy cells have higher expression of NMNAT2. Furthermore, the role of NNMT in epithelial-to-mesenchymal transition (EMT) has been recently established in squamous cell carcinoma cells [115]. Our results also showed that knockdown of NNMT in co-culture model indeed impairs the EndMT. (Figure 1.3). Overall, it demonstrated that the clearance pathway of NAM regulation enhanced by NNMT promotes the EndMT via the clearance, but not the salvage pathway in GBM. This fact explained that GSC induced EndMT, which is controlled by irreversible pathway in NAM energetic metabolism in BEC.

In an attempt to inhibit EndMT, we used inhibitor FK228 to investigate the potentials in NNMT-induced EndMT mechanism due to its available clinical therapeutic application. FK228 has been confirmed to effectively suppress the expression of NNMT in human papillary thyroid cancer cell and inhibit the tumor angiogenesis but haven't been tested in GBM [104, 112]. Our results here showed that FK228 can effectively inhibit the expression of NNMT in TuBEC. Furthermore, it blocked the EndMT in the co-culture system without cytotoxicity (Figure 1.5). Specifically, to understand whether the blocking mechanism is from BEC with the biological meaning, we demonstrated that the significant blocking of EndMT was presented by only pre-treating the BEC alone with FK228 compared to the FK228 treatment in BEC+USC02 (Figure 1.7). These results illustrated

that FK228 can effectively impair the EndMT in BEC. Recently, FK228 was approved by FDA in treating the T-cell lymphoma and leukemia [118, 119]. By identifying the cytotoxicity and the involvement of EndMT, the application of FK228 in tumor angiogenesis provides an extra evidence on the NAM regulation in GBM.

To further clarify the direct biological role of BEC in EndMT, the pre-treatments of knockdown assay for NNMT were tested in only BEC and then co-culture with USC02. Interestingly, the results indicated that EndMT was still impaired when the knockdown treatments were only treated in BEC (Figure 1.6, 1.7). It revealed that endothelial cell itself involved in regulation of the EndMT process. From the view of treating healthy BEC in our co-culture model, the strategy by decreasing the expression of the protein NNMT has the potential to apply in advance in health cell in order to preventing the cancer development before tumorigenesis.

In addition to applying the *in vitro* co-culture model for prescreening the NNMT, we also aimed to identify the potential route in order to illustrate that NNMT indeed involve in regulating EndMT. The AKT-MMP2 pathway was reported as one of the EndMT pathways that controls vascular endothelial growth factor secretion and angiogenesis [120]. NNMT also has been demonstrated to induce proliferation and invasion through MMP2 pathway in other cancer cells [114, 115]. In our study, the results confirmed that MMP2 and vimentin was located at the downstream of NNMT regulation (Figure 1.8). It provides the evidence that the NNMT indeed controls the EndMT and further angiogenesis through MMP2 as well as affect the structural protein vimentin. This fact can further strengthen our previous results that BEC existed the mechanism of NNMT-induced EndMT in GBM. The

MMP2 pathway was confirmed to be one of the routes. More complete network of EndMT inducing by NNMT will be valuable to investigate in GBM.

Glioblastoma has the hallmark feature with its extremely high vascularity. Recently many reports illustrated the mechanism of NAM metabolic-involved enzyme NNMT in glioma cells which missed the picture of discussing the nearby cells, such as the key vessel role endothelial cell, in tumor microenvironment [91, 97]. This present report demonstrated that NNMT contributed to induce the EndMT in BEC during the GBM progression. Most preclinical research in GBM use the mouse model or the cell lines which did not consider the critical role of tumor microenvironment, specifically the tumor vasculature. In our study, the GSC-BEC interaction co-culture model for preclinical screening not only considers the aspect with tumor vasculature but also identified the novel regulation from NAM metabolism in patient-derived cells with the clinical significance. Down-regulation of NNMT expression blocked the EndMT. The expression of NNMT was confirmed to control the EndMT, such as MMP2 and vimentin from BEC.

In this study, we have shown again the importance of EndMT as a target for anti-GBM therapy. In nicotinamide metabolism, we provide the explanation that one representative blockade—the irreversible clearance pathway was participating in EndMT (Figure 1.9, orange rectangle). The reversible salvage pathway of NAM did not present the higher expression in both protein and RNA level in TuBEC (Figure 1.2 and Figure 1.9). The identification of two main blockade in NAM lead us to understand the overall nicotinamide-mediated response in cancer therapy. Additionally, FK228, as available

clinical drug, provide the aspects of the angiogenic therapy by understanding the critical function of NNMT against glioma.

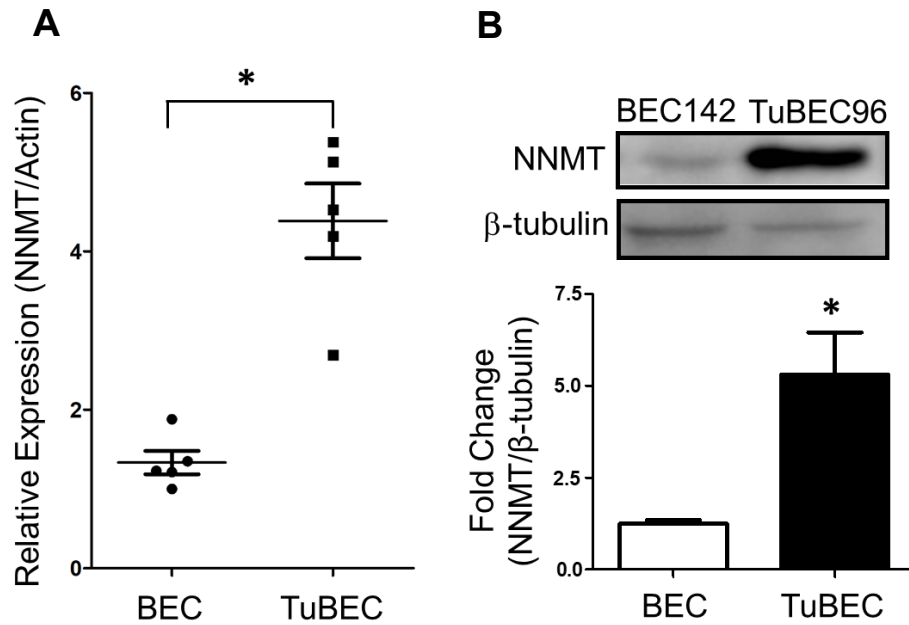


Figure 1. 1 Expression of NNMT increased in TuBEC compared to BEC.

(A) The mRNA levels of NNMT measuring by quantitative real-time PCR (healthy brain endothelial cell vs tumor-associated endothelial cell). (B) Protein levels of NNMT in western blotting assay. Each individual point representative single sample by 3 biological replicates. Fold change values are expressed as mean \pm standard deviation (* $p < 0.05$)

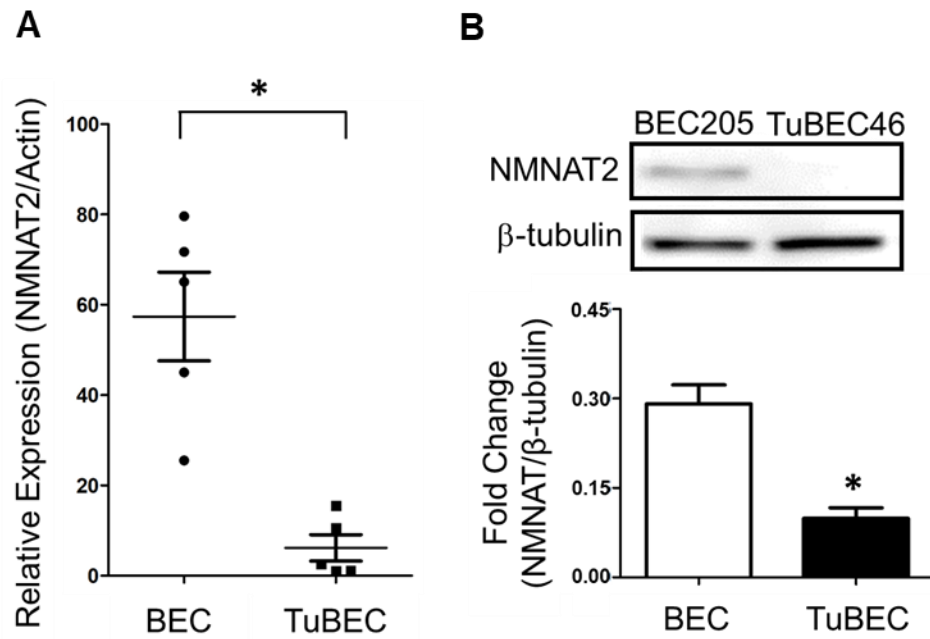


Figure 1. 2 Expression of NMNAT2 decreased in TuBEC compared to BEC.

(A) The mRNA levels of NMNAT2 measuring by quantitative real-time PCR (healthy brain endothelial cell vs tumor-associated endothelial cell). (B) The protein levels of NMNAT2 in western blotting assay. Each individual point representative single sample by 3 biological replicates. Fold change values are expressed as mean \pm standard deviation (*p <0.05)

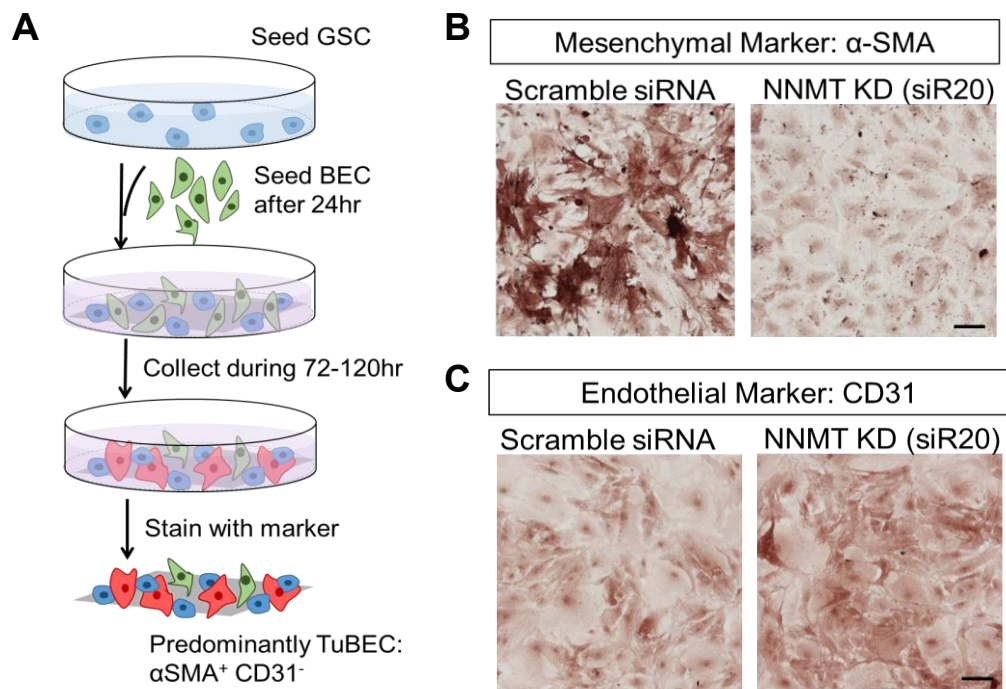


Figure 1.3 Knock-down NNMT in co-culture system in the siRNA transfection assay.

(A) The flow chart represents the process of our co-culture model. GSC (labeled as blue color) is seeded first and then cover with the BEC (green color). The transformed cells (red color) from BEC presented positive signal of α SMA and negative signal of CD31. The following experiments are based on this design with different treatment at certain time points. (B) The knockdown of NNMT blocked EndMT in co-culture model. The upper figures were stained by mesenchymal marker α -SMA. The bottom figures were stained by endothelial marker CD31. The control is the scramble siRNA (Left). The experiment group for NNMT knockdown is the NNMT KD (siR20) (Right). Each individual point representative single sample by 3 biological replicates. The bar was 100 μ m.

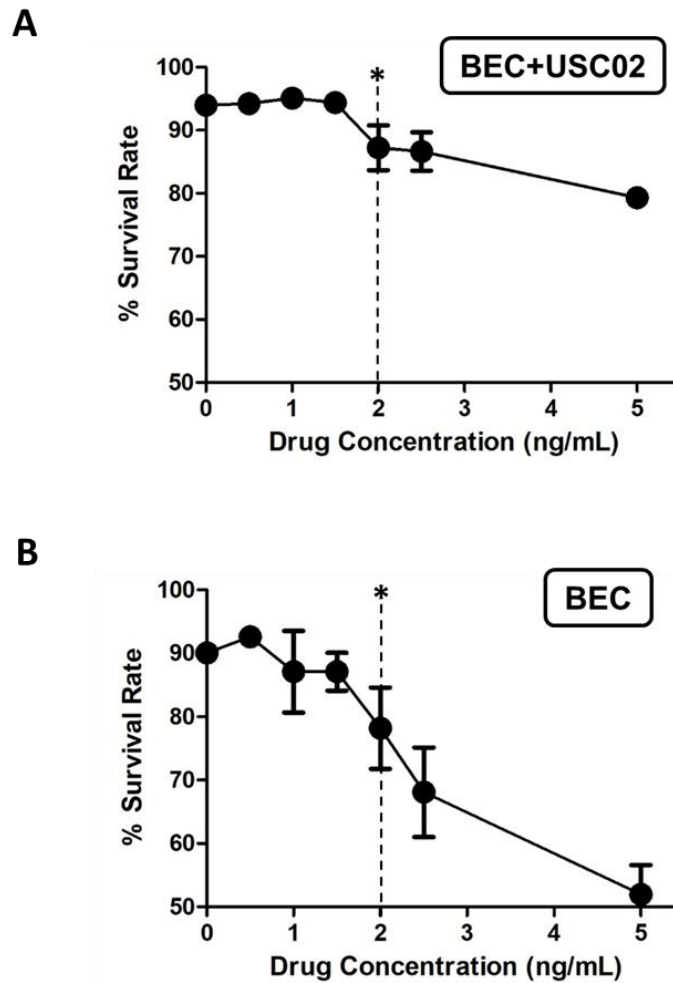
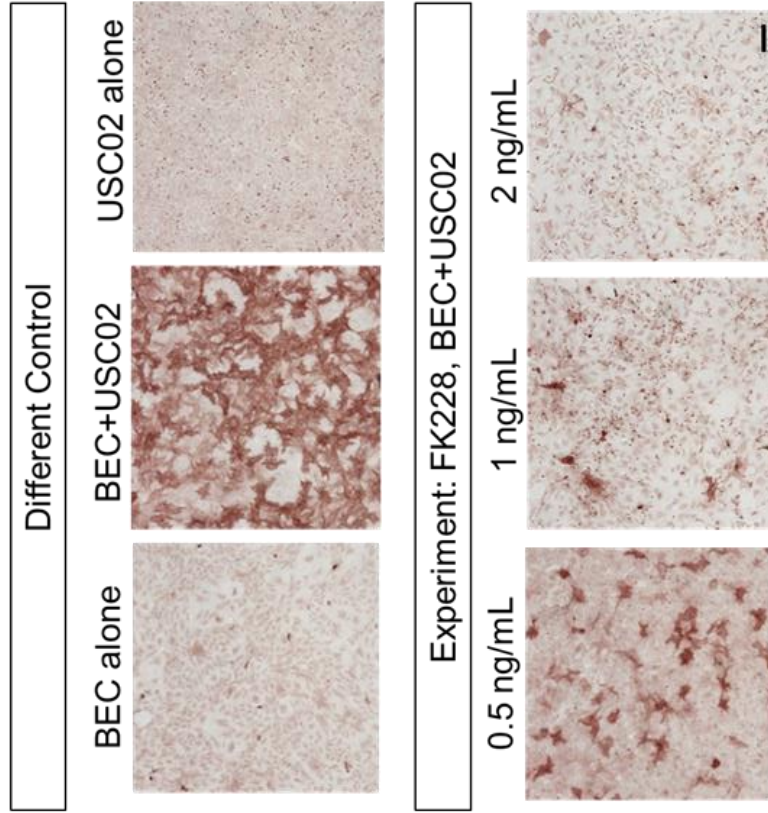


Figure 1. 4 Cytotoxicity effect of the inhibitor FK228 was measured in cell viability assay.

BEC lines and BECs+USC02 lines were treated with the inhibitor FK228 at the concentration of 0, 0.5, 1, 1.5, 2, 2.5 and 5 ng/mL. Cells were collected after 72 hr post drug treatment. (A) The cytotoxicity assay performed in both USC02 and BEC. (B) BEC line alone. Three biological repeats were measured here. *P<0.05 vs. concentration 0 ng/mL.

A



B

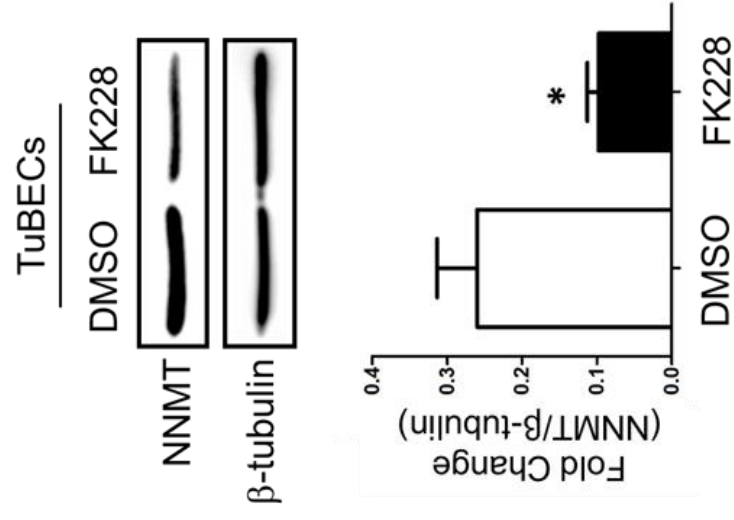


Figure 1. 5 Inhibitor FK228 lead the expression of mesenchymal marker in the co-culture system.

(A) The concentration effect of the inhibitor FK228 in the co-culture system. The upper figures were different controls from right to left. The right figure was the blank control with BEC alone. The second figure was the negative control which were co-culturing with USC02 without the inhibitor treatment after 72 hr. The third figure was the blank control with USC02 alone. The lower figures were the BECs with USC02 in the inhibitor FK228 concentration of 0.5 ng, 1 ng, and 2 ng/mL. All the cells were staining by the immuno-marker α -SMA. The representative images were measured by three biological repeats. The bar represented 100 μ m. (B) The protein level of NNMT in TuBEC when applying the inhibitor FK228 for 48 hr. Each individual bar representative single sample by 3 biological replicates. Fold change values are expressed as mean \pm standard deviation (*p <0.05)

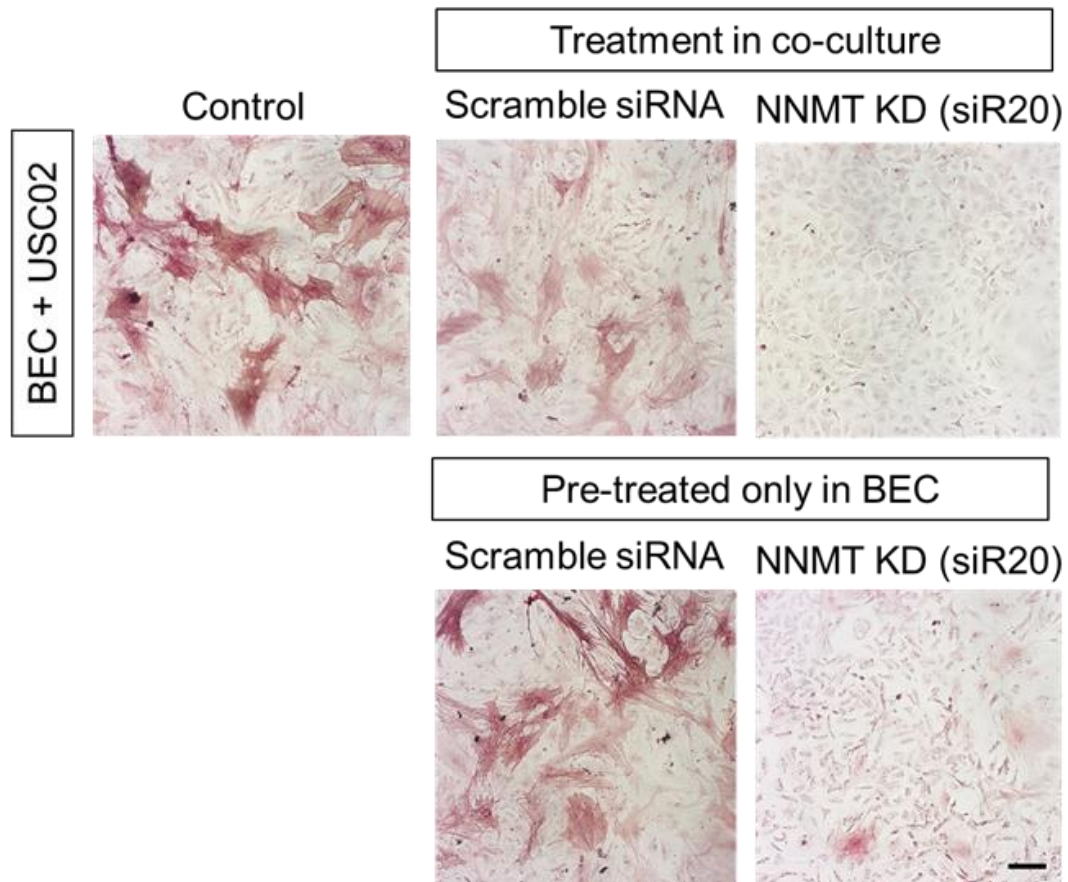


Figure 1. 6 Downregulation on NNMT only in BEC block the EndMT.

Representative images were BEC alone which transfected with siRNA- siR20 for 4-6 hr. The USC02 was then seeded for co-culturing with fresh medium next day. The control represented standard co-culture assay after 72 hr. The DMSO treatment was another control for monitoring the effect of treatment and chemical solvent. Cells were stained for α -SMA. The representative images were measured by three biological repeats. The bar was 100 μ m.

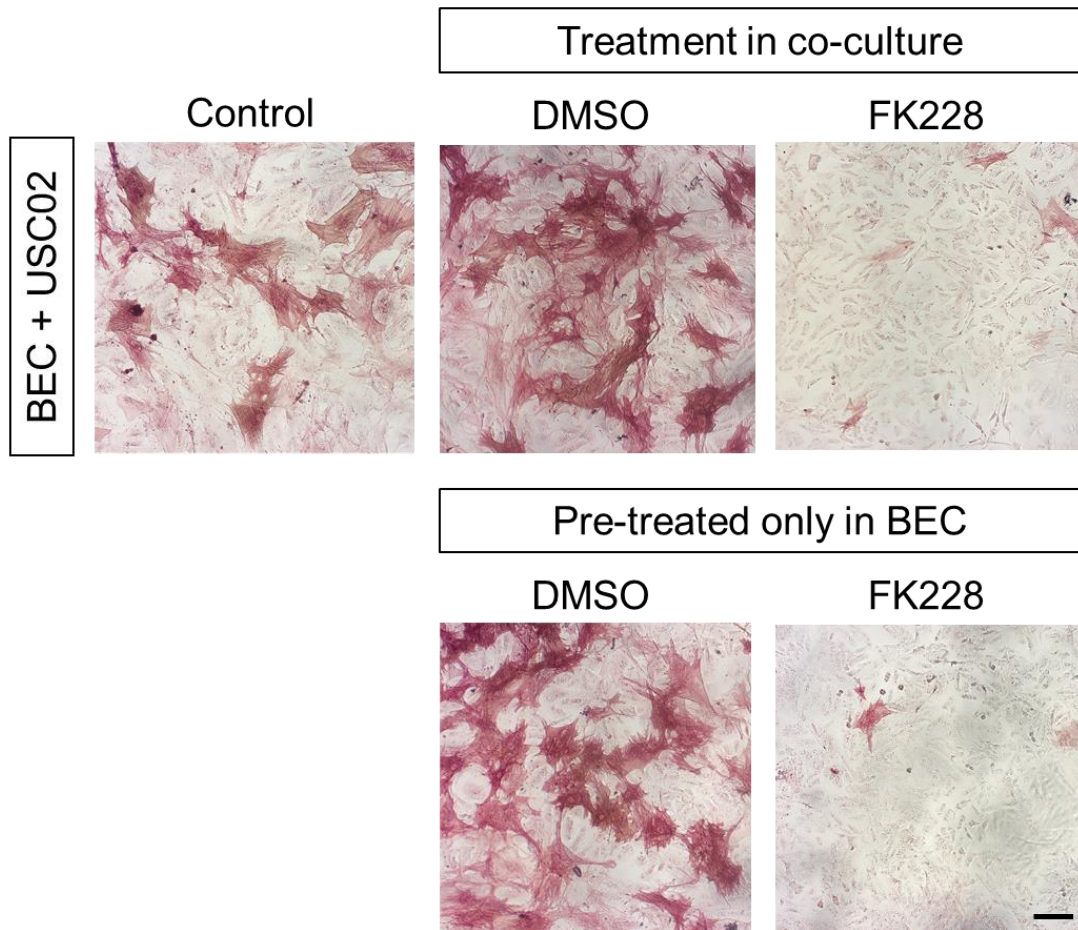


Figure 1. 7 Chemical inhibition of NNMT only in BEC block the EndMT.

Representative images were BEC treated with inhibitor FK228 alone without USC02 for 48 hr. After 48hr, BEC was seeded for co-culturing with USC02 in the fresh medium. USC02 was pre-seeded at 24 hr ago before the BEC was ready. The control represented standard co-culture assay after the FK228 treatment 72 hr. The DMSO treatment was another control for monitoring the effect of treatment and chemical solvent. Cells were stained for α -SMA. The representative images were measured by three biological repeats. The bar was 100 μ m.

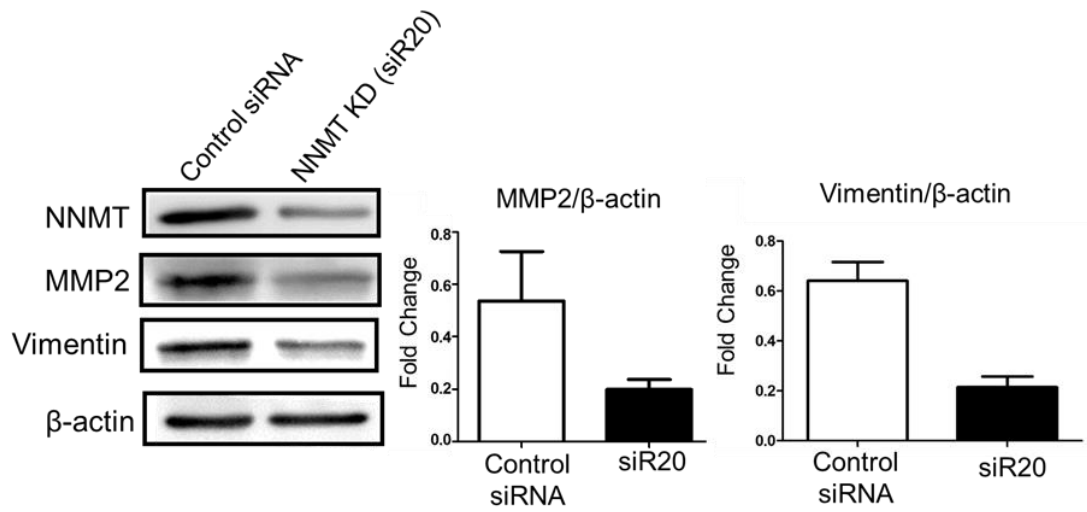


Figure 1. 8 NNMT regulated the downstream markers of EndMT in TuBEC.

The western images were the collection of knockdown NNMT in TuBEC in protein level. Knockdown the NNMT expression lead the downregulation of MMP2 and Vimentin gene. The representative images were measured by three biological repeats. *P<0.05 vs. Control RNA.

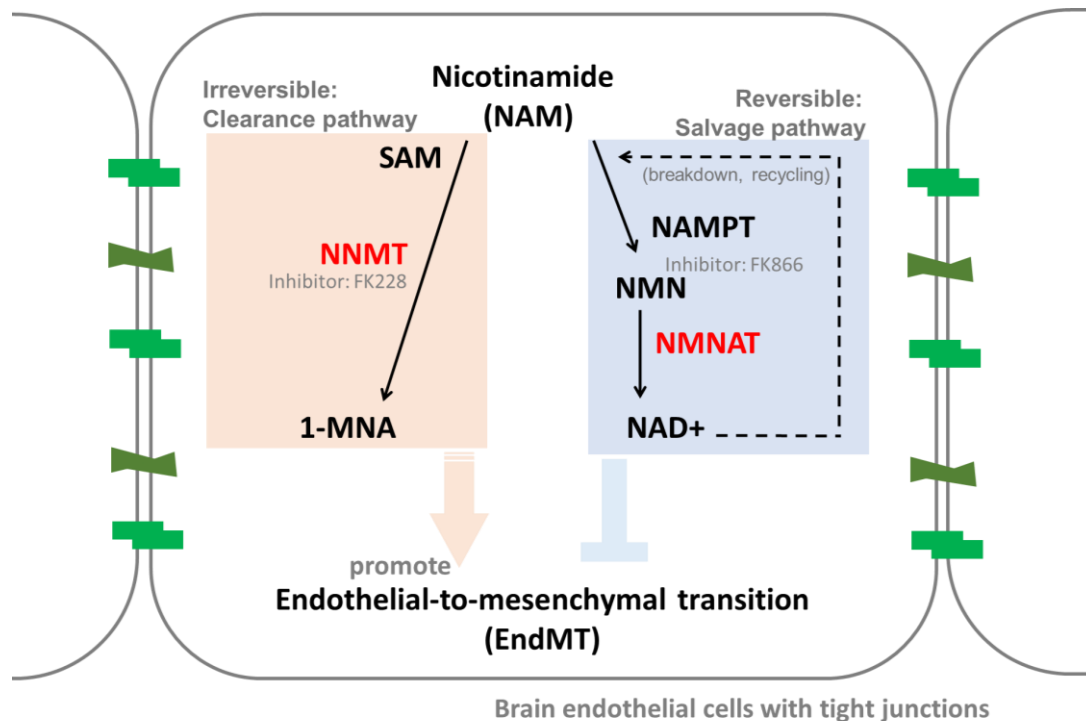
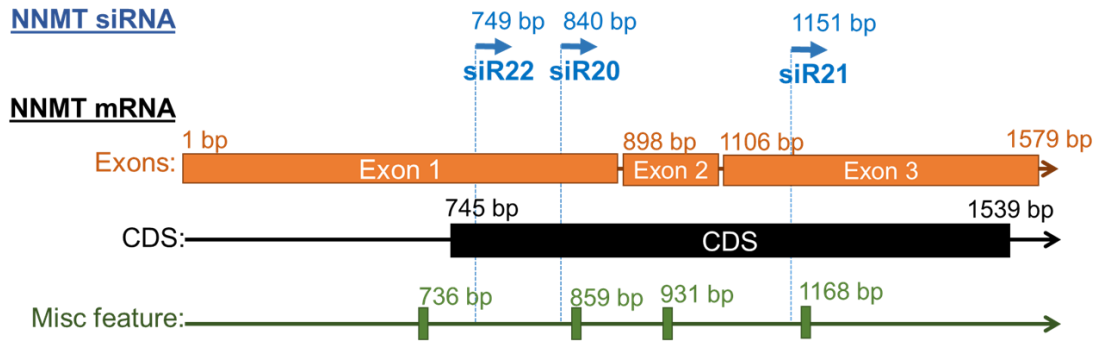


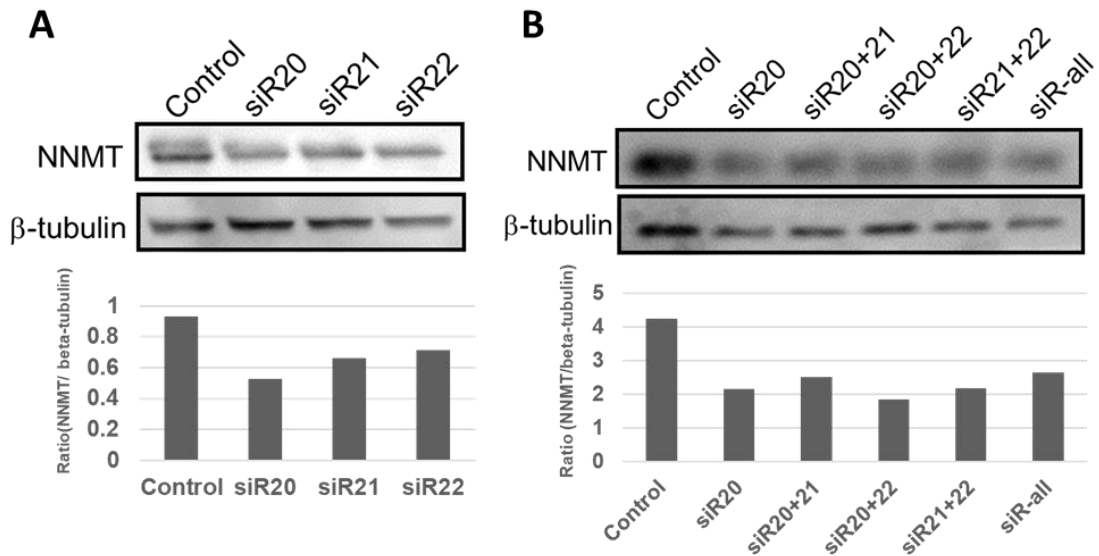
Figure 1. 9 Summary of nicotinamide blockades in this study.

Orange rectangle represented the blockade of NNMT-mediated pathway clearance pathway in NAM metabolism. Blue rectangle was the blockage of NMNAT-mediated pathway (reversible salvage pathway) in NAM metabolism. Brain endothelial cells (larger rectangle with grey outlines) were joined together with the tight junctions (green labels).



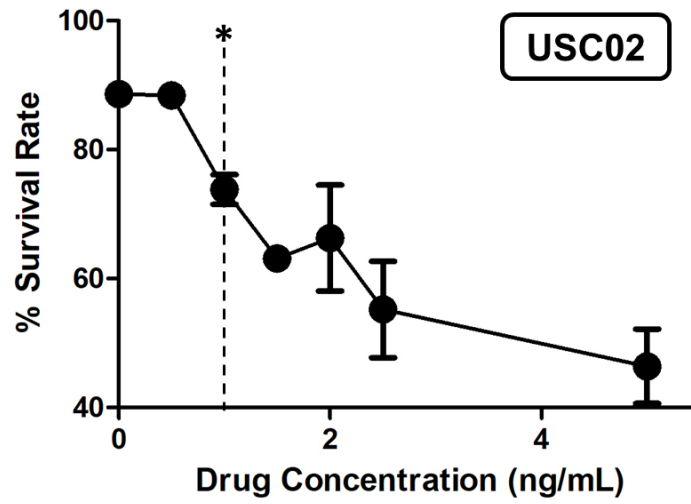
Supplementary Figure 1. 1 Supplementary Figure 1 Different region of siRNA to knockdown the NNMT expression.

The different exon regions of NNMT were showed in blue and design for knocking down from commercial siRNA in order to test the best area. Blue arrow is the region of siRNA mapped to NNMT. Orange box is exon map on NNMT. Black box is the open reading frame of NNMT. Gene map is downloaded from NCBI.



Supplementary Figure 1. 2 The efficiency of knockdown NNMT in different region and different combination of siRNA.

(A) Western blot of NNMT knockdown with different region of siRNA. In order to find the best region to knockdown NNMT in TuBEC, we tested three different regions. Control is the scramble siRNA. siR20, 21, and 22 is based on the map in supplementary figure 1. siR20 has the lowest efficiency. (B) Western blot of NNMT knockdown with different combination with siR20 in TuBEC. No significant difference was found with the combination of siRNAs.



Supplementary Figure 1. 3 Cytotoxicity effect of the inhibitor FK228 was measured in USC02 in cell viability assay.

USC02 line was treated with the inhibitor FK228 at the concentration of 0, 0.5, 1, 1.5, 2, 2.5, and 5 ng/mL. Cells were collected after 72 hr post drug treatment. Three biological repeats were measured here. *P<0.05 vs. concentration 0 ng/mL.

CHAPTER 2.

Novel candidate genes potentially participate in endothelial-to-mesenchymal transition in glioblastoma.

ABSTRACT

Current data on endothelial-to-mesenchymal transition (EndMT) in pathological conditions is summarized into several classical networks, including the TGF beta, Notch, Wnt/ β -catenin, Hedgehog, and BMP-driven network. Unknown pathways to support or eliminate the angiogenesis and tumorigenesis are still existed in overall biological process. Biological metabolism can also connect with these process during the EndMT in pathological condition. In this chapter, we aim to identify several potential novel candidates to participate in EndMT in glioblastoma and understand their metabolism/relationship in the role of cancer. According to the gene-set-analysis and the summary of reference, there are five candidates, including periostin (POSTN), nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), metallothionein (MT), dickkopf homolog 3 (DKK3) and cGMP-specific phosphodiesterase type 5 (PDE5A), selected from different representative network. Their function from the reference and expression in our EndMT model will be further addressed. Novel candidates are discussed with its metabolism network in EndMT. Several of them have been considered as the tumor indicator or therapeutic agents. This chapter provides the better understanding with the involvement of EndMT in glioma while setting up the new therapeutic strategies in the future.

2.1 Periostin (POSTN)

2.1.1 *POSTN: Introduction*

Periostin (POSTN), also called osteoblast-specific factor 2 (OSF2), is a 93.3-KDa secreted extracellular matrix (ECM) protein and is classified as the TGF- β -inducible protein [121]. POSTN was reported to be regulated in various types of cancers, including breast, colon, ovarian, prostate, bladder, renal cell carcinoma, and glioma [122-128]. The overexpression of POSTN in many of cancer type is generally associated with malignant phenotypes. The signaling pathways which activated by POSTN has been confirmed to promote cellular survival, angiogenesis, cell invasion, migration and resistance to hypoxia-induced cell death [128, 129].

The protein structure of POSTN interestingly leads the different cell functions (Figure 2.1). The C-terminal region includes the hydrophilic domain (HDP) which can form the different isoforms by alternative splicing and promote the cell-matrix interactions [130]. The alternative splicing of isoforms leads the different regulation of intercellular adhesion via interactions with other ECM proteins including fibronectin, tenascin, collagen and POSTN itself [130]. (Further study if confirm with invasion and metasis) The N-terminal region is highly conserved which regulate the cell function by binding to integrins by its four fasciclin homologous repeats (FAS) domain [121]. POSTN was shown to be able to bind the integrins $\alpha\beta3$, $\alpha\beta5$, and $\alpha6\beta4$, promoting the recruitment of the epidermal growth factor receptor (EGFR) and the activation of the Akt/PKB, Ras, and FAK-mediated signaling pathways [125, 131]. Further reports indicated that this region binds the integrins

which interact with cancer cells and cancer-associated endothelial cells. The FAK-mediated pathway which trigger by POSTN was confirmed to drive the angiogenesis and further trigger the invasion and metastasis especially in cancer-associated endothelial cell.

Endothelial-to-mesenchymal transition (EndMT), as the main topic in this research, plays as an important role during metastatic intravasation, invasion, and angiogenesis in cancer [56, 59]. To our best knowledge, current studies in POSTN was focus on epithelial-to-mesenchymal transition (EMT) and still not yet to have the complete study in EndMT. POSTN has been shown in general to promote EMT in several type of cancer and consider as the mesenchymal marker. In lung cancer and prostate cancer, POSTN was considered to increase the expression of EMT genes, such as N-cadherin, vimentin, twist and snail [129, 132]. In breast cancer, there is not yet to have the direct study but indicate that the POSTN promote the angiogenesis by recognizing with the VEGFR2 [133] However, it is opposite effect in bladder cancer and lung metastasis of melanoma cells. Upregulation of POSTN induced the expression of epithelial marker E-cadherin instead of mesenchymal markers [134, 135]. POSTN was confirmed to suppress the invasiveness via AKT/mTOR signaling pathways *in vivo* in the mouse orthotropic model of bladder cancer [128]. The role of EndMT has not yet been surveyed clearly in POSTN while the EMT leaves the opposite effect in POSTN in some of the cancer. POSTN could be considered as the cell-type-dependent regulation although POSTN promote EMT in general in many cancers.

POSTN was investigated to regulate the glioma malignancy. Upregulation of POSTN was consider in higher grade glioma compared to the lower grade which can be potentially considered as the diagnosis marker [136]. POSTN increased the resistance of

anti-angiogenesis therapy by bevacizumab treatment in glioma stem cells (GSC) [137]. It was also demonstrated that POSTN secreted from GSC activate the TGF β 1-induced pathway in tumor-supportive macrophages (M2 subtype) [122]. The role of POSTN in GSC was illustrated to promote the glioma progression and proliferation. However, there are not yet to have the studies directly in endothelial cell (BEC) in glioma.

2.1.2 POSTN: Result

As the potential target and unknown role in EndMT, the expression level of POSTN was first investigated in glioma. In Figure 2.1, quantitative PCR provided the evidence that the expression level of POSTN decreased in TuBECs compared to BECs after three biological repeats. The protein level was also investigated by Western blotting (Figure 2.2). Representative image showed that BECs had the clear band compared to subBEC and TuBEC even in individual patient (Figure 2.2A). Quantitative the image illustrated that the POSTN expression in BEC was indeed significantly higher than subBEC and TuBEC (Figure 2.2B). Overall, both of the results from RNA and protein level indicated that the expression of POSTN in BEC is higher than TuBEC after multiple repeats.

The results were controversial to the general understanding in POSTN from the reference which the POSTN was always overexpressed in malignant tumor and used as the mesenchymal marker in epithelial cell. We further investigated the immunostaining in the overall distribution of cell culture in our endothelial cell. The result showed that confBEC got the obvious staining of POSTN compared to TuBEC from immunostaining assay (Figure 2.3). Interestingly, the subBEC, which represented as the morphology change in

our model and means same cell but only 70% confluency compared to confBEC with 100%, also showed the less POSTN expression (Figure 2.3). This result led us to consider the different definition of POSTN in our system.

POSTN is a secreted ECM protein [121]. Another question is raised whether the secreted POSTN or unknown compounds could affect the expression of POSTN in TuBECs. The simple assay was designed in TuBEC with two different mediums, including the one from healthy BEC as experiment and another one originally from TuBEC as the control. After culturing for 48hrs and staining with POSTN marker, the results indicated that the experiment group which received the medium from BEC increased the expression of POSTN (Figure 2.4) compared to the medium from TuBEC. This result illustrated that the secreted compounds from BEC were able to induce the POSTN expression in TuBEC. Overall, based on our results, the expression of POSTN in endothelial cells in glioma was not sufficient to support the malignant phenotype but the opposite results in healthy BEC.

2.1.3 POSTN: Discussion

This is the first study to investigate the role of POSTN directly in tumor-associated endothelial cells. From previous studies, POSTN mediates the invasion, proliferation, angiogenesis, and EMT process via AKT/TGF β 1 pathway in many types of cancer cells. In glioma, POSTN in EMT has not yet been investigated but was confirmed to link with higher grades of glioma. POSTN, as the macricellular fascilin-like protein, is believed to be secreted from glioma stem cells and interact with the integrin α v β 5 to recruit tumor-supportive

macrophages [122, 138]. The mechanism from endothelial cell is still unknown in glioblastoma.

Our results presented the opposite effect in brain endothelial cell in glioblastoma. Both evidence from RNA and protein levels indicated that the normal healthy BEC has the relatively higher expression of POSTN compared to the TuBEC (Figure 2.1 and 2.2). Because of the purification from patient-derived sample, the distribution of those expressing cells was also questioned and investigated in order to assist our founding. In Figure 2.3, the immunostaining results demonstrated that POSTN marker was equally distributed in the population of these BECs or TuBECs. As same as the Figure 2.1 and 2.2, this staining result clearly also reflected that the expression of POSTN in BEC is higher than in TuBEC. This is not the first time to show that the POSTN has the opposite result in different cancer. Kim et al. presented several reports to support that POSTN increased the expression in healthy cell compared to the cancer cell in bladder cancer *in vitro* and small lung cancer cell *in vivo* [128, 134]. They indicated that the expression of POSTN may be cell-type dependent which is support our findings in BEC in glioma.

In tumor microenvironment, POSTN was the secreted ECM protein with the N-terminal region to recognize different integrins on receiving cell [138]. The mechanism in glioblastoma has been described in GSC where POSTN was secreted from GSC and received by the macrophages [122]. Pericytes in glial tumor cell was also reported to expression high amount of POSTN and promote angiogenesis [139]. In endothelial cells, the medium exchange test was tested from the higher amounts of POSTN in healthy BEC in our system (Figure 2.4). The results indicated that the compound mixture secreted from

healthy BEC indeed induced the expression of POSTN in TuBECs. This result was different to glioma stem cell and pericytes in previous report. It should be worth to confirm with more population of cells in the culture environment where we simply have the purified endothelial cell from patient. These findings illustrated that different molecular mechanisms do exist for the regulation of EndMT and invasiveness not only in bladder cancer cells but also in glioma.

POSTN has the prognostic significance as the diagnostic marker in many type of cancer, including the breast cancer, ovarian, and esophageal squamous cell carcinoma [140, 141]. It has been reported to increase the expression in recurring tumor and resistance to antiangiogenics therapy [137, 142]. In this research, we firstly focus on the endothelial cell itself derived from patients in glioblastoma. The results were opposite from the general understanding in POSTN as the mesenchymal marker or prognostic indicator. Research in bladder cancer currently support what we found in glioblastoma. Also, differ from carcinomas, relatively little is known about the prognostic or functional significance of POSTN in glioblastoma. This research provides the evidence that it should be careful to directly consider POSTN as the mesenchymal marker in glioma. More comprehensive study is required in order to consider POSTN in the marker of antiangiogenics therapy in glioma in the future.

2.2 Nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2)

2.2.1 NMNAT2: Introduction

Nicotinamide mononucleotide (NMN) adenylyltransferase 2 (NMNAT2), belongs to the rate-limiting enzyme, catalyzes the biosynthesis of Nicotinamide (NAM) from nicotinamide mononucleotide (NMN) and ATP [109, 143, 144]. Additional two isoforms are found to mediate this metabolic mechanism, including NMNAT1 and NMNAT3 [145, 146]. Among these three NMNAT isoforms, NMNAT2 is known to be most sensitive to NAD and can act as a sensor to intracellular metabolic state in NAD metabolism in pathological condition [147]. High levels of NMNAT2 transcripts are detected to express predominately in the mammalian brain [109]. NMNAT1 associates with nuclear, and NMNAT3 is in mitochondria [146]. Whereas, NMNAT2 is localized in Golgi vesicles, and synaptic compartments in neuron [109]. Because of the high requirements of the energy in cancer cell and tumor microenvironment, it may be helpful to know the role of NMNAT2 in our system.

NMNAT2 was known to involve in cancer and neurodegeneration with limited information. By using the a high-throughput screening platform to investigate endogenous NMNAT2 levels, overexpressing NMNAT2 provides neuroprotection in various neurodisorders [95]. In colorectal cancer, NMNAT2 was firstly reported to regulate the expression level and play an important functional role in p53-mediated cancer suppression process. As the well-known tumor suppressor gene, p53 was considered to be the critical role in cancer signaling pathways, metabolisms and cellular process. NMNAT2 has been

investigated to join the downstream of p53 regulation [96]. NMNAT2 also decreased the activity in the drug-resistance cancer cell in colorectal cancer [148]. Sirtuin (SIRT) needs NAD⁺ as a cofactor in regulating p53 activity. NMNAT2 was also indicated to interact with SIRT3 in non-small cell lung carcinoma [117]. These evidence indicated that NMNAT2 involved in cancer suppression in colorectal cancer and lung cancer. Moreover, NMNAT2 was considered to be a promising therapeutic indicator in colorectal cancer. Currently, the role of NMNAT2 as a tumor suppressor or an oncogene in glioblastoma is still unclear. Function of NMNAT in tumorigenesis is still need to be fully explored.

NMNAT belongs to one of the key enzyme in the representative pathways in nicotinamide adenine dinucleotide (NAD⁺) biosynthesis. NAD⁺ metabolism in cancer cell generally display the aberrant [92]. NAD is a high-energy molecule that can be reduced to form NAD dehydrogenase (NADH) in various cellular processes. In NAD synthesis, two major pathways involved from other NAD precursors, including the clearance pathway and salvage pathway. The clearance pathway is mediated by NNMT which was discovered in Chapter 1 in this dissertation. NAD synthesis via the salvage pathway is conducted by two key enzymes: nicotinamide phosphoribosyltransferase (NAMPT) and NMNAT. As the high-energy regulator with tumorigenesis, there are no much information in NMNAT.

For endothelial cell, no more connection also has even built to understand the NAD biosynthesis and EndMT. In this research, we survey the expressional level of NMNAT2. We expect to understand the relationship of salvage pathway in our glioma system. The aim is to provide more information for people to study this potential pathway and further

illustrate the possible perspective with salvage pathway compared to the clearance pathway in chapter 1 in this dissertation.

2.2.2 NMNAT2: Result

The expression level of NMNAT2 was firstly investigated in brain endothelial cell in glioma. In salvage pathway of NAD biosynthesis, previous reference focused more on NAMPT instead of NMNAT in cancer [149, 150]. NAMPT is inhibited while the cancer driven-factor CD38 is activated in pancreatic cancer [151, 152]. NMNAT2 serve as the same pathway with NAMPT. In our work, we identified NMNAT2 as the high-confidence candidate in EndMT in glioblastoma. We further need to investigate the results in both RNA and protein level.

In Figure 2.5, the result showed that NMNAT2 increased the expression in healthy BEC compared to TuBEC by using the quantitative real time-PCR. The protein level was further investigated by Western blotting with three biological repeats. The representative blotting image presented that BEC has more amounts of NMNAT2 compared to TuBEC (Figure 2.6A). The statistic results confirmed that NMNAT2 was significantly higher in BEC compared to TuBEC (Figure 2.6B). Overall, we confirmed that NMNAT2 increased the expression level in BEC compared to TuBEC in the EndMT after testing in our model.

Distribution of NMNAT2 was also surveyed in our culture system by using the immunostaining with three types of endothelial cells. In previous study, NMNAT2 has the relatively higher expression in the neuron in normal brain but it is not yet to describe in brain endothelial cell. BEC play as the critical role in metastasis and drug delivery in blood-

brain barrier. It is necessary to know the cell distribution in BEC in the pathological condition. We therefore conducted the immunostaining by using the maker of NMNAT2 in healthy BEC, subBEC and TuBEC (Figure 2.7). Although we can detect the difference of NMNAT2 by using qRT-PCR and Western blotting, immunostaining results still did not bring the clear staining and significant difference in these cells after multiple testing (Figure 2.7). Based on these results, the amount of NMNAT2 in brain could be tiny and hard to detect.

2.2.3 NMNAT2: Discussion

This is the first report to investigate the role of NMNAT2 in glioblastoma. Also, this is the first study to link the NMNAT2 with EndMT. The results in Figure 2.5 and 2.6 presented that NMNAT2 increased the expression level in BEC compared to TuBEC. This means the regulation of NMNAT2 was activated in health situation in brain but not in brain cancer. In previous research, NMNAT2 activated the expression in p53-dependent suppression in colorectal cancer [96, 116, 117]. They mainly tested the assay in cancer cell and the expression pattern of NMNAT2 was matched to our results which the healthy cell has higher expression of NMNAT2. In their report, the expression of NMNAT2 also linked as the drug-resistance indicator. As the first testing in endothelial cell, NMNAT2 belongs the benefits for preventing the glioblastoma. It could be further used as the therapy indicator in glioblastoma in the future.

Our results in the immunostaining of NMNAT2 did not show the significant difference among confBEC, subBEC and TuBEC (Figure 2.7) after multiple replicates. From the reference, overexpression of *Nmnat2* is toxic to neurons, suggesting that endogenous protein levels must be tightly regulated and providing a rationale for its low abundance [117, 153]. It could be applied in our case to explain the reason that brain endothelial cell was hard to detect in immunostaining assay. Also, this results relatively means that the NMNAT2-mediated pathway should be tightly regulated in brain endothelial cell. The results in Figure 2.5 and 2.6 presented that the subBEC, which is from healthy sample but no tightly junction, already loss the expression of NMNAT2 compared to the healthy BEC (confBEC). This result suggest NMNAT2 involve in relatively earlier change in our EndMT model. In sum, these properties could lead the NMNAT2-mediated pathway become hard to apply in drug in brain but provide as the therapy indicator in clinical early detection in glioblastoma.

NAD biosynthesis was recently considered as the potential target in cancer therapeutics [92]. NAD biosynthesis mainly divided into two individual routes, including the clearance pathway and salvage pathway. The clearance pathway was discussed in chapter 1 in this dissertation. The key enzyme NNMT increased the expression level in TuBEC (Chapter 1, Figure 1.3 and 1.4). We further confirmed the expression of NNMT support the progression of EndMT by using siRNA knockdown assay and chemical inhibitor. For the salvage pathway, results from the represented enzyme NMNAT2 gave the opposite feedback to us. The expression of NMNAT2 increased in the healthy BEC but loss the function in TuBEC. Overall, based on the results we have, it suggested that one of

the NAD pathway, clearance pathway could be the new solution for drug development, meanwhile, salvage pathway have the potential to be the novel indicator in EndMT in glioblastoma.

2.3 Metallothionein (MT)

2.3.1 MT: Introduction

Metallothioneins (MTs), which was identified over 50 years, belongs to the low-molecular-weight (less than 10 KDa) and cysteine-rich (range from 15%-30%) proteins [154, 155]. There are four classes of mammalian MT isoforms, including MT-1, MT-2, MT3 and MT-4. In human, eight members of MT-1 (MT-1A, 1B, 1E, 1G, 1H, 1M and 1X) in MT-1 category and one member MT-2A in MT-2 were discovered in individual tissues [156]. Generally, many of them can bind up to 20 monovalent and 7 divalent heavy metal ions [154, 157]. The main function of MTs is the regulation of homeostasis and detoxification, including the protection against oxidative stress or metals. Both heavy and trace metals can be chelated via sulfur-based clusters on cysteine residue [158]. Cytokine-induced induction in MT also provides a long-lasting protection to avert oxidative damage [159]. Moreover, MTs also display different functions in angiogenesis and pathological conditions.

The most critical field of MTs is focused on oncological application recently. Gene patterns of MTs is considered to be helpful to identify the tumor function, resistance to the

drugs, and create the suitable chemotherapy in the future [160]. Several solid tumors have the MT studies but presented different patterns, including tumor of head and neck, prostate carcinoma, breast tumors, ovarian carcinoma, hepatic tumors and colorectal carcinoma. For instance, MT-1H was found to decrease the expression in cancer tissue compared to the normal tissue in multiple type of cancers, including breast, colorectal, and neuroblastoma, etc. [161]. MT-1H was further confirmed as the tumor suppressor by targeting the promoter region of euchromatin histone methyltransferase 1 in prostate cancer [161]. The expression of MT-1F, MT-1G, MT-1X and MT-2A also displayed the down-regulation in colon cancer cell compared to the normal cell in both RNA and protein level [162]. However, MT-1G and MT-1A were induced in human myeloid leukaemia cells [163].

MTs have been investigated in several classical glioma cell lines but not yet in endothelial cell in GBM. The expression of MT1E was statistically correlated with the abilities of migration and invasion in the human glioma cell lines [164]. Higher amount of MTs was consider as the indicator of poor survival rate in glioblastoma. Increased the expression of MT-3 was confirmed to eliminate the p53 in GBM U87 cell line [165]. The expression of MT1A, MT1X, MT2, MT3 genes increased in result of the shorter patient survival in glioblastoma (grade IV) as compared to I-III grade astrocytomas [166]. It is believed that the impact of MTs are associated with the tumor progression in glioblastoma.

Whether MTs regulate the EndMT is not yet reported. The closer study is in EMT where the upstream regulator metal regulatory transcription factor 1 of MT-1F is confirmed to control the mesenchymal marker snail2 and β -catenin in ovarian cancer cell [167]. The

connection of MTs with endothelial cell was firstly reported to enhance the angiogenesis by activating the MMP9 and VEGF-mediated pathway in mice [168]. In this report, we are aiming to understand the potential role of MT in EndMT in glioblastoma. Based on previous EMT-related reference [169] and the gene-set analysis result (Table I.1), MT-1X presented the highest potential in our EndMT model among human MTs. We hypothesis that MT-1X is contributed to EndMT. Its RNA expression level, protein expression level, and immunostaining in overall cell distribution will be investigated in endothelial cell in GBM.

2.3.2 *MT: Result*

As the first investigation of MT in EndMT, we processed the analysis on MT1X with healthy BEC and patient-derived TuBEC in the *in vitro* model. From previous reference, the expression of MT1X decreased in several type of cancers, including liver cancer [157, 170]. However, in glioblastoma, research indicated that the downregulation of MT1X is associated the lower survival rate of patient [166]. The RNA expression level was presented in Figure 2.9. In our model, the MT1X presented the lower expression in TuBEC compared to BECs by using qRT-PCR. However, we further tested the protein level by using Western blotting (Figure 2.10). After multiple trials, the results did not present the significant banding for MT1X. The results from RNA expression did not reflect to its protein expression in MT1X.

The MT1X distribution of the cell also further tested in our model and presented the interesting findings with different distribution. In Figure 2.10, the representative results presented that the healthy BEC has the darkest staining of MT1X marker compared to TuBEC and subBEC. Compared with subBEC, TuBEC still got more staining of MT1X which even presented the brown color instead of red in immunostaining assay. The majority of the staining in subBEC and TuBEC is accumulated in or nearby cell nucleus which is matched to the normal situation in the reference records. Specifically, for healthy BEC, the staining of MT1X was distributed among the cells. The area where those BECs with tight junction presented the darker color. It is clear that MT1X present higher expression all over the whole cell. This could infer that MT1X was activated in BEC and regulated in this stage.

2.3.3 MT: Discussion

MTs functions in detoxification and the regulation of homeostasis for the metal ion. Previous reference mainly considered MTs as the tumor suppressor with anti-carcinogenic ability though it relied on different type of cancer [171]. However, several studies also indicated the opposite aspect where lower survival rate is closely correlated with the higher amounts of MTs in glioblastoma [166, 172]. In this study, MT1X was first investigated in endothelial cell in glioblastoma. The results illustrated that the amount of MT1X decreased around 5-10 folds in the patient-derived TuBEC compared to BEC in RNA expression level (Figure 2.8). This result is conflicted with recent studies in glioma but matched to some

of the cases in other type of cancers. In these studies, none of them focus on endothelial cell. In glioma, molecular mechanism was discussing in MT-3 with p53 in classical cell line U87 but not MT-1 [172]. In other pathological situation in brain, MT-1 and MT-2 was indicated to elevate the expression to support the angiogenic response especially in endothelial cell in *in vitro* experiment [173]. More researches are required to confirm the potential of MT family in EndMT in tumorigenesis.

The results from immunostaining with marker MT1X can partially support our results in qRT-PCR (Figure 2.10) though Western blotting did not reflect us the clear image (Figure 2.9). The supportive staining result indicated that MT1X raised the strongest expression in BEC compared to other two stages during the EndMT. However, if we compared to these two stages, subBEC and TuBEC, the staining situation was not continuously regulated in linear decreasing during the EndMT process. The subBEC has the lowest staining relative to confBEC and TuBEC which means MT1X presented the differ regulation during this stage. Based on these findings, MT1X indeed has the regulation during EndMT; moreover, MT1X is not simply suppressing or inducing by one-step during the entire EndMT. More researches to clarify the mechanism are required in the future.

Overall, the expression of MTs are currently considered as no universal rule in all human tumors and rely on differentiation status of each type of tumors [160]. In our case, we investigated the MT1X which provide the evidence to downregulate in TuBEC but suffer the differ control during the transition from healthy BEC. To our best knowledge, MT can mediate at least three fundamental processes as following: 1) the release of gaseous

mediators such as hydroxyl radical or nitric oxide; 2) mechanism in apoptosis, proliferation and angiogenesis, and 3) the binding and exchange of heavy metals such as zinc or copper [154, 157, 160]. The detail mechanisms should be achieved further in order to evaluate the clinical safety of potential strategies to modulate the MT expression. This report provided the first light of the MT1X in angiogenesis and EndMT in glioma.

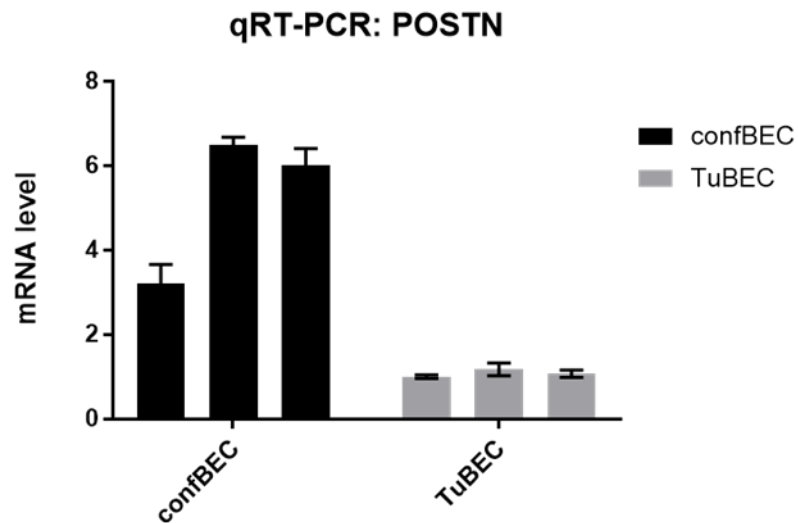


Figure 2. 1 Expression of POSTN decreased in glioma tumor associated endothelial cell compared to BEC in RNA level.

Bar chart represents the mRNA levels of POSTN measuring by quantitative real-time PCR (healthy brain endothelial cell vs tumor-associated endothelial cell). Each individual bar represents single sample by 3 replicates. Fold change values are expressed as mean \pm standard deviation (*p < 0.05).

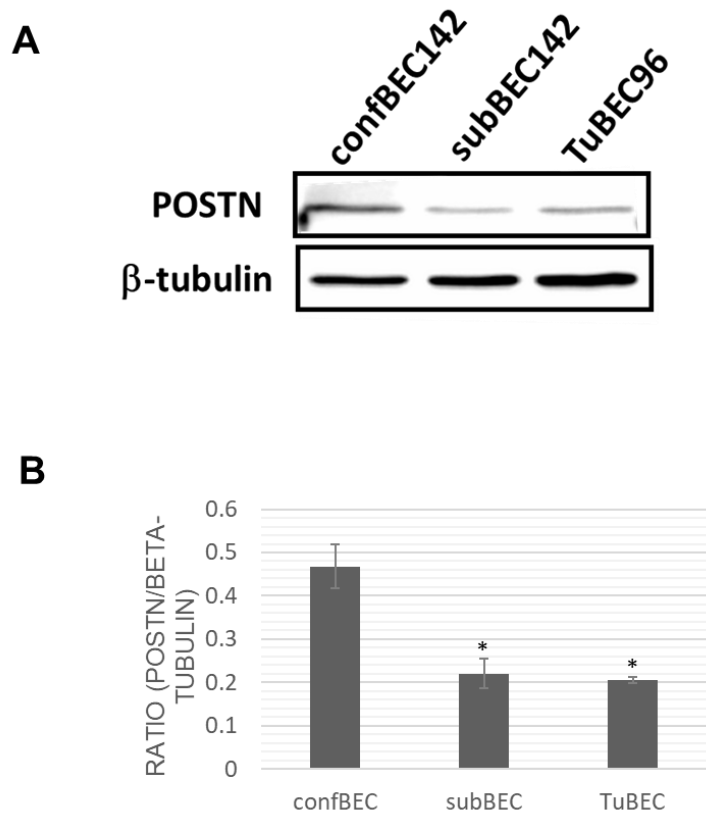


Figure 2. 2 Expression of POSTN decreased in glioma tumor associated endothelial cell in Western blotting.

Panel A was the representative image for Western blotting by using the POSTN antibody. Panel B was the statistical results from panel A in Western blotting assay. Each individual bar represents by 3 biological replicates. Image intensity was measure and converted by ImageJ. Statistical results were analysis compared to the confBEC by student's t test. P-value <0.05: *.

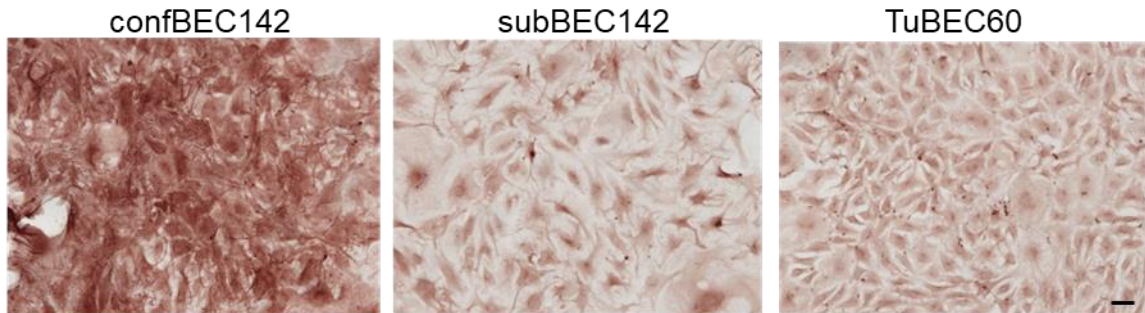


Figure 2. 3 POSTN staining showed the higher expression in healthy brain endothelial cell (BEC) compared to the glioma tumor-associated endothelial cell (TuBEC).

Representative images are stained with the immuno-marker POSTN. Three type of cells from left to right were confBEC (healthy BEC which arrived 100% confluency), subBEC (healthy BEC which were 70% confluency), and TuBEC (glioma patient-derived endothelial cell). Cells were cultured in EBM medium in 24-well plate for 48 hrs. Each image was performed at least three biological replicates. Bar, 100 μ m.

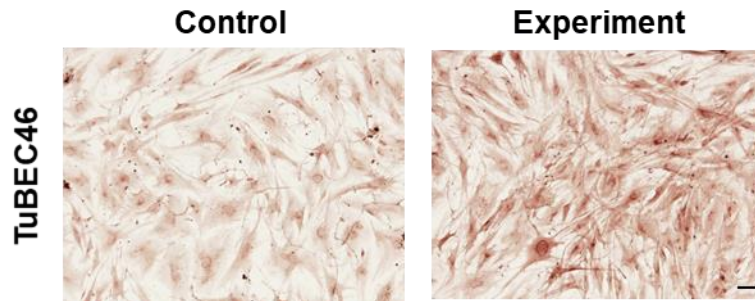


Figure 2. 4 Medium exchange from confBEC229 to TuBEC46 was observed the POSTN accumulation and morphology change.

Representative images are stained with the immuno-marker POSTN. Control was the TuBEC46 cultured for 48hrs. Experiment group was the TuBEC46 cell applied with the BEC medium for 48hrs. The BEC medium was the used medium collected from BEC229. All the cells were cultured in EBM medium in 24-well plate for 48 hrs. Bar, 100 μ m.

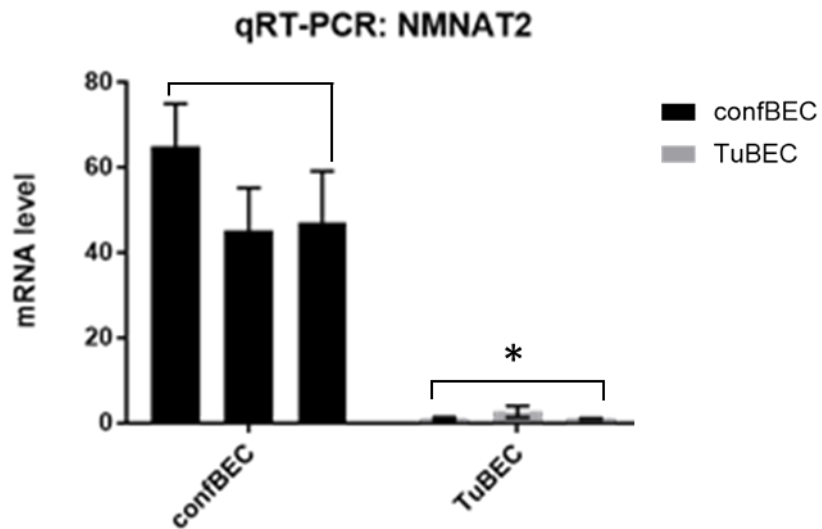


Figure 2.5 Expression of NMNAT2 decreased in glioma tumor associated endothelial cell compared to BEC in RNA level.

Bar chart represents the mRNA levels of NMNAT2 measuring by quantitative real-time PCR (healthy brain endothelial cell vs tumor-associated endothelial cell). Each individual bar represents single sample by 3 replicates. Fold change values are expressed as mean \pm standard deviation (*p < 0.05).

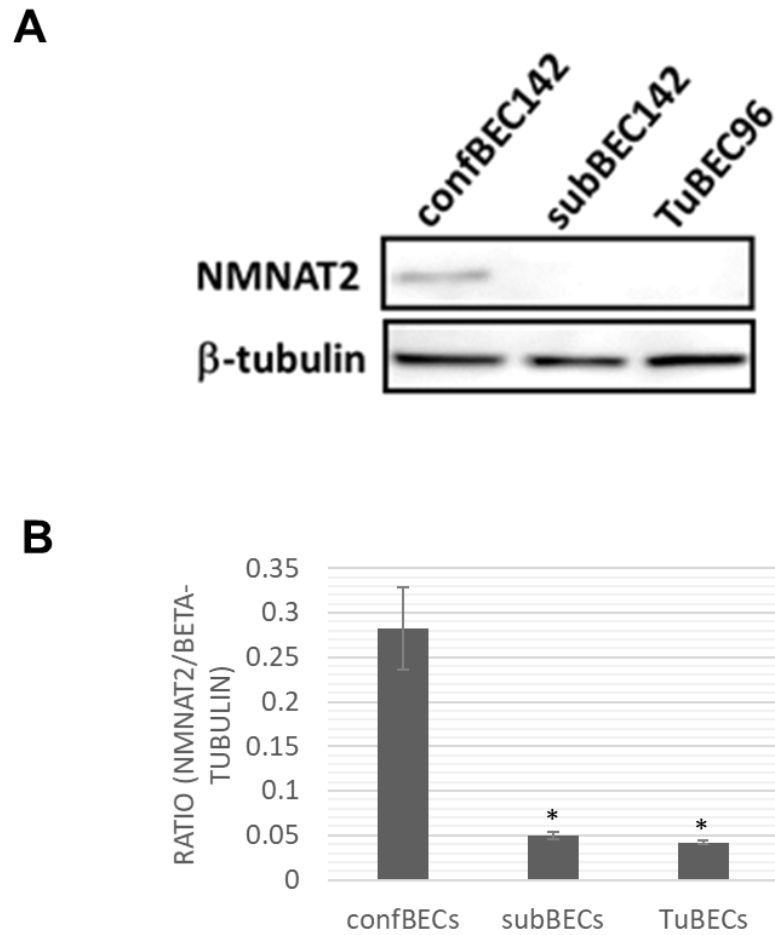


Figure 2.6 Expression of NMNAT2 decreased in glioma tumor associated endothelial cell in Western blotting.

Panel A was the representative image for Western blotting by using the NMNAT2 antibody. Panel B was the statistical results from panel A in Western blotting assay. Each individual bar represents by 3 biological replicates. Image intensity was measure and converted by ImageJ. Statistical results were analysis compared to the confBEC by student's t test. P-value <0.05: *.

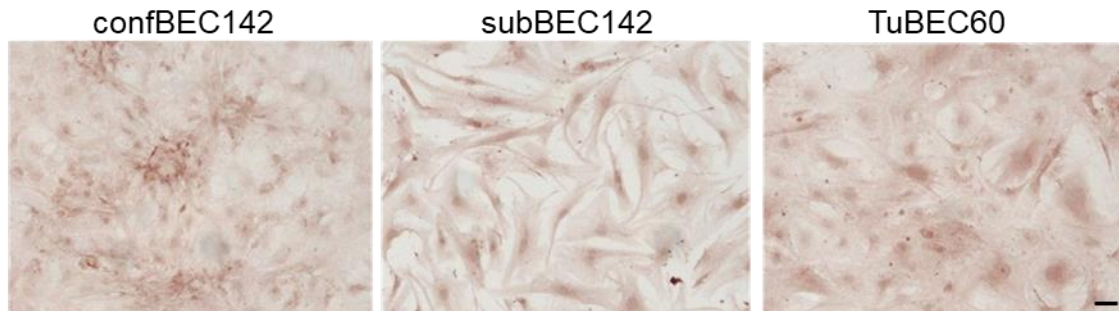


Figure 2.7 NMNAT2 staining showed no obvious difference in healthy brain endothelial cell (BEC) compared to the glioma tumor-associated endothelial cell (TuBEC).

Representative images were stained with the immuno-marker NMNAT2. Three type of cells from left to right were confBEC (healthy BEC which arrived 100% confluency), subBEC (healthy BEC which were 70% confluency), and TuBEC (glioma patient-derived endothelial cell). Cells were cultured in EBM medium in 24-well plate for 48 hrs. Each image was performed at least three biological replicates. Bar, 100 μ m.

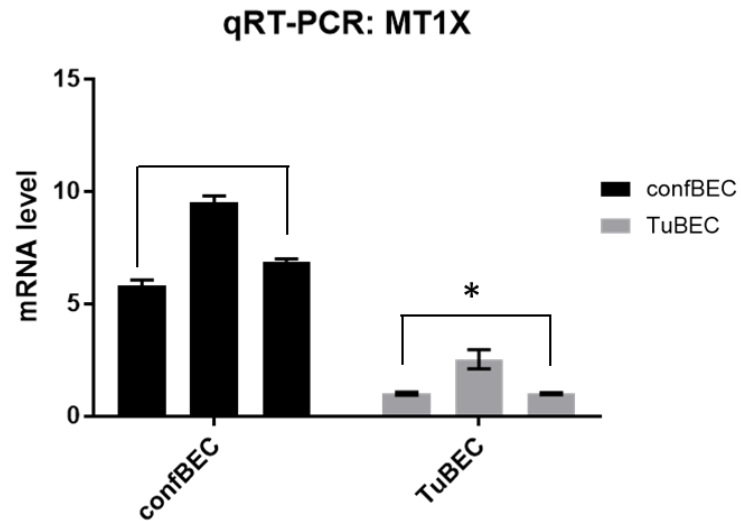


Figure 2. 8 Expression of MT1X decreased in glioma tumor associated endothelial cell compared to BEC in RNA level.

Bar chart represents the mRNA levels of MT1X measuring by quantitative real-time PCR (healthy brain endothelial cell vs tumor-associated endothelial cell). Each individual bar represents single sample by 3 replicates. Fold change values are expressed as mean \pm standard deviation (*p < 0.05).

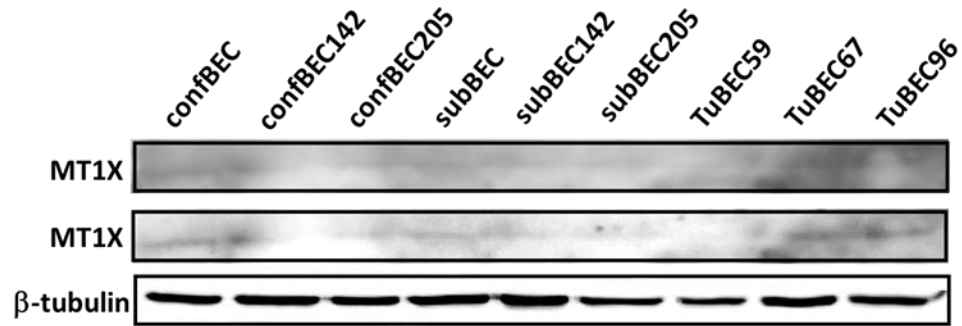


Figure 2. 9 Expression of MTX presented no difference in TuBEC compared to BEC in Western blotting.

Representative image was for Western blotting by using the MT1X antibody. There were no significant band has been identified after multiple replicates. (Not reflect the difference by using Western blotting approach)

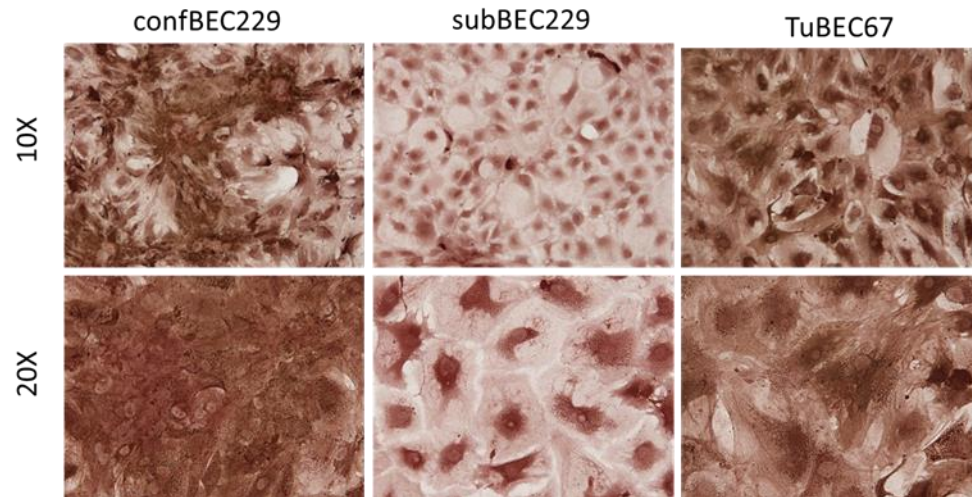


Figure 2. 10 MT1X staining showed increased the expression in healthy brain endothelial cell (BEC) compared to the glioma tumor-associated endothelial cell (TuBEC).

Representative images are stained with the immuno-marker NMNAT2. Three type of cells from left to right were confBEC (healthy BEC which arrived 100% confluency), subBEC (healthy BEC which were 70% confluency), and TuBEC (glioma patient-derived endothelial cell). Cells were cultured in EBM medium in 24-well plate for 48 hrs. Each image was performed at least three biological replicates. Bar, 100 μ m.

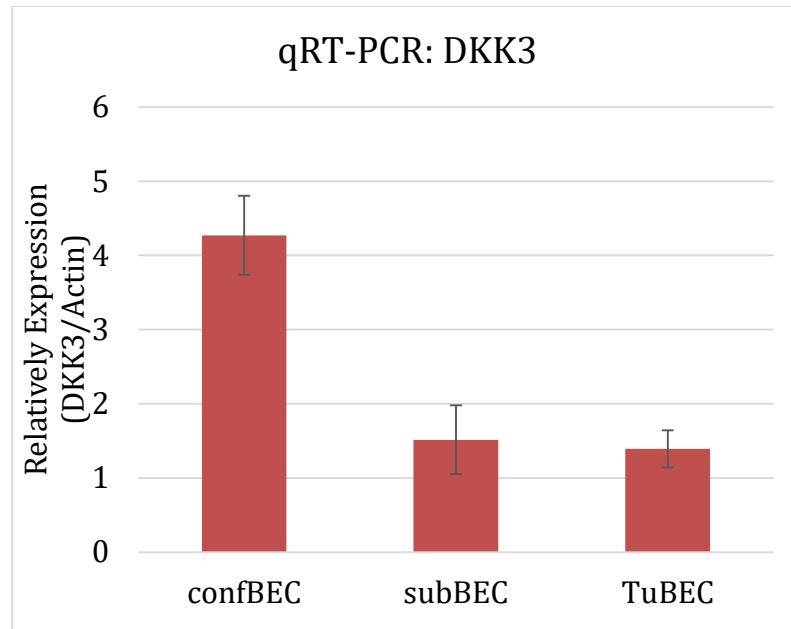


Figure 2. 11 Expression of DKK3 decreased in glioma tumor associated endothelial cell compared to BEC in RNA level.

Bar chart represents the mRNA levels of MT1X measuring by quantitative real-time PCR (healthy brain endothelial cell vs tumor-associated endothelial cell). Each individual bar represents single sample by 3 replicates. Fold change values are expressed as mean \pm standard deviation (*p <0.05).

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