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Molecular Characterization of Glioblastoma Vessels:
Identification of Insulin-like Growth Factor Binding Protein 7 (IGFBP7)
as Modulator of Angiogenesis and Tumor Growth

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MOLECULAR CHARACTERIZATION OF GLIOBLASTOMA VESSELS: IDENTIFICATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 7 (IGFBP7) AS MODULATOR OF ANGIOGENESIS AND TUMOR GROWTH

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To my family and friends

"Every great advance in science has issued from a new audacity of imagination."

-John Dewey (1859-1952)

AUTHORIZATION

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ABSTRACT

Angiogenesis is a major requirement for tumor growth. Inhibition of angiogenesis is considered a promising strategy for the treatment of highly vascularized tumors, such as glioblastoma multiforme (GBM). Tumor vessels exhibit an aberrant molecular and functional phenotype; therefore, identification and functional characterization of molecular differences between normal and tumor vessels could lead to the development of more targeted and effective anti-angiogenic therapies. The objective of this thesis was to identify molecular biomarkers of GBM vessels and evaluate their potential role in angiogenesis. Molecular differences between GBM and non-malignant brain vessels were identified using a combination of laser capture microdissection of brain vessels, RNA amplification and transcriptional profiling using microarray technology. Among differentially expressed genes, insulin-like growth factor binding protein 7 (IGFBP7) was validated as a selective biomarker of GBM vessels, induced in and secreted by tumor endothelial cells, and deposited into the vascular basement membrane. Analyses of tumor microenvironment properties and mediators revealed that IGFBP7 induction in human brain endothelial cells (HBEC) is triggered by members of TGF-β family released by GBM cells through ALK5/Smad-2 signaling pathway, associated with the late phase angiogenesis. Functional *in vitro* and *in vivo* studies using a chicken chorioallantoic membrane (CAM) tumor model indicated that IGFBP7 has pericyte-recruiting and tumor suppressive roles in GBMs. The findings presented in this thesis demonstrated novel functions of IGFBP7 in angiogenesis and tumorigenesis that may lead to future development of diagnostic or anti-angiogenic/anti-tumorigenic therapeutic approaches to treat GBMs.

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α-SMA	Alpha-smooth muscle actin
γ-GT	Gamma-glutamyl transpeptidase
ABC	ATP-binding cassette
AJ	Adherens junction
ALK1, 5	Activin receptor-like kinase 1, 5
Ang-1, -2	Angiopoietin-1, -2
ANOVA	Analysis of variance
API5	Apoptosis inhibitor 5
aRNA	Amplified ribonucleic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCL2	B-cell CLL/lymphoma 2
bFGF	Basic fibroblast growth factor
BM	Basement membrane
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
Bp	Base pair
°C	Degree Celsius
CAM	Chick chorioallantoic membrane
CCL21	Chemokine (C-C motif) ligand 21
CD36	Cluster of Differentiation 36 (Thrombospondin-1 receptor)
CD105	Endoglin
cDNA	Complementary deoxyribonucleic acid
CLIC4	Chloride intracellular channel 4
CLT	Capillary-like tube
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CTL	Control
DAPI	4',6-Diamidino-2-Phenylindole
dB-cAMP	Dibutyryl cyclic adenosine monophosphate
DEPC	Dimethylpyrocarbonate
D-MEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EDG-1	Endothelial differentiation sphingolipid G-protein-coupled receptor-1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EJ-1	Human bladder carcinoma cell line
ELISA	Enzyme-Linked ImmunoSorbent Assay
ERK	Extracellular regulated kinase
FBS	Fetal bovine serum

FDA	Food and Drug Administration
FGF-2	Fibroblast growth factor-2
FVIII-rAg	Factor VIII-related antigen (von Willebrand Factor)
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GC	Giant cell
GFAP	Glial fibrillary acidic protein
Glut-1	Glucose transporter type 1
H&E	Hematoxylin and Eosin
HBEC	Human brain endothelial cell
HBSS	Hank's buffered salt solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HI	Heat-inactivated
HIF-1	Hypoxia-inducible factor-1
HRE	Hypoxia-responsive element
HRP	Horseradish peroxidase
HUGO	Human Genome Organization
IBS	Institute for Biological Sciences
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGFBP-rP	IGFBP-related protein
IgG	Immunoglobulin G
IIT	Institute for Information Technology
IL-8	Interleukin-8
IQGAP1	IQ motif containing GTPase activating protein 1
JAK1	Janus kinase 1
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
KRT19	Keratin 19
LCM	Laser capture microdissection
LDL	Low-density lipoprotein
mac25	Meningioma-associated cDNA 25
MAPK	Mitogen-activated protein kinase
MAO	Monoamine oxidase
MBP	Myelin basic protein
MDM2	Mouse double minute 2 homolog
MEF2C	Myocyte enhancer binding factor 2C
MEK	MAPK-ERK kinase
MEM	Minimum Essential Medium
MMP	Metalloprotease
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein

MT-MMP	Membrane type-MMP
mTOR	Mammalian target of rapamycin
MVD	Microvessel density
NaCl	Sodium chloride
N-cadherin	Neural-cadherin
NCAM	Neural cell adhesion molecule
NCBI	National Center for Biotechnology Information
NRC	National Research Council
NuMA	Nuclear Mitotic Apparatus
OCI	Ontario Cancer Institute
OD	Optical density
PA	Plasminogen activator
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
Penta-His	Penta-Histidine
PGI2	Prostacyclin
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PlGF	Placenta-like growth factor
PSF	Prostacyclin secreted factor
PSMA	Prostate-specific membrane antigen
P-Smad	Phospho-Smad
PTEN	Phosphatase/tensin homolog protein
PUM2	Pumilio homolog 2
PV-1	Plasmalemmal vesicle associated protein-1
Q-PCR	Quantitative- Polymerase chain reaction
R	Correlation coefficient
RGD	Arginine-Glycine-Aspartic acid
Rho-A	Ras homolog member A
RIPA	Radioimmuno precipitation assay
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase- Polymerase chain reaction
S1P	Sphingosine-1-phosphate
SCID	Severe combined immunodeficiency
SCYE1	Small inducible cytokine subfamily E, member 1
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
s.e.m	Standard error of the mean
SMC	Smooth muscle cell
SOD-2	Superoxide dismutase-2
SOX-9	SRY (sex determining region Y)-box 9

SPARC	Secreted protein, acidic, cystein-rich
SYNGAP1	Synaptic Ras GTPase activating protein 1 homolog
T98G	Glioblastoma cell line
T98G-CM	T98G-conditioned media
TAF	Tumor-derived adhesion factor
TβR-I, -II	Transforming growth factor β receptor type I, II
TBST	Tris buffered saline-Tween
TEM	Tumor endothelial marker
Tf	Transferrin
TGF-β1, -β2, -β3	Transforming growth factor-beta type 1, 2, 3
TIMP	Tissue inhibitor of metalloprotease
TJ	Tight junction
TKI	Tyrosine kinase inhibitor
tPA	Tissue-type PA
TRA2A	Transformer-2α
TRAF7	TNF receptor-associated factor 7
TRIM2	Tripartite motif-containing 2
Thy-1	Thymus cell surface antigen 1
U87MG	Glioblastoma cell line
U87MG-CM	U87MG-conditioned media
UEA 1	Ulex Europeaus Agglutinin I
uPA	Urokinase-type PA
vBM	Vascular basement membrane
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR-1/Flt1	VEGF receptor type 1
VEGFR-2/KDR	VEGF receptor type 2
VIM	Vimentin
WHO	World Health Organization
ZFP36L2	Zinc finger protein 36, C3H type-like 2
ZIC2	Zic family member 2
ZNF224	Zinc finger protein 224
ZO	Zonula occludens

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Angiogenesis

Blood vessels supply oxygen and nutrients to tissues in the body. New blood vessel formation occurs through two different processes, vasculogenesis and angiogenesis (Carmeliet, 2000). Vasculogenesis is the formation of blood vessels by endothelial progenitors, whereas angiogenesis is the sprouting of new blood vessels from pre-existing ones. Angiogenesis is tightly regulated and occurs physiologically during embryonic development, wound healing, and during the female reproductive cycle (i.e. corpus luteum formation in the ovary and endometrial regeneration during the menstrual cycle) (Pepper, 1997). Angiogenesis is also activated in pathological conditions. Several diseases are characterized by excessive angiogenesis, including arthritis, psoriasis, atherosclerosis, obesity, and most importantly cancers (Carmeliet, 2003). Angiogenesis in tumors was described a century ago (Goldman, 1907), but it was only in 1971 that Judah Folkman proposed that tumors cannot grow more than few millimetres without the supply of nutrients and oxygen, and that tumor growth is angiogenesis-dependent. Therefore, blocking angiogenesis was suggested as a potential strategy to inhibit tumor growth (Folkman, 1971).

1.1.1 Phases of angiogenesis

The process of angiogenesis is regulated by the net balance of pro- and anti-angiogenic mediators. The 'angiogenic switch' is turned on when positive regulators dominate (e.g. VEGF, bFGF, EGF, PDGF), whereas it is turned off (i.e. endothelial quiescence is achieved) when negative regulators predominate (e.g. thrombospondin-1,

endostatin, angiostatin) (Bergers and Benjamin, 2003; Pepper, 2001). Angiogenesis is a multi-step process that can be divided into two phases: the activation (early) phase and the resolution (late) phase (Jain, 2003; Pepper, 1997).

A) Early phase of angiogenesis

Quiescent vessels are composed of endothelial cells (ECs) that are surrounded by pericytes or smooth muscle cells (SMCs), both embedded in a basement membrane (BM). In specialized brain ECs, both adherens junctions (AJs), consisting of vascular endothelial (VE)-cadherin dimer which bind to the actin cytoskeleton via α- and β-catenin proteins, and tight junctions (TJs) tighten the vessels, thus providing mechanical strength and controlling vessel permeability (Carmeliet, 2003). In response to angiogenic stimuli such as hypoxia, inflammation or mechanical forces, ECs become activated through the release of angiogenic factors. Vascular endothelial growth factor (VEGF) is the most important growth factor regulating angiogenesis. VEGF induces EC vasodilation and increases EC permeability via Src kinases (Eliceiri *et al.*, 1999). As a consequence, plasma proteins such as fibrin extravasate into the interstitial milieu and serve as a provisional matrix for EC migration (Carmeliet, 2000). Nitric oxide is also involved in EC vasodilation (Kubes, 1995). To enable EC migration, vessel walls need to disassemble. Angiogenesis activation results in the loosening of AJs. Tyrosine phosphorylation of the intracellular protein catenin has been reported to dissociate the junctional complex, thereby loosening cell-cell contacts and allowing EC migration (Hoschuetzky *et al.*, 1994). Angiopoietin-2 (Ang-2) causes pericyte and SMC detachment from EC, thus inducing instability in the vascular network (Gale and

Yancopoulos, 1999). Several proteases such as metalloproteases (MMPs), plasminogen activators (PAs), heparinases, chymases, tryptases and cathepsins degrade the BM matrix components to stimulate EC migration and sprouting into the surrounding interstitial space (Jackson, 2002; Pepper *et al.*, 1991). MMPs are zinc-dependent proteases divided into two groups, secreted MMPs and membrane-type (MT)-MMPs; both are able to degrade several extracellular matrix (ECM) proteins and are regulated by tissue inhibitors of metalloproteases (TIMPs) (Pepper, 2001). For example, MMP-2 and MMP-9 degrade type IV collagen, the major component of the BM, and promote EC invasion (Koivunen *et al.*, 1999). PAs are divided into two groups, tPAs (tissue-type PAs), which function intravascularly and mainly degrade fibrin clots (Stubbs *et al.*, 1998), and uPAs (urokinase-type PAs), which function extravascularly and are important for cell migration, invasion and tissue remodeling (Binder *et al.*, 2007; Pepper, 2001). Both PA families are involved in catalyzing the conversion of plasminogen to plasmin. The active protease plasmin directly digests various components of the ECM and activates MMPs which further degrade the ECM. Plasmin and PAs are regulated by specific inhibitors such as anti-plasmin and PA inhibitors-1, and -2 (Andreasen *et al.*, 2000). MMP- and active plasmin- mediated degradation of the ECM leads to a decrease of ECM protein density, exposes cryptic sites for adhesion molecules like integrins, and releases matrix-bound angiogenic factors such as heparin binding forms of VEGF or fibroblast growth factor-2 (FGF-2) to promote EC proliferation and migration (Gengrinovitch *et al.*, 1999; Park and Lee, 1999; Saksela and Rifkin, 1990).

Integrins are heteromeric transmembrane receptors composed of α and β subunits. These subunits form different heteromeric combinations that interact with ECM

components (fibronectin, laminin, vitronectin, collagens) in a selective manner. Integrins recognize specific motifs such as the arginin-glycine-aspartic acid (RGD) sequence and are responsible for anchoring cells to the ECM, which triggers intracellular signaling events necessary for the invasion, migration and survival of EC during angiogenesis (Guo and Giancotti, 2004). For example, the expression of αvβ3 and αvβ5 increases EC proliferation and positively regulates angiogenesis (Nisato *et al.*, 2003).

During the early phase of angiogenesis, VEGF induces EC proliferation and survival through the MAPK and PI3K/AKT signaling pathways (Gerber *et al.*, 1998; Zachary, 2003). The PI3K/AKT pathway, in turn, regulates several cell cycle genes such as cyclin D1, p27 and $BclX_L$ (Milkiewicz *et al.*, 2006). Translocation of β-catenin to the nucleus is mediated through to dissociation of adherens complexes, and results in binding to T-Cell Factor and induction of genes involved in EC proliferation such as c-myc (Ben-Ze'ev *et al.*, 2000).

As ECs migrate, the sprouting tip of the EC protrudes into the interstitium and forms a lumen either through fusion of cytoplasmic vesicles or intercellular canalization (Egginton and Gerritsen, 2003; Milkiewicz *et al.*, 2006). Integrins, VEGF (121 and 165), angiopoietin-1 (Ang-1), myocyte enhancer binding factor 2C (MEF2C) and MAPK all contribute to the process of the lumen formation (Carmeliet, 2000; Davis *et al.*, 2002; Davis and Camarillo, 1996).

B) Late phase of angiogenesis

After the lumen of a new vessel is formed, the process enters into the late phase of angiogenesis, which consists of inhibition of EC proliferation and migration, and

induction of vessel stabilization and maturation. Vessel stabilization is characterized by a) recruitment of mural cells and their integration into the vascular wall of the newly formed vessel, leading to quiescence of both mural cells and ECs, b) BM reconstitution and c) tightening of cell junctions (Carmeliet, 2003). Vessel walls and network structures undergo further remodeling processes including tissue- and organ-specific specialization, development of a hierarchical vessel network (Jain, 2003), and coordination of vessel regression (Dor *et al.*, 2003); collectively, these processes are called maturation.

Pericytes are found around capillaries, arterioles and venules whereas vascular SMCs coat larger vessels such as arteries and veins (Armulik *et al.*, 2005). Both cell types are referred to as mural cells (Banfi *et al.*, 2005). Pericytes have long processes and are embedded within the vascular basement membrane (vBM) of microvessels (Armulik *et al.*, 2005). Until recently they were thought to serve as an inert scaffold for ECs. Genetic mouse mutant studies demonstrated their importance in the regulation, stabilization, maturation and remodeling of vessels, mainly through direct physical contact and paracrine signaling to ECs (Bergers and Song, 2005). Indeed, recruitment of pericytes into the microvessel wall was revealed to be critical for vessel stabilization and maturation (Chantrain *et al.*, 2006; Jain, 2003). Four main pathways are involved in pericyte recruitment (Chantrain *et al.*, 2006): platelet-derived growth factor B (PDGFB)/PDGF receptor β (PDGFR- β); sphingosine-1-phosphate (S1P)/endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1); Ang-1/Tie2; and transforming growth factor β type I (TGF-β1).

BM reformation occurs through the deposition of perivascular ECM and is essential for vessel stabilization and maturation (Carmeliet, 2004; Jain, 2003). The BM

provides structural and organizational stability for vascular endothelium (Davis and Senger, 2005). Proteases (MMPs) and their inhibitors (TIMPs) further remodel the ECM, thus revealing anti-angiogenic fragments such as endostatin (O'Reilly *et al.*, 1997), angiostatin (O'Reilly *et al.*, 1994), arresten (Colorado *et al.*, 2000) and canstatin (Kamphaus *et al.*, 2000) or releasing anti-angiogenic matrix-bound molecules such as thrombospondin-1 (Jimenez *et al.*, 2000), resulting in the inhibition of cell migration and proliferation and EC stabilization. TIMP-3, a heparin sulfate binding protease inhibitor 3, is highly expressed in pericytes and facilitates EC-pericyte interaction resulting in vessel stabilization (Baker *et al.*, 2002). Some of the regulators of pericyte recruitment such as S1P/EDG-1 and TGF-β1/activin receptor-like kinase 5 (ALK5) also induce the secretion of ECM components. Adhesion of ECs to ECM via integrins is important for EC-pericyte interaction. Laminin (likely 10 and 8) interaction with integrins (α6β1 and α3β1) has been implicated in vessel stabilization (Davis and Senger, 2005). Laminins/integrins also promote EC quiescence by suppressing EC proliferation (Mettouchi *et al.*, 2001).

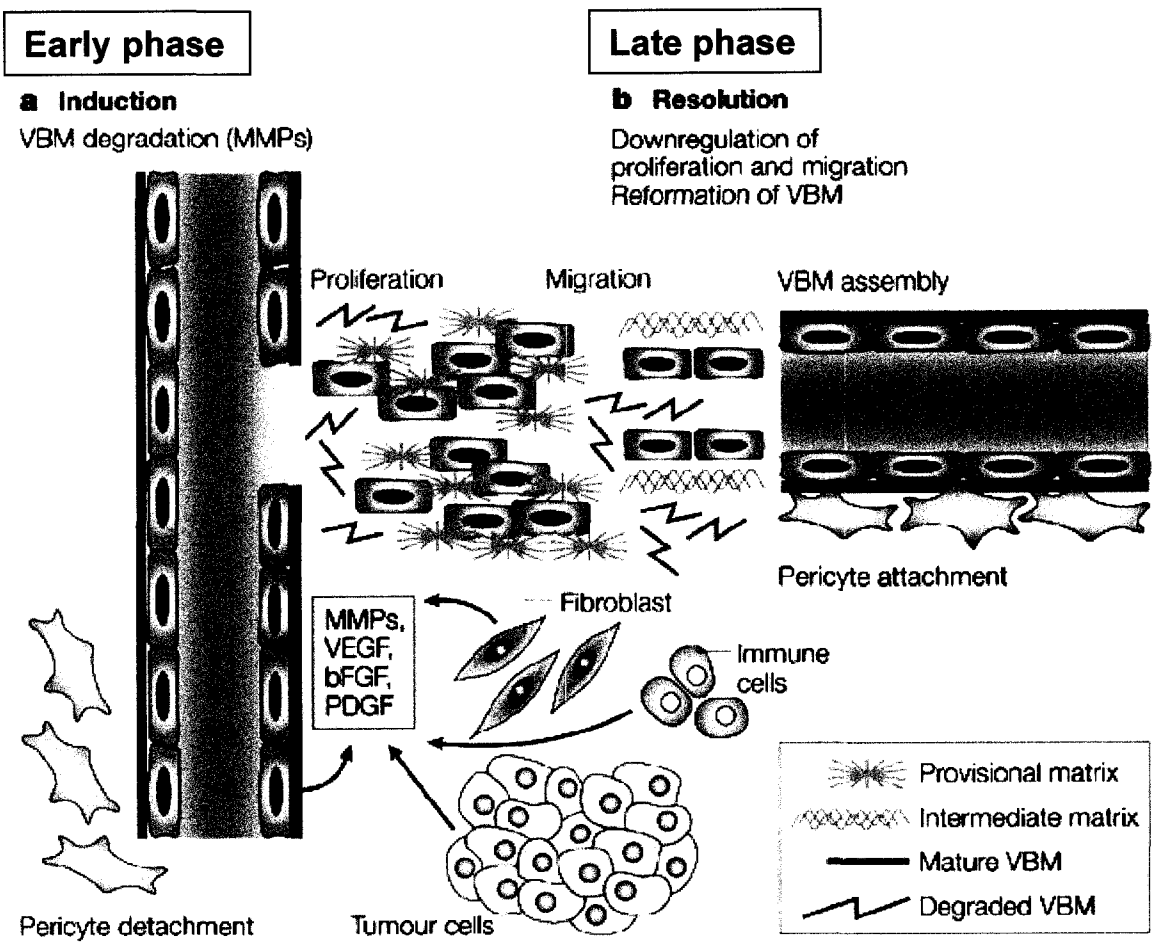
The tightening of endothelial cell-cell junctions plays an important role in vessel stabilization and maturation (Jain, 2003). This step maintains the integrity of the endothelium and controls vessel permeability. VE-cadherin and neural (N)-cadherin allow EC-EC and EC-mural cell communication, respectively. During the last stage of vessel maturation, ECs specialize into tissue- and organ- network structures (Jain, 2003). The Notch pathway and ephrin determine the arterial-venous fate of ECs and guide vascular branching (Rossant and Howard, 2002). Blood flow determines hierarchical position within the vasculature network.

The process of angiogenesis is summarized in Fig. 1.

Figure 1

Process of angiogenesis

Schematic illustration of the principal components of the angiogenic process: (a) In the early phase, growth factors (e.g. VEGF, bFGF, PDGF) and MMPs derived from the tumor microenvironment induce pericyte detachment and degradation of the VBM. The formation of a 'provisional matrix' from extravasated plasma proteins allows EC migration and proliferation. Eventually, the ECs organize to form a new lumen. (b) In the late phase, EC proliferation and migration are inhibited, a new mature vBM is formed and pericytes are recruited to stabilize newly formed vessels. Reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Kalluri, 2003), copyright by Nature Publishing Group (2003).



1.1.2 Regulation of angiogenesis

Angiogenesis is a complex multi-step process that requires a complex signaling pathway and a high degree of spatial and temporal orchestration of multiple angiogenic factors. One of the primary forces driving the onset of angiogenesis is hypoxia. Oxygen deprivation activates hypoxia-inducible factor-1 (HIF-1), a transcription factor that binds to hypoxia-responsive elements (HRE) and activates the transcription of various target genes involved in angiogenesis (Pugh and Ratcliffe, 2003). VEGF is a central regulator of the angiogenic process (Dvorak, 2006) and is induced by HIF-1 (Kaur *et al.*, 2005). The VEGF family is composed of five members: VEGF-A, -B, -C, -D, and placenta-like growth factor (PlGF) (Ferrara, 2004). VEGF-C and -D are known to be important in lymphangiogenesis- the development of new lymph vessels (Karkkainen *et al.*, 2002). VEGF-A, also termed VEGF is the most important factor that stimulates both physiological and pathological angiogenesis. VEGF binds to two tyrosine receptors, VEGF receptor-1 (VEGFR-1/Flt1) and VEGF receptor-2 (VEGFR-2/KDR) and signals through VEGFR-2 on the endothelial surface to mediate EC permeability, proliferation, migration and survival (Dvorak, 2006; Ferrara, 2004; Zachary, 2003). Genetic ablation studies showed that a lack of a single VEGF allele results in embryonic lethality due to abnormal vessel development (Carmeliet *et al.*, 1996). VEGF is over-expressed in several cancers, and anti-VEGF or anti-VEGFR-2 strategies have been shown to reduce tumor growth and angiogenesis (Benjamin *et al.*, 1999). PlGF is a ligand for VEGFR-1 that contributes to pathological angiogenesis through the displacement of VEGF from VEGFR-1, thereby resulting in increased VEGF available for VEGFR-2 (Carmeliet, 2004; Park *et al.*, 1994). PlGF-induced crosstalk between VEGFR-1 and VEGFR-2

amplifies VEGF-driven angiogenesis (Autiero *et al.*, 2003; Carmeliet *et al.*, 2001). PDGF, FGF-2, epidermal growth factor (EGF), hepatocyte growth factor (HGF), TGF-β and insulin-like growth factor (IGF) are also regulators of angiogenesis. In addition to the growth factors mentioned above, several other molecules are involved in angiogenesis, among them Ang-1/Ang-2/Tie2 receptor, neuropilins, and chemokines (i.e. interleukin (IL)-8, stromal cell-derived factor-1) (Carmeliet, 2004; Milkiewicz *et al.*, 2006).

1.1.2.1 Insulin-like growth factor (IGF) system

The IGF system is composed of IGFs (IGF-I and IGF-II), IGF receptors (IGF type 1 and type 2), IGF-binding proteins (IGFBP-1 to IGFBP-6) and IGFBP proteases (Jones and Clemmons, 1995). IGFs have multiple functions; they are mitogenic, anti-apoptotic, promote cell migration, regulate glucose metabolism and participate in angiogenesis (Jones and Clemmons, 1995; Wu *et al.*, 2002). Several studies confirmed the role of IGF-1 in angiogenesis. IGF-1 has been shown to promote rat aortic angiogenesis *in vitro* (Nicosia *et al.*, 1994) and enhance neovascularization *in vivo* (Su *et al.*, 2003). The IGFBP proteins serve as transporters and storage pools for IGF-1, and have recently been expanded to include IGFBP-related proteins (IGFBP-rP1 to IGFBP-rP10), thereby forming the IGFBP superfamily (Hwa *et al.*, 1999). Although all IGFBP members are secreted proteins with a conserved N-terminal cystein-rich domain IGFBP motif (GCGCCXXC), IGFBPs bind to IGFs with high affinity, whereas IGFBP-rPs bind to IGFs with low affinity (Hwa *et al.*, 1999) suggesting that their activities are IGF-independent.

A) IGFBP7

The human IGFBP-rP1 gene, also known as IGFBP7, is localized in chromosome 4q12-13 (Swisshelm *et al.*, 1995). IGFBP7 cDNA was first cloned by Murphy and colleagues (1993) as a gene preferentially expressed in normal leptomeningeal cells compared to meningiomas and was named mac25 (meningioma-associated cDNA 25). Independently, IGFBP7 was purified from the conditioned medium of human bladder carcinoma cell line EJ-1 as a cell adhesive glycoprotein and was designated TAF (tumor-derived adhesion factor) (Akaogi *et al.*, 1994). Yamauchi and colleagues (1994) purified IGFBP7 from plasma and, because it stimulated prostacyclin activity, named it PSF (prostacyclin secreted factor). IGFBP7 gene structure consists of 5 exons that are translated into IGFBP7 protein that contains three major domains: an amino-terminal cystein-rich domain containing IGFBP motif (which is conserved in all IGFBPs), a mid-region that includes an aldehyde dehydrogenase-like domain and a Kazal-type serine proteinase inhibitor-like domain, and a C-terminal immunoglobulin-like domain (Hwa *et al.*, 1999).

IGFBP7 is cleaved by a membrane-bound matriptase 1 into a two-chain form consisting of a N-terminal sequence #27-97 (8 kDa) and a C-terminal sequence # 98-282 (25 kDa) (Ahmed *et al.*, 2006). The cleaved (25 kDa) and uncleaved (33 kDa) forms of IGFBP7 have been shown to modulate IGF-dependent and -independent biological functions (Ahmed *et al.*, 2003). For example, cleaved IGFBP7 inhibits the IGF-dependent growth activity of uncleaved IGFBP7, and increases cell attachment activity (Ahmed *et al.*, 2003). Aside from proteolytic cleavage, several studies have also indicated that IGFBP7 is regulated by DNA methylation. IGFBP7 gene expression is

silenced by promoter/exon 1/intron 1 hypermethylation in cancers, including colon, lung, liver and skin tumors (Chen *et al.*, 2007; Komatsu *et al.*, 2000; Ruan *et al.*, 2007; Wajapeyee *et al.*, 2008); since IGFBP7 expression is down-regulated in these cancers, a tumor suppressor role for IGFBP7 has been proposed. In contrast, up-regulation of IGFBP7 has been reported in colorectal carcinoma (Adachi *et al.*, 2001; Umeda *et al.*, 1998) and in the cerebrospinal fluid of children with acute lymphoblastic leukemia (How *et al.*, 1999).

IGFBP7 regulates several biological processes, including cellular proliferation and adhesion (Ahmed *et al.*, 2003). IGFBP7 interacts with ECM components such as collagen IV to promote cell adhesion and morphological changes (Akaogi *et al.*, 1996) through binding to cell surface heparin sulphate (Kishibe *et al.*, 2000; Sato *et al.*, 2007). While studying the distribution of IGFBP7 in tumors, Akaogi and colleagues (1996) observed that IGFBP7 accumulates in blood vessels of numerous tumors, suggesting that IGFBP7 may play a role in angiogenesis; this was subsequently referred as angiomodulin. The knowledge on IGFBP7 is still limited (Pubmed; key word: IGFBP7 OR IGFBP-rP1 = 113 published studies). IGFBP7 functions in both angiogenesis and tumorigenesis remain to be clarified.

1.1.3 Brain angiogenesis

During embryonic development, after the formation of the primitive vascular system through vasculogenesis, most blood vessels, including those of the brain, arise through angiogenesis (Plate, 1999). During brain angiogenesis, which is driven in part by the metabolic demands of the growing nervous system (Tuor *et al.*, 1994), newly formed

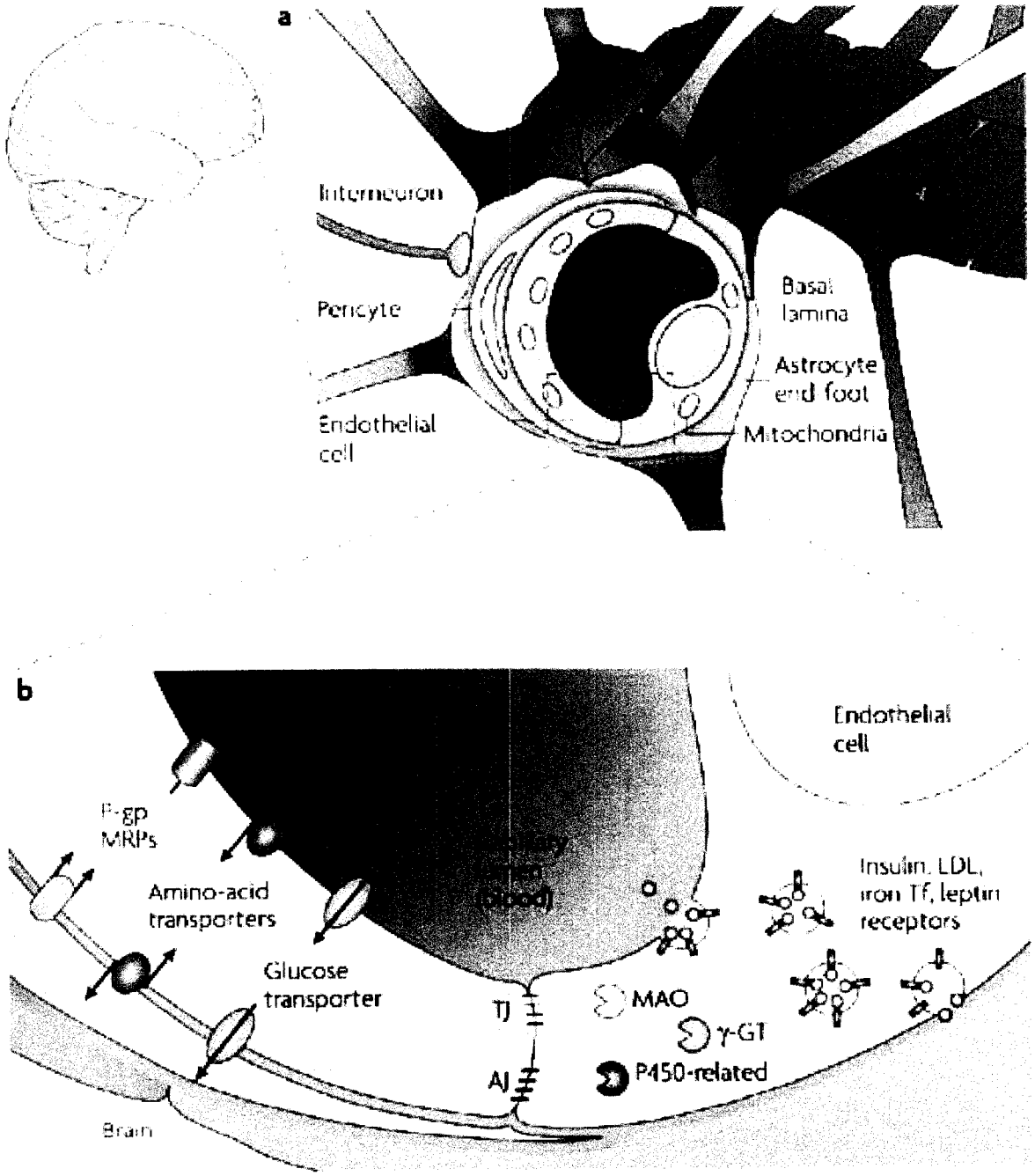
vessels originating from the perineural vascular plexus invade the neuroectodermal tissue radially and converge towards the ventricular zone in which cerebral branches are formed (Greenberg and Jin, 2005). The rate and timing of brain angiogenesis are tightly regulated by specific angiogenic factors, many of which also participate in brain development (i.e. neurogenesis) (Ward and Lamanna, 2004). For example, capillary sprouts in the ventricular zone express VEGFR-2 and invade towards a gradient of VEGF secreted by ventricular cells; this signaling allows for precise spatio-temporal organization of the brain vascular development as well as axonal growth and neuronal survival (Greenberg and Jin, 2005). The early embryonic brain capillaries are permeable to substances that are excluded in the adult brain (Johanson, 1980). As brain angiogenesis in the embryo progresses, the vessels organize and mature. Unlike the peripheral endothelium, the ECs under the influence of the brain environment acquire a selective and highly specialized phenotype, known as the blood-brain barrier (BBB) (Engelhardt, 2003). The key characteristic of the EC BBB phenotype is the presence of TJs formed by occludins, claudins, zonula occludens (ZO-1, ZO-2, and ZO-3), junctional adhesion molecules (JAMs), cingulin and 7H6 (Kniesel and Wolburg, 2000). These TJs are organized in a complex way to provide high electrical resistance (~2000 Ω X cm^2) (Crone and Olesen, 1982), thereby strictly controlling the permeability of brain vessels (Wolburg and Lippoldt, 2002). Endothelial cells of the BBB also express transporters including Glut-1 and P-glycoprotein at the abluminal and luminal membrane of the vessel, respectively, to facilitate nutrient uptake and restrict the access of neurotoxins into the brain (Engelhardt, 2003; Schinkel *et al.*, 1994). Brain ECs are enriched in mitochondria (Oldendorf *et al.*, 1977), have few fenestrae (Fenstermacher *et al.*, 1988)

and mininimal pinocytic activity (Sedlakova *et al.*, 1999) compared to the capillaries of other organs. Proximity of EC to brain cells including astrocytes, pericytes and neurons is important for the induction of the BBB properties. Several studies suggested that astrocytic processes, in close apposition to the cerebral capillaries are critical in the development of the BBB (Stewart and Wiley, 1981; Willis *et al.*, 2004). Neurons, in conjunction with astrocytes, can synergistically mediate BBB differentiation (Schiera *et al.*, 2003). Pericytes contribute to increases in the barrier properties of brain vessels through induction of TJs (Hori *et al.*, 2004). The vascular basal lamina, which contains several ECM proteins, has also been reported to induce BBB differentiation by interacting with the cerebral endothelium through integrin receptors (Engelhardt, 2003). Interestingly, αV-integrin-deficient mice develop cerebral haemorrhages and abnormal vasculature due to inappropriate adherence of brain parenchymal cells to the BM of cerebral vessels (McCarty *et al.*, 2002). When the BBB is established, the neurovascular unit composed of ECs, astrocytes, neurons, pericytes and basal lamina tightly regulate cerebral microvascular permeability and blood flow (Hawkins and Davis, 2005) (Fig. 2). In various brain diseases including brain tumors, the vessels lose the BBB properties and leak fluid and plasma proteins into the brain, therefore contributing to cerebral oedema (Long, 1970; Papadopoulos *et al.*, 2004).

Figure 2

The blood-brain barrier

(a) Schematic representation of a cerebral capillary. The circumference of the brain capillary lumen is composed of one or two ECs. Pericytes are attached to the abluminal side of the ECs. Both ECs and pericytes share the same basal lamina. The brain capillary is surrounded by astrocytic end-feet processes. (b) The brain vasculature is characterized by the presence of TJs between the cerebral ECs which form a diffusion barrier and selectively exclude molecules in the blood from the brain, unless they are transferred by transporters localized in the ECs (P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein family). AJs, which stabilize cell–cell interactions, are also found between the ECs. In addition, the presence of intra- and extra-cellular enzymes endows this dynamic interface with metabolic activity (MAO, monoamine oxidase; γ-GT, γ-glutamyl transpeptidase; cytochrome P450). Large molecules can also be transferred to the central compartment by transcytosis. The receptors for insulin, low-density lipoprotein (LDL), iron transferrin (Tf) and leptin are all involved in transcytosis. Reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery] (Cecchelli *et al.*, 2007), copyright (2007).



1.2 Gliomas

Gliomas are a heterogeneous group of neoplasms derived from glial cells that account for 40-45% of all intracranial tumors (Kleihues *et al.*, 1995). According to the World Health Organization (WHO) classification system, gliomas of astrocytic origin (astrocytomas) are classified into four clinical grades based on their histopathological characteristics: pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma multiforme (GBM) (grade IV) (Kleihues *et al.*, 1995). Grade I tumors are benign and can be cured if they are surgically removed; grade II tumors are infiltrative and total cure after surgical resection is infrequent; grade III tumors are invasive, have increased cellularity and mitotic activity, and evolve towards grade IV (GBM); GBM is the most malignant type of gliomas (Kleihues *et al.*, 1995). GBMs can progress either from lower-grade gliomas (secondary GBMs) or arise in a rapid *de novo* manner (primary GBMs). Current standard treatments for GBM patients consist of surgery, radiotherapy and chemotherapy. Despite these treatments, GBMs generally recur with a more aggressive phenotype; the mean survival time for patients is approximately one year (Dai and Holland, 2001).

1.2.1 Cellular components of GBM

GBM is a highly heterogeneous tumor; the cellular composition of GBM is often varied and mixed. GBM mainly contains glial cells that include fibrillary and/or gemistocytic cells (Kleihues *et al.*, 1995; Miller and Perry, 2007). The fibrillary astrocytes are enlarged, elongated and irregular in shape, whereas gemistocytic astrocytes display a large cytoplasm with hyperchromatic nuclei. Both cells types are generally

positive to glial fibrillary acidic protein (GFAP) (Miller and Perry, 2007). However, a population of undifferentiated GFAP-negative cells (small anaplastic cells) are also found (Burger and Kleihues, 1989). Occasionally, multinucleated giant cells (GC) are located within the same tumor (Kleihues *et al.*, 1995). Vessels in GBM contain active ECs, SMCs or pericytes and inflammatory cells (e.g. leukocytes) that are closely associated with small and pathologic vessels (Stevens *et al.*, 1988). Recently, the presence of stem cells in GBM has been suggested (Galli *et al.*, 2004; Ignatova *et al.*, 2002; Singh *et al.*, 2003; Singh *et al.*, 2004; Yuan *et al.*, 2004). These GBM stem cells were able to regenerate new neurospheres and differentiate into cells with neuronal or glial cell markers *in vitro* (Galli *et al.*, 2004; Singh *et al.*, 2003; Singh *et al.*, 2004; Yuan *et al.*, 2004). *In vivo*, GBM stem cells can establish a tumor in SCID mouse brain with morphology and lineage markers resembling the original tumor, even after serial transplantations (Galli *et al.*, 2004). These studies led to the hypothesis that neural stem cells or early glial progenitors could be the cell-of-origin of transformation in gliomagenesis (Reynolds and Weiss, 1992). Although the identification of GBM stem cells (potentially a transformed variant of normal neural stem cell) supports this hypothesis, the cellular origin of glioma remains enigmatic. Given that GBM stem cells could be responsible for populating and repopulating the tumors as they develop and grow, these cells could have major therapeutic implications (Louis, 2006).

1.2.2 Molecular pathology of GBM

Genetic alterations in GBM result in either abnormal signal transduction and/or disruption of cell cycle arrest pathways (Dai and Holland, 2001). Several growth factors

and their cognate receptors are up-regulated or mutated in gliomas including PDGF-A, -B/PDGFR-α, -β (Hermanson *et al.*, 1992), and EGF/EGFR (Wong *et al.*, 1987). The EGFR gene is amplified in 40% of GBMs and these tumors often acquire additional genomic rearrangement of EGFR gene resulting in the expression of truncated receptor that shows constitutive tyrosine kinase activity, aberrant receptor signaling and processing (Nagane *et al.*, 2001). The most common rearrangement of the EGFR gene (occurs in 50-60% of those that have the amplified EGFR) is an in-frame deletion of 801 bp, which make up exons 2-7 in the mRNA (known as EGFRvIII) that confers a dramatically enhanced tumorigenicity to GBM cells (Nishikawa *et al.*, 1994). The PDGF/PDGFR loop is also reported to increase glioma proliferation (Guha *et al.*, 1995). In contrast to EGFR, amplification or rearrangement of PDGFR is very rare. EGFR or PDGFR receptor activation results in stimulation of downstream signals including the PI3K/AKT pathway, RAF/MAPK/ERK pathway and PLC-γ/PKC pathway. The loss of function of the tumor suppressor gene PTEN (phosphatase and tensin homologue on chromosome 10) is also frequently found in GBMs (Choe *et al.*, 2003). Other commonly seen gene mutations in human GBMs are deletion of INK4A-ARF (occurs in 60% GBMs) (Fulci *et al.*, 2000) and alteration of p53 and MDM2 protein (Newcomb *et al.*, 1998; Watanabe *et al.*, 1996), all involved in the regulation of cell cycle.

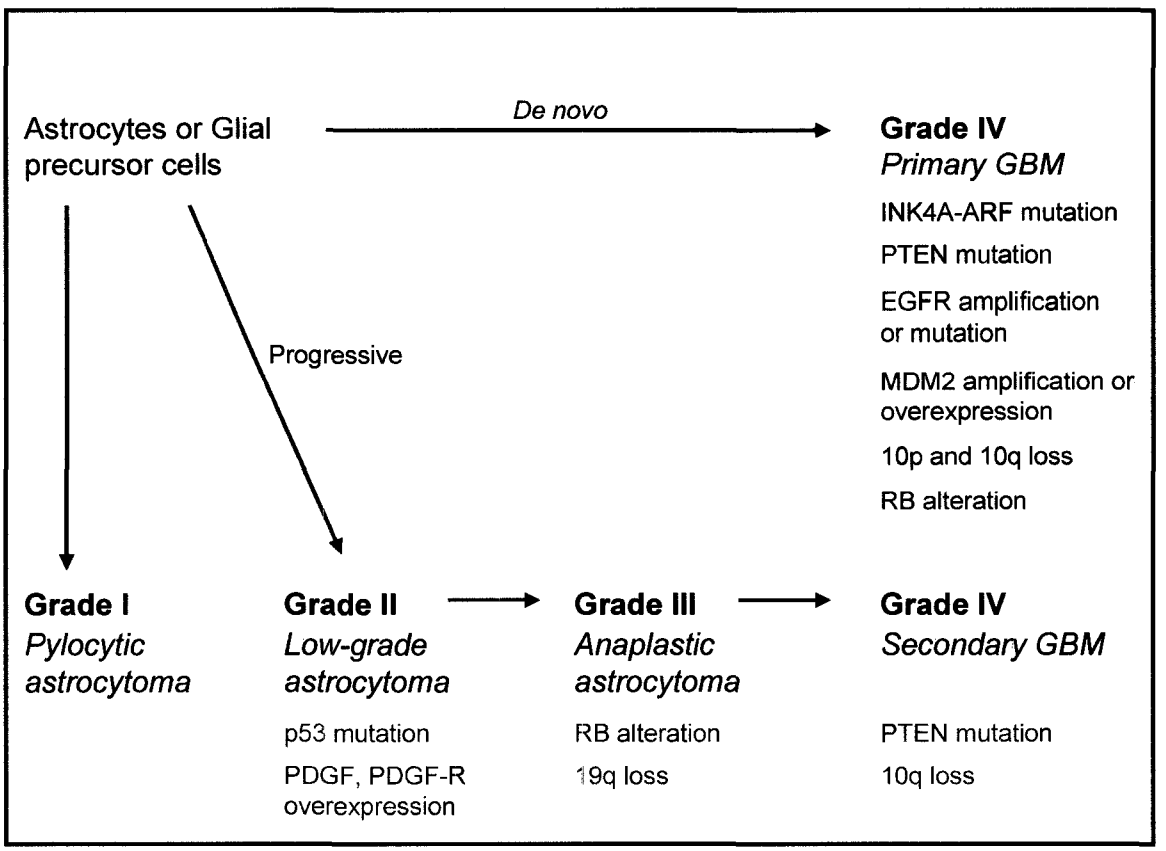
Primary and secondary GBMs are clinically and genetically different (Lang *et al.*, 1994). Primary GBMs usually develop in older patients and tumor presentation is aggressive and fatal. *De novo* GBMs are characterized by EGFR amplification/overexpression, deletion of PTEN and INK4A-ARF, and over-expression of MDM2 protein. In contrast, secondary GBMs occur in younger patients and involve

serial accumulation of genetic alterations often associated with PDGFR amplification/overexpression and p53 mutation (Furnari *et al.*, 2007; Kleihues and Ohgaki, 1999; Watanabe *et al.*, 1996). A summary of the genetic aberrations involved in gliomagenesis is shown in Fig. 3.

Figure 3

Genetic pathways leading to primary and secondary GBMs

In *de novo* primary GBMs, EGFR amplification/overexpression, and PTEN and INK4-ARF mutations are often observed. Secondary GBMs frequently contain p53 mutation and PDGF/PDGF-R overexpression. See text and reviewed papers for details (Furnari *et al.*, 2007; Kleihues and Ohgaki, 1999).



1.2.3 Biological features of GBM

Several key biological features of GBM, including infiltration and invasion of surrounding brain tissue, excessive proliferation, necrosis, and high angiogenesis rate, limit the success of therapeutic approaches and make GBM treatment a difficult challenge. Although GBM cells infiltrate and grow rapidly, they rarely metastasize outside the CNS. However, the infiltrative nature of glioma cells that invade normal brain parenchyma is the principal reason for failure of neurosurgical resections (Lefranc *et al.*, 2005). The major migration route of glioma cells is along the white matter tract and the BM of blood vessels (Giese *et al.*, 1998). Glioma cells migrate through the corpus callosum and often, tumor cells cross this region to form butterfly lesions (Bernstein *et al.*, 1989). It appears that GBM cells have a tropism for specific sites and require selective interactions with specific cells to invade (Bernstein and Woodard, 1995; Kleihues *et al.*, 1995). The process of glioma cell invasion into normal brain tissue involves cell interaction with adjacent cells and with the ECM. Several ECM proteins, including tenascin-C and SPARC, and adhesion molecules, including integrins, cadherins, NCAM and gap junctions, have been associated with glioma invasion (Giese *et al.*, 1996; Mariani *et al.*, 2001; Rempel *et al.*, 1998). For example, activation of integrin $\alpha 2\beta 1$ through binding to its ECM ligand tenascin promotes cell movement (Uhm *et al.*, 1999). Furthermore, GBM-derived proteases degrade ECM proteins to remodel the extracellular environment and to facilitate cell migration and tumor growth (Demuth and Berens, 2004). Members of the MMP family, such as MMP-2, MMP-9 and their TIMPs, uPA, and cathepsin B have been shown to increase glioma invasiveness; blocking the function of these proteases results in decreased invasive properties (Rao, 2003).

Necrosis is the most powerful histological feature that predicts a highly malignant state of glioma because it can affect more than 80% of the total tumor mass (Kleihues *et al.*, 1995). Insufficient blood supply and anoxia due to microthrombosis cause necrosis in GBMs (Brat and Van Meir, 2004). Vascular occlusion and thrombosis lead to tumor cell migration away from the dysfunctional vessel; tumor cells surrounding these necrotic zones are called 'pseudopalisading cells' (Kleihues *et al.*, 1995). Several studies showed that these 'pseudopalisading cells' are highly hypoxic since they over-express the nuclear transcription factor HIF-1 (Brat *et al.*, 2004; Brat and Mapstone, 2003; Zagzag *et al.*, 2000). Hypoxia can lead to the selection of cells that survive and resist the hypoxic tumor environment (i.e. apoptosis-resistant cells), therefore allowing the emergence of highly malignant tumor cell clones (Graeber *et al.*, 1996).

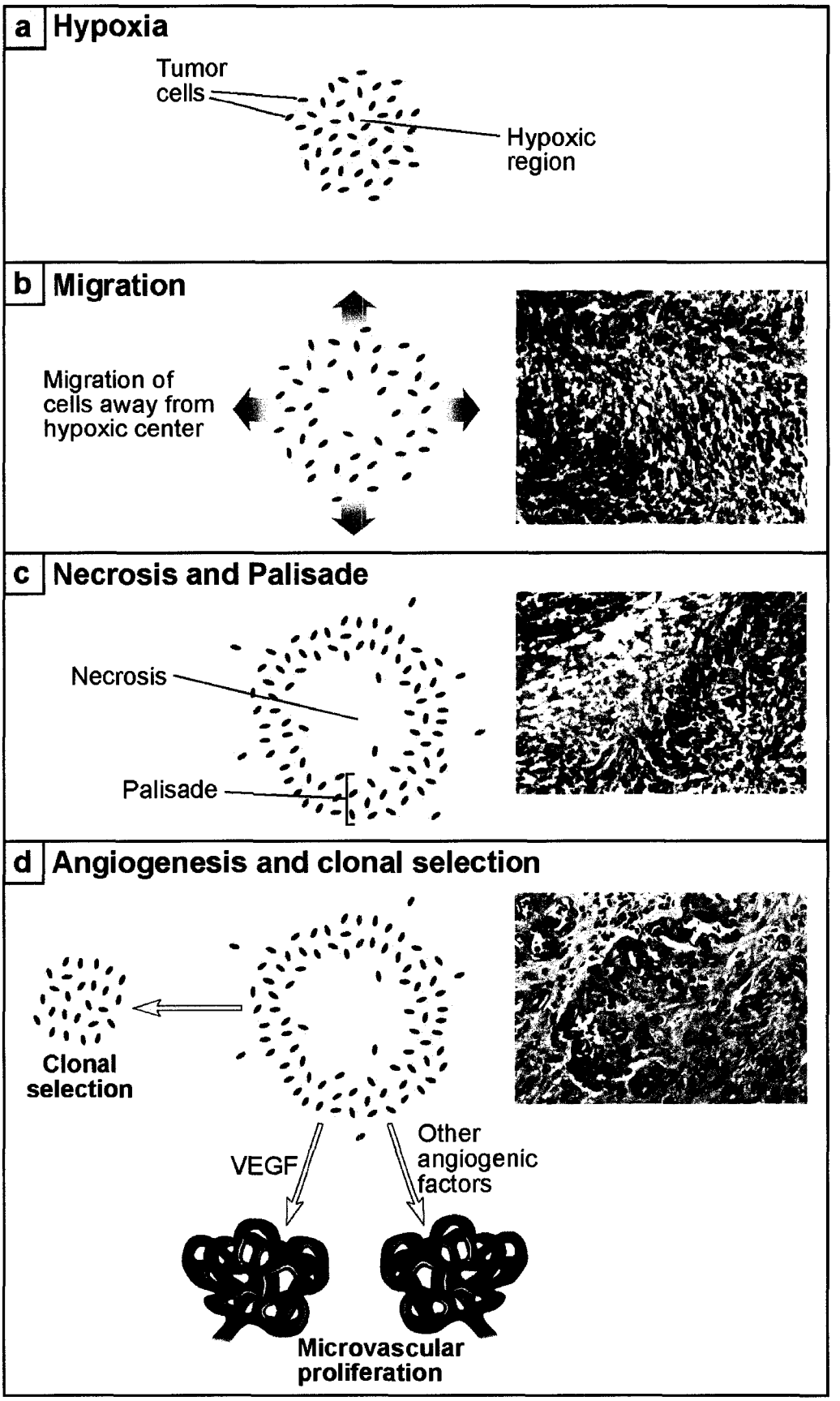
Although necrosis is characteristic of GBM, rapid tumor progression would not be possible without vascular endothelial proliferation; in fact, necrosis is intimately related to vascular hyperplasia. Vascular proliferation in GBM occurs in two pathological forms: 1) a diffuse increase of small vessel density throughout the tumor and 2) microvascular proliferation observed in proximity of pseudopalisade structures (Louis, 2006). The 'pseudopalisading cells' secrete pro-angiogenic factors, such as VEGF (Plate, 1999; Shweiki *et al.*, 1992) and IL-8 (Brat *et al.*, 2005; Desbaillets *et al.*, 1997) to initiate angiogenesis. This angiogenic response attempts to form new vasculature to support a rapid tumor expansion, but the process often cannot be completed, thus creating regions of microvascular hyperplasia, which is characterized by highly proliferative ECs that form tufted microaggregates–glomeruloid bodies, surrounded by a BM and an incomplete layer of pericytes (Brat and Van Meir, 2001; Stiver *et al.*, 2004). These

glomeruloid bodies are often non-functional and lack blood flow, resulting in a vicious cycle involving hypoxia-induced necrosis and angiogenesis that herald the onset of a rapid tumor growth (Louis, 2006) (Fig. 4).

Figure 4

Mechanisms of hypoxia-induced angiogenesis and GBM tumor growth

The center of the tumor mass has insufficient blood supply and is hypoxic (a). This region induces associated genes that lead to the migration of tumor cells away from this hypoxic center (b) (see also photomicrograph inset; H&E, 200X) causing the formation of necrosis in the central region and the development of a palisade of dense tumor cells (c) (see also photomicrograph inset; H&E, 200X). These 'pseudopalisading cells' express angiogenic factors (e.g. VEGF) that induce angiogenesis including glomeruloid microvascular proliferation (d) (see photomicrograph inset; H&E, 200X). Hypoxia also leads to the selection of more malignant tumor cells that survive and are resistant to the tumor microenvironment (d). See text for details. Reprinted with permission from the Annual Review of Pathology: Mechanisms of Disease, Volume 1 ©2006 by Annual Reviews (www.annualreviews.org), (Louis, 2006).



1.2.4 Mechanisms of GBM vascularization and angiogenesis

GBMs are among the most vascularized solid tumors. Brain tumors use four mechanisms to recruit blood vessels: co-option, angiogenesis, intussusception and vasculogenesis (Jain *et al.*, 2007). Co-option of pre-existing vessels is widely recognized as the first process used by tumor cells to gain access to oxygen and nutrients (Holash *et al.*, 1999; Leenders *et al.*, 2002). As the tumor grows, tumor cells lining the coopted vessel interact with the ECs leading to increased EC apoptosis and triggering hypoxia, which in turn induces VEGF expression and initiates the sprouting angiogenesis (Carmeliet, 2000). Intussusception is a variant of angiogenesis involving the insertion of interstitial tissue columns into the lumen of pre-existing vessels (Djonov *et al.*, 2002; Patan *et al.*, 1996), enabling the tumor to acquire blood supply. This process is metabolically less demanding than sprouting since ECs do not proliferate, but only increase their volume (Auguste *et al.*, 2005). The molecular regulators involved in this process are still not clearly understood, but shear stress and increased blood flow have been proposed to play major roles (Djonov *et al.*, 2002). Another mechanism by which tumors can acquire vasculature is vasculogenesis. Vasculogenesis is distinguished from angiogenesis in that it involves differentiation of the precursor cells, angioblasts, into ECs forming *de novo* vascular network instead of sprouting from existing vessels (Risau, 1997). Evidence suggests that bone-marrow derived endothelial cells can enter into the blood circulation and incorporate into tumor vessels to support tumor angiogenesis (Duda *et al.*, 2006; Lyden *et al.*, 2001; Santarelli *et al.*, 2006).

Although not described in brain tumors, mosaic vessels and vascular mimicry are two other mechanisms contributing to tumor angiogenesis (Auguste *et al.*, 2005). The

mosaic vessel process consists of tumor cells lining the wall of tumor vessels together with ECs, forming functional vessels (Chang *et al.*, 2000). Tumor cells themselves can organize into vascular channel structures to provide blood supply for tumor growth, which is a mechanism called vascular mimicry (Maniotis *et al.*, 1999).

1.2.4.1 GBM vasculature

A) Structural abnormalities

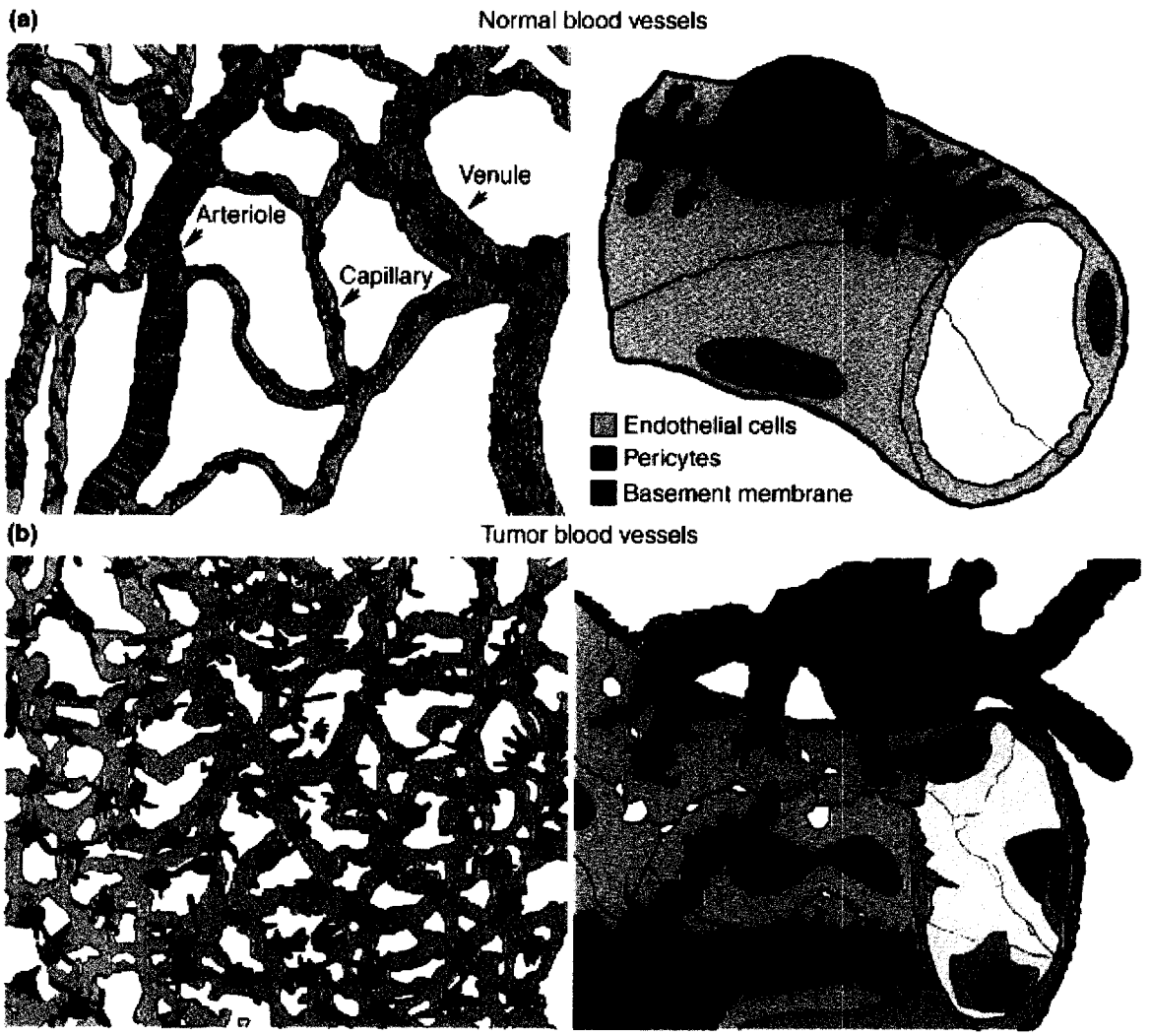
The vasculature of GBM is structurally different from 'normal' cerebral vessels (Fig. 5). For instance, GBM vessels are heterogeneous, disorganized, tortuous and display an irregular diameter (Jain *et al.*, 2007). Tumor vessels show significant abnormalities in cellular (ECs and pericytes) and acellular constituents (BM). EC hyperplasia results in increase in vessel wall thickness (Schlageter *et al.*, 1999). Tumor vessel walls have several 'holes' or 'openings' such as endothelial fenestrae, vesicles and transcellular holes (Deane and Lantos, 1981), and contain wide interendothelial junctions, resulting in increased permeability (Carmeliet and Jain, 2000; Hashizume *et al.*, 2000). GBM vessels are also characterized by abnormal pericyte coverage. Whereas several reports described an extensive number of pericytes in direct contact with ECs within the glioma vasculature (Stewart *et al.*, 1991; Wesseling *et al.*, 1995), others found lower pericyte coverage compared to normal cerebral vessels based on alpha-smooth muscle actin (α-SMA) marker (Benjamin *et al.*, 1999; Bergers and Song, 2005; Eberhard *et al.*, 2000). Although pericytes are present in tumor vessels, they lack the normal contractile capacity (Eberhard *et al.*, 2000). In addition to abnormal pericytes, GBM vessels display a discontinuous BM. The BM is irregular in thickness and, in some regions, projects into

the tumor parenchyma (Baluk *et al.*, 2003; Jain *et al.*, 2007; Schlageter *et al.*, 1999). The tumor vascular network exhibits complex branching patterns and lacks hierarchy (Konerding *et al.*, 1999). As a result of structural abnormalities, tumor vessels are often described as immature.

Figure 5

Schematic illustrating differences between normal and tumor blood vessels

(a) Normal endothelium differentiates into macro- and micro-vasculature. The normal blood vessels are embedded by SMCs or pericytes and surrounded by a thin BM. (b) Tumor vasculature is organized without hierarchical branching. SMCs or pericytes are abnormal and loosely attached to the ECs. The BM is irregular in thickness. Reprinted from Current Opinion in Genetics & Development, 15, Baluk et al., Cellular abnormalities of blood vessels as targets in cancer, 1801-1815, Copyright (2005), with permission from Elsevier.



B) Functional abnormalities

The abnormal structure of GBM vessels leads to abnormal vascular function. Although the BBB is disrupted in GBM vessels, some features of the BBB are retained. The loss of BBB is not uniform and the level of tumor vessel leakiness is heterogeneous (Jain *et al.*, 2007). The loss of BBB properties and increased permeability in tumor vessels augment fluid and plasma protein leakage in the periphery of the tumor and surrounding brain (Jain *et al.*, 2007). Consequently, interstitial fluid pressure (IFP) within the tumor increases and fluid and plasma proteins accumulate in the surrounding brain, leading to vasogenic brain oedema (Boucher *et al.*, 1997). IFP forms a barrier to drug delivery and cerebral oedema is related to morbidity in patients with GBMs (Jain, 1994).

C) Molecular characteristic of GBM vessels

The altered tumor vasculature is associated with abnormal gene expression. For example, VEGF/VEGFR-2 signaling is responsible for the increase in tumor vessel permeability (Machein *et al.*, 1999). Down-regulation of anti-thrombotic molecules, such as anti-thrombin III, and overexpression of thrombomodulin in tumor vessels may explain the tendency for intratumoral haemorrhage and intravascular thrombosis in malignant gliomas (Isaka *et al.*, 1994). GBM vessels lose the expression of several junctional molecules including VE-cadherin, claudin-1, -3, -5, and occludin (Charalambous *et al.*, 2006; Liebner *et al.*, 2000; Papadopoulos *et al.*, 2001; Wolburg *et al.*, 2003), which likely contributes to the leakiness of the BBB in GBM. GBM vessels express some BBB markers such as ZO-1 (Sawada *et al.*, 2000) and members of the ATP

binding cassette (ABC) transporters, including P-glycoprotein (ABCB1/MDR1) (Demeule *et al.*, 2001; Takamiya *et al.*, 1997) and ABCG2 (Zhang *et al.*, 2003), therefore limiting the delivery of drugs into the brain. VEGF/VEGFR-2 complexes (Brekken *et al.*, 1998; Machein *et al.*, 1999), integrins αvβ3 (Max *et al.*, 1997), α5β1 (Kim *et al.*, 2000), endoglin (CD105) (Burrows *et al.*, 1995), Thy-1 (Lee *et al.*, 1998), prostate-specific membrane antigen (PSMA) (Chang *et al.*, 1999) and tumor endothelial markers (TEMs) (St Croix *et al.*, 2000) have all been documented at higher levels in tumor vessels compared to normal vessels. Since normal and tumor vessels express different 'markers', molecules selectively expressed in tumor vessels including transporters or angiogenic factors can be used to overcome the BBB and/or selectively target anti-cancer drugs into brain tumors. Genomic profiling technologies have extensively contributed to the identification of novel markers in tumor endothelium that could be exploited for diagnostic or therapeutic purposes (Bhati *et al.*, 2008; Buckanovich *et al.*, 2007; Ghilardi *et al.*, 2008; Seaman *et al.*, 2007; St Croix *et al.*, 2000).

1.2.5 Anti-angiogenic strategies for brain tumors

The anti-angiogenic therapies are based on strategies that block the function of specific growth or angiogenic factors, their receptors or downstream signaling. The anti-VEGF antibody bevacizumab (Avastin®) was the first anti-angiogenic drug approved by the U.S. Food and Drug Administration (FDA) to treat metastatic colorectal cancer, non-small cell lung cancer, and breast cancer. Avastin inhibits tumor angiogenesis by selectively neutralizing VEGF-A (Ferrara, 2004). VEGF-receptor tyrosine kinase inhibitors (TKIs) (sorafinib and sunitinib) are also used for the treatment of metastatic

renal cell carcinoma (Hiles and Kolesar, 2008). Targeting EC in combination with other components of the vasculature such as pericytes and BM components could also be helpful for anti-angiogenic therapies (Baluk *et al.*, 2003). For instance, the combined administration of SU5416 and SU6668, which inhibit VEGFR/2/FGFR on ECs and PDGFR-β on pericytes, respectively, significantly improved therapeutic efficacy in a Rip1Tag2-transgenic mouse model of pancreatic β cell carcinogenesis (Bergers *et al.*, 2003). Endostatin, a proteolytic fragment of collagen type XVIII, is a BM-derived anti-angiogenic peptide that has been reported to inhibit angiogenesis and tumor growth (O'Reilly *et al.*, 1997). The combination of angiogenic inhibitors with chemotherapy is increasingly used because the anti-angiogenic therapy can normalize the vasculature, therefore enhancing the delivery and efficacy of the administered cytotoxic drugs (Jain, 2001; Jain, 2005).

Although preclinical studies demonstrated the benefit of anti-angiogenic agents in the treatment of brain tumors and GBMs, no anti-angiogenic agent has yet been approved for the clinical treatment of brain tumors. Cediranib (AZD2171; a pan-VEGF receptor tyrosine kinase) has been demonstrated by magnetic resonance imaging (MRI) to induce a transient 'normalization window' in tumor vessels, thereby alleviating vasogenic oedema in patients with recurrent GBMs (Batchelor *et al.*, 2007). Beneficial effects have also been confirmed with Avastin® and chemotherapy (Pope *et al.*, 2006; Vredenburgh *et al.*, 2007), and with sunitinib (de Bouard *et al.*, 2007) in high grade gliomas. Temozolomide, an orally administered alkylating agent that crosses the BBB, is used clinically to treat GBMs (Mason and Cairncross, 2005). Given that the failure of drug delivery to the brain is due to the BBB, aggressive hyperosmolar (Doolittle *et al.*, 2000;

Fortin *et al.*, 2005) or chemical (Lesniak *et al.*, 2001) BBB disruption protocols are applied to enhance the transfer of cytotoxic agents into malignant brain tumors. Approaches to render the cytotoxic drugs more permeable (e.g. encapsulation in nanoparticles or liposomes, modification with hydrophobic side groups, conjugation to BBB carriers such as transferrin) have been tested but did not significantly impact the clinical treatment of gliomas (Misra *et al.*, 2003).

1.3 Research hypotheses and objectives

Since GBM is a highly vascularized tumor, anti-angiogenic therapy represents an attractive approach to suppress tumor growth. However, anti-angiogenic treatments attempted in GBMs have so far produced marginal improvements, suggesting that better understanding of the molecular differences between normal and GBM vessels and their interactions with the tumor microenvironment are required to develop more efficacious treatments.

The overall hypothesis of this doctoral thesis is that GBM tumor vessels express highly specific biomarkers, some of which are functionally implicated in tumor angiogenesis and tumor growth.

The first hypothesis examined in this thesis was that the differential transcriptional profiling of the GBM and normal brain vascular compartments will enable identification of novel biomarkers of tumor vessels. The first objective therefore was to develop methods for selective isolation of brain vasculature using LCM and its subsequent analyses using cDNA microarrays. These methods were then used to identify

differentially expressed genes between vessels derived from GBM tumors and those from non-malignant brains. This study is described in Chapter 2.

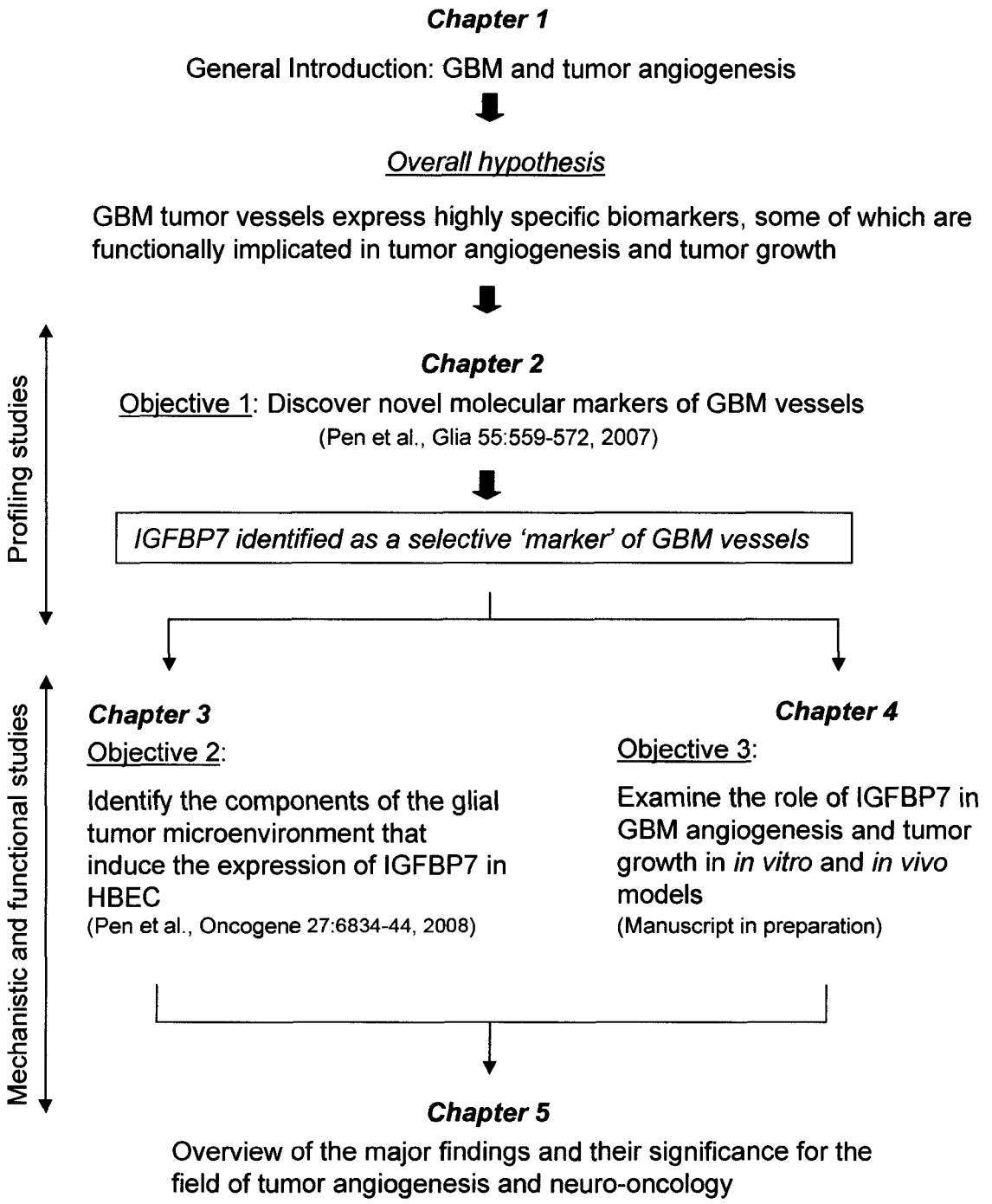
From the profiling study presented in Chapter 2, IGFBP7 is identified as highly up-regulated vascular biomarker of GBM vessels. We then hypothesized that the high expression of IGFBP7 in GBM vessels is induced by specific components of the tumor microenvironment. The second objective of this thesis was to identify the components of the glial tumor microenvironment that induce the expression of IGFBP7. These findings are presented in Chapter 3.

Finally, we hypothesized that IGFBP7 plays functional roles in tumor angiogenesis and tumor growth. The third objective of this thesis was to examine the role of IGFBP7 in GBM angiogenesis and tumor growth in *in vitro* and *in vivo* models. These studies are described in Chapters 3 & 4.

An overview of all major findings and their significance for the field of tumor angiogenesis and neuro-oncology are presented in Chapter 5. The outline of this doctoral thesis is summarized in Fig. 6.

Figure 6

Outline of the doctoral thesis



CHAPTER 2

Molecular Markers of Extracellular Matrix Remodeling in Glioblastoma Vessels: Microarray Study of Laser-Captured Glioblastoma Vessels

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Running title: Gene profiling of glioblastoma vessels

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Contribution of the co-authors:

- Dr. Joel Martin created the LitMiner™ software and offered assistance in the use of this custom software.
- Dr. Maria Moreno helped with the microarray analyses, literature mining and provided support and guidance throughout this research work. She has also revised this manuscript.
- Dr. Danica Stanimirovic provided scientific guidance and supervision throughout this research project. She has also edited this manuscript.

ABSTRACT

Glioblastoma multiforme (GBM) are the most malignant and vascularized brain tumors. The aberrant vascular phenotype of GBM could be exploited for diagnosis or therapeutic targeting. This study identified new molecular markers of GBM vessels, using a combination of laser capture microdissection (LCM) microscopy, RNA amplification, and microarray analyses to compare vessels from non-malignant human brain and GBM tumors. Forty-two genes were differentially expressed in GBM vessels compared to non-malignant brain vessels. Validation of differentially expressed genes was performed by literature mining, Q-PCR, and immunohistochemistry. Among the differentially expressed genes, only 64% were previously associated with vessels, angiogenesis, gliomas, and/or cancer. The up-regulation of genes encoding secreted extracellular proteins IGFBP7 and SPARC was confirmed by Q-PCR in LCM-captured vessels. Whereas SPARC and IGFBP7 protein were absent in non-malignant brain vessels, distinct immunoreactivity patterns were observed in GBM sections whereby SPARC was strongly expressed in perivascular cells adjacent to GBM vessels while GBM endothelial cells were immunostained for IGFBP7. IGFBP7 immunoreactivity was also detected on the abluminal side of GBM vessels deposited between strands of vascular basal lamina. The study discerns unique molecular characteristics of GBM vessels compared with non-malignant brain vessels that could potentially be used for diagnostic or therapeutic purposes.

INTRODUCTION

Gliomas are a heterogeneous group of neoplasms derived from glial cells that account for 40–45% of all intracranial tumors (Kleihues et al. 1995). The most malignant type of gliomas, glioblastoma multiforme (GBM) (World Health Organization-WHO grade IV), is an infiltrating and highly vascularized tumor with the mean survival time of ~9–12 months from diagnosis (Vajkoczy and Menger 2000). Angiogenesis contributes to GBM malignancy by providing oxygenation and nutritional supply needed for tumor growth and invasion. Therefore, microvessel density is a marker of GBM invasiveness and a prognostic indicator (Leon et al. 1996).

GBM vessels exhibit an altered phenotype, including tortuous morphology, microvascular hyperplasia with microaggregates of proliferating endothelial cells accumulating at the edge of parental blood vessels (Schlageter et al. 1999), endothelial gaps and fenestrations resulting in increased permeability, and a thin basement membrane (Schlageter et al. 1999) allowing pericytes to establish direct contacts with tumor endothelial cells (Benjamin et al. 1999). In addition to morphological changes, GBM vessels exhibit up-regulation of genes/proteins involved in angiogenesis, including vascular endothelial growth factors (VEGFs) (Miyagami et al. 1998) and $\alpha v\beta 3$ integrin (Gladson 1996). GBM vessels also acquire a multidrug resistance phenotype and thereby reduce the access of drugs to tumor cells (Zhang et al. 2003).

Genomic high-throughput technologies such as DNA microarrays have enabled a more comprehensive molecular profiling of human gliomas (Sallinen et al. 2000). These studies demonstrated that GBMs overexpress various gene families related to cell adhesion, motility, invasion, and angiogenesis (Sallinen et al. 2000). However, only a

fraction of the identified mRNA expression changes has been confirmed at the protein level and functional interactions among these genes resulting in various malignant phenotypes remain unknown. GBM are highly heterogeneous and harbor various cell types in different stages of differentiation within the tumor mass (Mischel et al. 2003). Therefore, analyses in whole GBM tissue are likely to miss marker genes/proteins specifically expressed in defined, often low abundant cell populations that may be critically important for tumor pathogenesis.

Specific vascular markers are good molecular candidates for diagnostic, tumor delivery, and tumor therapy applications (Jansen et al. 2004). Several vessel-targeting strategies, including administration of VEGF antagonists (Saleh et al. 1996) and blocking $\alpha v\beta 3$ integrin using RGD (Arg-Gly-Asp) peptides (MacDonald et al. 2001), have shown a limited efficacy in GBM.

Endothelial tumor markers have been analysed by genome-wide screening of cell cultures (Abe and Sato 2001; Wang et al. 2000), or in freshly isolated endothelial cells purified from enzymatically digested colorectal cancer tissues using either antibody mixtures (St Croix et al. 2000) and/or gradient centrifugation (van Beijnum et al. 2006). To identify molecular markers of GBM vessels, we performed a high-density microarray analysis on GBM vessels extracted using a laser capture microdissection (LCM) method, which preserves transcriptome profile of cells *in situ*. Subsequent validation of differentially expressed genes by quantitative-PCR (Q-PCR) and immunohistochemistry confirmed strong up-regulation of insulin-like growth factor binding protein-7 (IGFBP7) and SPARC in GBM endothelium and perivascular cells, respectively. These studies demonstrated that GBM and normal brain endothelium are molecularly distinct, a finding

that may have significant implications for the development of anti-angiogenic therapies and could enable targeting of GBM vessels for diagnostic imaging or therapeutic delivery.

MATERIALS AND METHODS

Experimental Design

A common reference design was used for this study (Konig et al. 2004). RNA obtained from LCM-captured vessels from five GBM and five non-malignant brains, each treated as individual, was each hybridized separately against a 'reference RNA' extracted from non-malignant homogenized brain tissue on a 19K human single-spot cDNA microarray chips (OCI). Six replicates were performed for each biological sample (60 microarrays total).

The common reference design was applied because factors such as age, sex, ethnicity, and therapy are difficult to control in a small sample pool. This design has been shown to have high reliability in similar microarray studies (Konig et al. 2004).

Tissue preparation and sectioning

Frozen blocks of surgically removed brain tissue were obtained from the Ottawa General Hospital Neuropathology Brain Bank. Histopathologically diagnosed GBM and non-malignant brain tissues were embedded in Tissue-Tek freezing medium (Miles Laboratories, Elkhart, IN), sectioned on a cryostat (Jung CM3000; Leica, Richmond Hill, ON, Canada) at 10 μm thickness, mounted on Superfrost Plus microscope slides (Fisher Scientific, Nepean, ON, Canada), and kept at -80°C until used.

Vessel staining and LCM capture

Vessels in brain sections were stained, as described previously (Mojsilovic-Petrovic et al. 2004), with the lectin, Ulex Europeaus Agglutinin I (UEA 1) (Vector Laboratories, Burlington, ON, Canada). Slides were observed using a Pixcell II Laser Capture Microdissection microscope (Arcturus, Mountain View, CA). Vessel dissection was performed using a 7.5 μm laser spot size, 0.75–0.90 ms laser pulse, and 25–30 mV laser beam power. Size of the captured vessels varied from 20 to 150 μm. Approximately 500–700 laser spots (microvessel cells) were captured on one cap (CapSure LCM Caps; Arcturus, Mountain View, CA); 2–3 caps (1,000–1,500 spots) and 10–15 caps (~10,000 spots) were collected from each section for cDNA microarray and Q-PCR analyses, respectively. To control for purity of vessel isolations, vessel-free brain parenchyma was also collected for Q-PCR analyses of various vascular and non-vascular gene markers. LCM-captured tissues were lysed with 100 μL of RNA lysis buffer and 0.7 μL of β-mercaptoethanol (Stratagene, La Jolla, CA) and kept at -80°C until RNA isolation.

RNA isolation and amplification for microarray analyses

Total RNA was extracted from LCM-captured vessels (1,000 spots) and LCM-captured brain parenchyma, using an Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) as described in the manufacturer's protocol. RNA was eluted in dimethylpyrocarbonate [DEPC]-treated Milli-Q distilled water and concentrated to a total volume of 10 μL using a Speedvac concentrator (Vacufuge™ Eppendorf, Hamburg, Germany). To obtain the required quantity of RNA for microarray analyses, concentrated

total RNA from LCM-captured vessels was amplified using the HS RNA Amplification Kit (Arcturus, Mountain View, CA). Two rounds of *in vitro* amplification were performed with LCM-captured samples according to manufacturer's protocol. To control for the linearity of RNA amplification, RNA was amplified from different amounts of captured endothelial cells using HS RiboAmp Amplification kit and quantity of amplified RNA (aRNA) was measured at optical density (OD) 260 nm.

Total RNA was isolated from non-malignant homogenized brain tissue for the reference RNA using TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the recommended protocol. To eliminate potential bias introduced by amplification protocol, reference RNA was amplified using the same protocols applied to LCM-extracted samples. The RNA (1 μg) was primed with oligo $(dT)_{12-18}$ primers (0.5 μg/μL; Gibco BRL) and reverse transcribed with 1–3 U of avian myeloblastosis virus reverse transcriptase (Promega Biosciences, San Luis Obispo, CA).

cDNA Microarrays

19Kv7 human single-spot cDNA glass microarrays (Ontario Cancer Institute, University of Health Network, Toronto, ON, Canada) were used in this study. cDNA labeling was performed using a modified version of the Amino-Allyl Indirect labeling protocol available at http://www.microarrays.ca. Briefly, 2.5 μg of aRNA was reverse transcribed using 200 units of Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) and cDNA was purified using PCR Microcon Filter (Millipore, Bedford, MA). Three microliters of 0.3 M sodium bicarbonate (pH 9) were added to each cDNA sample. A volume of 4.5 μL of fluoro-Link Cy3 and Cy5 monofunctional dyes

(Amersham Pharmacia Biotech, Piscataway, NJ) was incubated with 8 μL of cDNA sample for 1 h at room temperature (RT) in darkness. Un-incorporated nucleotides were removed using a Qiagen PCR purification kit (QIAGEN, Mississauga, ON). OD of each probe was measured at 260 nm and equal quantities were combined for each set of reference and test samples. Microarray hybridization was performed as described previously (Moreno et al. 2006). cDNA microarrays were scanned at wavelengths adjusted to read Cy3 (535 nm) and Cy5 (635 nm) fluorescent images using dual-color confocal laser scanner ScanArray 5000 (GSI Lumonics, Billerica, MA). Images were analysed using QuantArray® Micorarray Analysis Software v.2.0 (GSI Lumonics). Relative cDNA expression levels were quantified by comparing fluorescent signals obtained from Cy3- and Cy5-labeled probes.

Statistical analysis

Microarrays were performed in six replicates, using reciprocal labeling in three of the trials. The background of each spot was evaluated by counting pixel intensities in an area surrounding the spot and the subarray median background was subtracted from the fluorescent intensity value of each spot using in-house custom-developed software (Normalizer^{TM}). Spots showing low fluorescence intensity (spot intensities below 8% of the range of intensities for each dye) were tagged and removed from the data set. The $\log_2$ raw net signals from each subarray channel were normalized using an intensity-dependent/spatial algorithm.

Log_2 ratios of technical replicates of both GBM vessels versus reference RNA and non-malignant brain vessels versus reference RNA were averaged. To determine changes

in gene expression between GBM and non-malignant brain vessels, biological replicates were averaged and the average $\log_2$ ratio of the five GBM vessel samples versus reference RNA was subtracted from the average $\log_2$ ratio of the five non-malignant brain vessel samples versus reference RNA. A two-tailed distribution and two-sample t test with unequal variance was applied to determine significant differences between GBM versus non-malignant brain vessels. Log_2 ratios with $P > 0.1$ were rejected and $\log_2$ ratios ≥ 0.6 and $\leq (-0.6)$ were considered differentially expressed.

Literature network

To analyze whether differentially expressed genes have been functionally linked to specific tissue or process of interest for this study, literature mining was performed using the National Research Council-Institute for Information Technology/National Research Council-Institute of Biological Sciences (NRC-IIT/NRC-IBS) designed software tool, LitMiner™ (www.litminer.ca). This program performs searches of 'knowledge terms' (tissue, process, disease) and links them to 'query genes' (and their aliases) in published literature. The program also creates hierarchical networks of primary (direct link between gene and knowledge term), secondary (gene-gene associations), and tertiary (association between genes not directly linked and those directly linked to knowledge term) links among genes and indicates their strength based on the number of publications that describes the link. LitMiner™ also enables easy generation of interactive tables (see Supplementary Table http://www.litminer.ca/htmltables/pen/) and schematics of literature networks among selected genes and knowledge terms. Published literature was searched for entries

containing knowledge terms 'angiogenesis,' 'vessel,' 'GBM,' or 'tumor' in conjunction with each differentially expressed gene. Molecular and biological roles of identified genes were also determined using a built-in Gene Ontology term function in the LitMiner™. All steps performed by LitMiner™ automatically can be replicated manually using Entrez Gene and HUGO Gene Nomenclature Committee to obtain synonyms/aliases for each gene, PubMed to find entries that describe each gene synonym and the selected knowledge term in the same document, and Gene Ontology to obtain molecular/biological function of a gene.

Real-time quantitative PCR

Q-PCR was used to validate gene expression changes observed in microarray analyses. Separate LCM-captured samples (10,000 spots each) cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Q-PCR reactions were performed with SYBR Green PCR Core Reagent kits and an ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Primers used for real-time Q-PCR were designed according to sequences in GenBank and are described in Table 1. cDNA from reference RNA serially diluted from 1:10 to 1:1,000 was used to generate standard curves. The thermal PCR conditions were 15-s denaturation at 95°C and 1-min annealing–extension at 60°C for 40 cycles. Fluorescence was detected at the end of each 60°C phase. The transcript of each gene was normalized to the transcript levels of the housekeeping gene β-actin. Statistical analysis was performed using Student's t test, and $P < 0.05$ between control and GBM vessels was considered significant.

Table 1

Primers designed to amplify selected genes by Q-PCR

Gene	Sense primers for Q-PCR	Antisense primers for Q-PCR
IGFBP7	5'-GCGAGCAAGGTCCTTCCATA-3'	5'-GGGATTCCGATGACCTCACA-3'
SPARC	5'-TCATCCCCATGGAGCATTG-3'	5'-CCAGGGCGATGTACTTGTCA-3'
API5	5'-TCAGGTTCACCACCCAAGAAAT-3'	5'-TTTCCCACTGGGAGGGTTATAA-3'
Endoglin	5'-TGCAGGTGTCAGCAAGTATGATC-3'	5'-ATGTTGAGGCAGTGCACCTTT-3'
β-actin	5'-TGTCCACCTTCCAGCAGATGT-3'	5'-AGTCCGCCTAGAAGCATTTGC-3'

Each set of forward and reverse primers was designed from NCBI published sequences using the Primer Express Software v2.0.

Immunohistochemistry

Brain tissue sections were incubated in methanol for 10 min at RT. Slides were rinsed with 0.2 M PBS (pH 7.3) supplemented with 0.1% Triton-X, followed by incubation with an universal blocking solution (Dako Diagnostics, Mississauga, ON, Canada) for 1 h at RT. Blocking solution was then removed and slides were incubated with a polyclonal goat anti-human IGFBP7 antibody (1:30; R&D System, Minneapolis, MN), or with a monoclonal mouse anti-human SPARC antibody (1:50; R&D System) for 3 h at RT. Sections were then washed with PBS and incubated with the secondary antibody, Cy3-labeled anti-goat IgG (1:500; Molecular Probes, Eugene, OR) or Alexa Fluor 555 donkey anti-mouse (1:500; Molecular Probes), respectively, for 1 h at RT. Slides were washed with PBS and counterstained with a rabbit polyclonal laminin antibody-1 (1:500; Neomarkers; Medicorp, Montreal, QC, Canada) or with a rabbit polyclonal anti-human Von Willebrand Factor (1:400; Dako Diagnostics) overnight at 4°C, followed by incubation with FITC-labeled goat anti-rabbit IgG (1:500; Molecular Probes) for 1 h at RT after washing with PBS. Subsequent staining of vessels or cell nuclei were performed by incubating sections with UEA1 (1:20; Vector Laboratories) for 3 min at RT or with Hoechst (1:1,000; Dapi; Sigma, Oakville, ON, Canada) for 5 min at RT, respectively. In control slides, the primary antibody was either omitted or replaced with an anti-human IgG antibody. Sections were then rinsed with milli-Q water and dehydrated by sequential exposure to increasing concentrations of ethanol (70, 96, and 100%, 30 s each), followed by a 5-min treatment with xylene (Anachemia Canada, Montreal, QC, Canada). Sections were air-dried and visualized under the fluorescent or confocal microscope. Confocal imaging was performed using a Zeiss LSM 410 confocal

microscope (Carl Zeiss, Göttingen, Germany) and 20X or 63X objectives with sections scanned using 0.5-μm increments. The size of the image was 512 pixels/512 pixels.

RESULTS

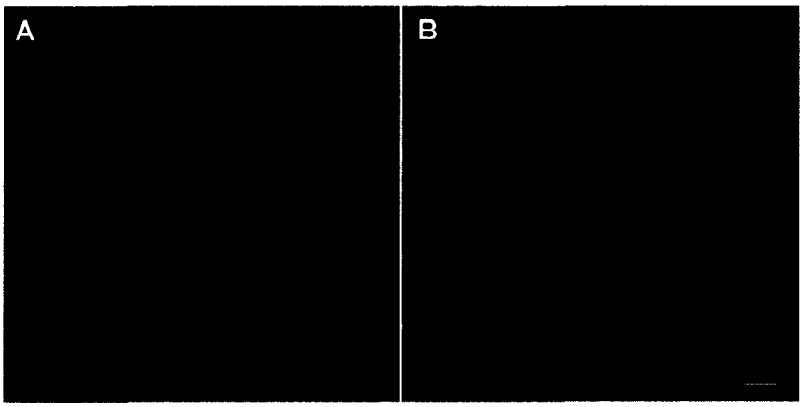
Characterization of tissue procured by LCM

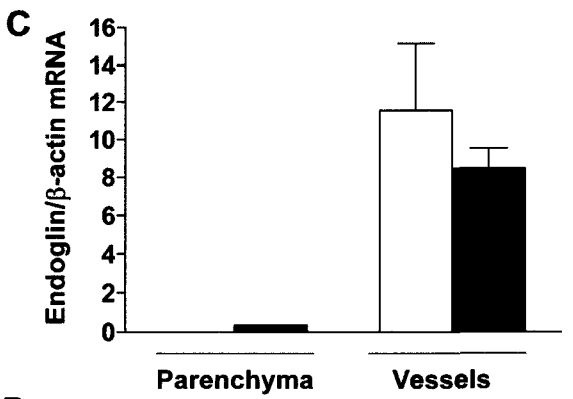
Differences in gene expression were analysed between LCM-captured vessels from five GBM and five non-malignant brains. Vessels from sections of GBM tumors and non-malignant brain were visualized using a fast staining with UEA 1 that specifically binds to fucose residues enriched in human brain endothelial cells (Hamid et al. 2003). Compared to vessels in non-malignant brain tissue, GBM sections reproducibly showed higher vessel density and aberrant tortuous vascular morphology (Figs. 1A,B). Vascular enrichment in LCM-assisted vessel dissections was confirmed by Q-PCR expression analysis of the endothelium-selective marker, endoglin (Ge and Butcher 1994). Endoglin mRNA was highly expressed in vessels from both non-malignant brain and GBM, but was not detected in the brain or tumor parenchyma (Fig. 1C). There was no difference in endoglin mRNA expression between GBM and non-malignant brain vessels (Fig. 1C). LCM-captured human brain vessels were also enriched in the endothelial marker, Factor-VIII related antigen (von Willebrand factor; FVIII-rAg) and junctional protein ZO-1, whereas GFAP mRNA, an indicator of astrocytic contamination, was expressed at low levels as previously shown (Mojsilovic-Petrovic et al. 2004).

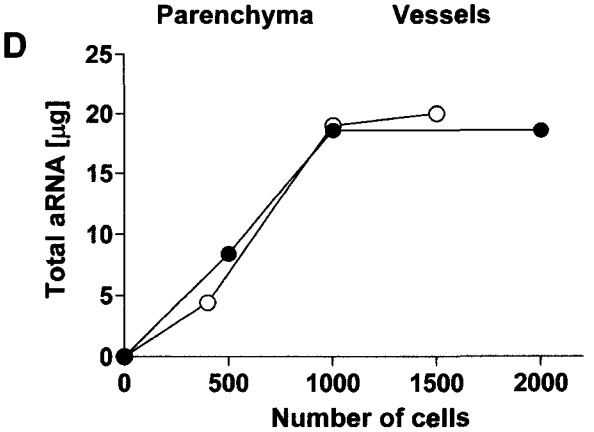
Figure 1

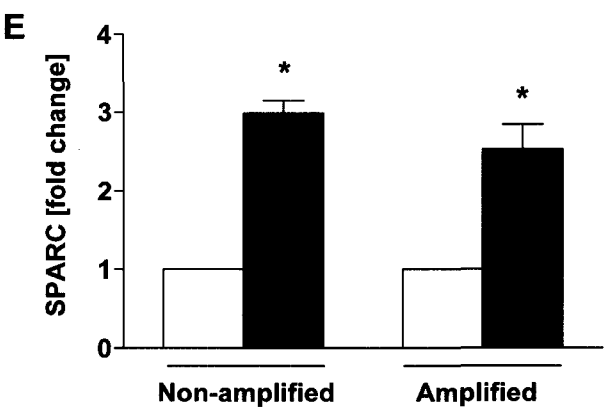
Extraction and RNA amplification of brain vessels using laser capture microdissection (LCM) microscopy

Brain vessels from non-malignant brain (A) and GBM (B) tissue sections were stained with the fluorescein-tagged UEA 1 and extracted by LCM. Scale bar = 100 μm. (C) Representative bar graph of endoglin mRNA expression in LCM-captured brain vessels and perivascular parenchyma from non-malignant brain (open bars) and GBM (black bars) determined by Q-PCR. (D) RNA from the indicated number of LCM-captured cells was extracted and amplified in two amplification cycles. The quantity of aRNA from control brain (open circles) and GBM (black circles) was determined at optical density 260 nm. (E) Expression of SPARC mRNA in LCM-captured vessels prior to and after RNA amplification determined by Q-PCR. Each bar represents the mean ± s.e.m. of duplicate determinations in two LCM-samples expressed as fold difference between endoglin (C) or SPARC (E) versus β-actin in the same sample. Asterisks indicate significant difference ($P < 0.01$; Student's t-test) between control and GBM vessels.









To obtain sufficient RNA quantities for microarray analyses, two RNA amplification cycles of LCM-captured vessels were performed. In prior studies (Iscove et al. 2002; Zhao et al. 2002), mRNA amplification techniques such as the one used in this study showed good accuracy and preservation of the RNA expression profiles. Increasing amounts of starting material resulted in linear increases of aRNA in both control and GBM vessels (Fig. 1D), reaching a maximum of ~20 μg aRNA derived from 1,000 captured cells; this number of cells was subsequently used for microarray experiments. To control for potential amplification bias towards certain genes, the common reference RNA was amplified in the same manner as LCM-captured samples and used as hybridization 'background' for each (non-malignant brain or GBM) vessel sample. Possible amplification bias was also analysed by Q-PCR for selected differentially expressed genes. For example, the ratio of SPARC mRNA expression between normal and tumor vessels was not significantly different prior to and after RNA amplification (Fig. 1E).

Differential gene expression between LCM-captured vessels from non-malignant brain and GBM

Microarray analyses between control and GBM vessels were performed using SS-Human 19Kv7 DNA arrays. After data normalization, scatter plots generated for each microarray showed linear regression range from R^2 = 0.8272-0.987 for GBM vessels versus reference RNA and from R^2 = 0.901-0.9721 for control vessels versus reference RNA. A threshold approach was used to select genes with fold change ≥ 1.5 and t test P value < 0.1, resulting in 69 differentially expressed genes and expressed sequence tags.

Forty-two differentially expressed genes with known function, 21 up-regulated (Table 2) and 21 down-regulated (Table 3), were identified in GBM vessels compared to control vessels. Molecular functions of identified differentially expressed genes ranged from structural roles to catalytic and signaling activities (Tables 2 and 3).

Table 2

List of up-regulated genes in LCM-captured glioblastoma vessels compared to vessels from non-malignant human brain tissue determined by high-density microarray analyses

Approved Gene name	Approved Gene Symbol	UniGene Cluster	Localization (GO Classification)	• Molecular Functions ○ Biological Functions (GO Classification)	Fold change	p value (t-test)
Insulin-like growth factor binding protein 7	IGFBP7	Hs.479808	extracellular region	• insulin-like growth factor binding ○ regulation of cell growth, negative regulation of cell proliferation	3.71	0.0514
Major histocompatibility complex, class I, B	HLA-B	Hs.77961	integral to plasma membrane	• MHC class I receptor activity ○ antigen presentation, endogenous antigen||antigen processing, endogenous antigen via MHC class I	2.44	0.0754
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	Hs.111779	extracellular space	• calcium ion binding, collagen binding ○ ossification	2.03	0.0815
Zinc finger protein 36, C3H type-like 2	ZFP36L2	Hs.503093	nucleus	• transcription factor activity ○ cell proliferation	2.02	0.0987
Vimentin	VIM	Hs.533317	intermediate filament	• structural constituent of cytoskeleton, protein binding ○ biological_process unknown	1.99	0.0946
Vimentin	VIM	Hs.533317	intermediate filament	• structural constituent of cytoskeleton, protein binding ○ biological_process unknown	1.98	0.0754
Keratin 19	KRT19	Hs.514167	intermediate filament	• structural constituent of cytoskeleton ○ ---	1.94	0.0627
Zic family member 2 (odd-paired homolog, Drosophila)	ZIC2	Hs.369063	nucleus	• DNA binding, zinc ion binding ○ brain development	1.90	0.0789
Synaptic Ras GTPase activating protein 1 homolog (rat)	SYNGAP1	Hs.520072	---	• GTPase activator activity ○ ---	1.83	0.0435

(Continued)

Approved Gene Name	Approved Gene Symbol	Unigene Cluster	Localization (GO Classification)	• Molecular Functions ○ Biological Functions (GO Classification)	Fold change	p value (t-test)
Filamin B, beta (actin binding protein 278)	FLNB	Hs.476448	integral to plasma membrane actin cytoskeleton	• actin binding ○ cytoskeletal anchoring, signal transduction, myogenesis, actin cytoskeleton organization and biogenesis	1.81	0.0427
Neurobeachin-like 1	NBEAL1	Hs.558535	---	• --- ○ ---	1.76	0.0271
Glyceraldehyde-3-phosphate dehydrogenase	GAPD	Hs.479728	cytoplasm	• glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity ○ glycolysis	1.69	0.0299
Transmembrane, prostate androgen induced RNA	TMEPAI	Hs.517155	integral to membrane	• molecular_function unknown ○ androgen receptor signaling pathway	1.67	0.0604
Actin related protein 2/3 complex, subunit 3, 21kDa	ARPC3	Hs.524741	Arp2/3 protein complex	• structural constituent of cytoskeleton ○ cell motility||regulation of actin filament polymerization	1.66	0.0649
Polypyrimidine tract binding protein 1	PTBP1	Hs.172550	Nucleoplasm, nucleolus, heterogeneous nuclear ribonucleoprotein complex	• poly-pyrimidine tract binding ○ nuclear mRNA splicing, via spliceosome	1.64	0.0295
Jumonji domain containing 1C	JMJD1C	Hs.413416	---(intracellular?)	• --- ○ ---	1.57	0.0846
Radical S-adenosyl methionine domain containing 2	RSAD2	Hs.17518	---	• catalytic activity, iron ion binding ○ ---	1.54	0.0919

(continued)

Approved Gene Name	Approved Gene Symbol	Unigene Cluster	Localization (GO Classification)	• Molecular Functions ○ Biological Functions (GO Classification)	Fold change	p value (t-test)
Zinc finger protein 224	ZNF224	Hs.549077	nucleus	• DNA binding, zinc ion binding ○ regulation of transcription, DNA-dependent	1.53	0.0582
Chromosome 20 open reading frame 17	C20orf17	Hs.473117	---	• --- ○ ---	1.53	0.0843
Attractin-like 1	ATRNL1	Hs.501127	membrane	• receptor activity, structural molecule activity, sugar binding ○ development	1.53	0.0470
Chloride intracellular channel 4	CLIC4	Hs.440544	membrane	• voltage-gated chloride channel activity ○ chloride transport	1.53	0.0909
Insulin-like growth factor binding protein 7	IGFBP7	Hs.479808	extracellular region	• insulin-like growth factor binding ○ regulation of cell growth, negative regulation of cell proliferation	1.51	0.0895
Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	SCYE1	Hs.480465	extracellular space	• tRNA binding, cytokine activity ○ tRNA aminoacylation for protein translation, chemotaxis, inflammatory response, signal transduction, cell-cell signaling	1.50	0.0422
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	Hs.111779	extracellular space	• calcium ion binding, collagen binding ○ ossification	1.50	0.0651

Only genes with known function are included. Redundant spots appear in the list as different entries to illustrate the reproducibility of the data. GO = Gene Ontology. --- not described by GO.

Table 3

List of down-regulated genes in LCM-captured glioblastoma vessels compared to vessels from non-malignant human brain tissue determined by high-density microarray analyses

Approved Gene name	Approved Gene Symbol	UniGene Cluster	Localization (GO Classification)	• Molecular Functions ○ Biological Functions (GO Classification)	Fold change	p value (t-test)
Apoptosis inhibitor 5	API5	Hs.435771	Cellular component unknown	• Molecular_function unknown ○ Transport, anti-apoptosis	2.95	0.0056
Janus kinase 1 (a protein tyrosine kinase)	JAK1	Hs.207538	cytoskeleton	• Janus kinase activity, ATP binding ○ protein amino acid phosphorylation, intracellular signaling cascade	2.86	0.0028
SEC5-like 1 (S. cerevisiae)	SEC5L1	Hs.484412	---	• --- ○ ---	2.77	0.0409
Chromosome 9 open reading frame 52	C9orf52	Hs.130086	---	• --- ○ ---	2.74	0.0031
Guanine nucleotide binding protein (G protein), beta polypeptide 1-like	GNB1L	Hs.105642	---	• --- ○ biological_process unknown	2.63	0.0039
Apoptosis inhibitor 5	API5	Hs.435771	Cellular component unknown	• Molecular_function unknown ○ Transport, anti-apoptosis	2.63	0.0062
Hypothetical gene supported by AF151042; NM_017761	PNRC2	Hs.449042	----	• receptor activity ○ ---	2.59	0.0058
Apoptosis inhibitor 5	API5	Hs.435771	Cellular component unknown	• Molecular_function unknown ○ Transport, anti-apoptosis	2.50	0.0014
SET binding protein 1	SETBP1	Hs.435458	nucleus	• DNA binding ○ regulation of transcription, DNA-dependent	2.42	0.0041
4-aminobutyrate aminotransferase	ABAT	Hs.336768	mitochondrial matrix	• 4-aminobutyrate transaminase activity, pyridoxal phosphate binding, (S)-3-amino-2-methylpropionate transaminase activity ○ aminobutyrate metabolism, neurotransmitter catabolism	2.33	0.0587

(Continued)

Approved Gene name	Approved Gene Symbol	UniGene Cluster	Localization (GO Classification)	• Molecular Functions ○ Biological Functions (GO Classification)	Fold change	p value (t-test)
Ubiquitin specific peptidase 40	USP40	Hs.96513	---	• cysteine-type endopeptidase activity, ubiquitin thiolesterase activity ○ ubiquitin-dependent protein catabolism	2.04	0.0173
Retinoic acid induced 14	RAI14	Hs.431400	---	• --- ○ ---	2.01	0.0916
Hypothetical protein FLJ20366	FLJ20366	Hs.390738	---	• --- ○ ---	1.93	0.0163
Synapsin II	SYN2	Hs.445503	synaptic vesicle, synapse	• --- ○ neurotransmitter secretion	1.83	0.0228
Scavenger receptor class A, member 5 (putative)	SCARA5	Hs.591833	Cytoplasm, membrane	• scavenger receptor activity ○ ---	1.82	0.0098
TNF receptor-associated factor 7	TRAF7	Hs.334479	ubiquitin ligase complex	• ubiquitin-protein ligase activity, zinc ion binding ○ activation of MAPKKK, regulation of transcription, DNA-dependent, protein ubiquitination, regulation of apoptosis	1.71	0.0080
Tripartite motif-containing 2	TRIM2	Hs.435711	ubiquitin ligase complex, cytoplasm	• ubiquitin-protein ligase activity, zinc ion binding, myosin binding ○ protein ubiquitination	1.70	0.0795
Myelin basic protein	MBP	Hs.551713	---	• structural constituent of myelin sheath ○ immune response, synaptic transmission, central nervous system development, nerve ensheathment	1.67	0.0028

(Continued)

Approved Gene name	Approved Gene Symbol	UniGene Cluster	Localization (GO Classification)	• Molecular Functions ○ Biological Functions (GO Classification)	Fold change	p value (t-test)
Transformer-2 alpha	TRA2A	Hs.445652	nucleus	• pre-mRNA splicing factor activity ○ nuclear mRNA splicing, via spliceosome	1.59	0.0139
SMAD, mothers against DPP homolog 5 (Drosophila)	SMAD5	Hs.167700	Nucleus, integral to membrane	• receptor signaling protein activity, transcriptional activator activity ○ regulation of transcription, DNA-dependent, transforming growth factor beta receptor signaling pathway	1.53	0.0690
Ankyrin repeat domain 44	LOC91526	Hs.432706	---	• --- ○ ---	1.52	0.0425
Pumilio homolog 2 (Drosophila)	PUM2	Hs.467824	---	• --- ○ ---	1.52	0.0167
Peroxisomal LON protease like	LONPL	Hs.643528	---	• --- ○ ---	1.51	0.0429

Only genes with known function are included. Redundant spots appear in the list as different entries to illustrate the reproducibility of the data. GO = Gene Ontology. --- not described by GO.

Gene networks by literature mining

To determine whether the identified genes have been previously associated with the selected knowledge terms, published literature was analysed using LitMiner™. Twenty-seven differentially expressed genes, 18 up-regulated and 9 down-regulated, have been previously reported in relation to knowledge terms, angiogenesis, vessels, GBM/glioma, or tumor (see Supplementary Table; http://www.litminer.ca/htmltables/pen/). Sixteen genes were linked to vessels, whereas 13 (IGFBP7, SPARC, ZFP36L2, VIM, KRT19, ZIC2, SYNGAP1, GAPD, CLIC4, SCYE1, JAK1, MBP, SMAD5) were associated with angiogenesis through a direct literature link. The network of primary, secondary, and tertiary literature association of differentially expressed genes to angiogenesis is shown in Fig. 2A.

Supplementary Table

Literature links of differentially expressed genes with indicated knowledge terms identified by LitMiner™

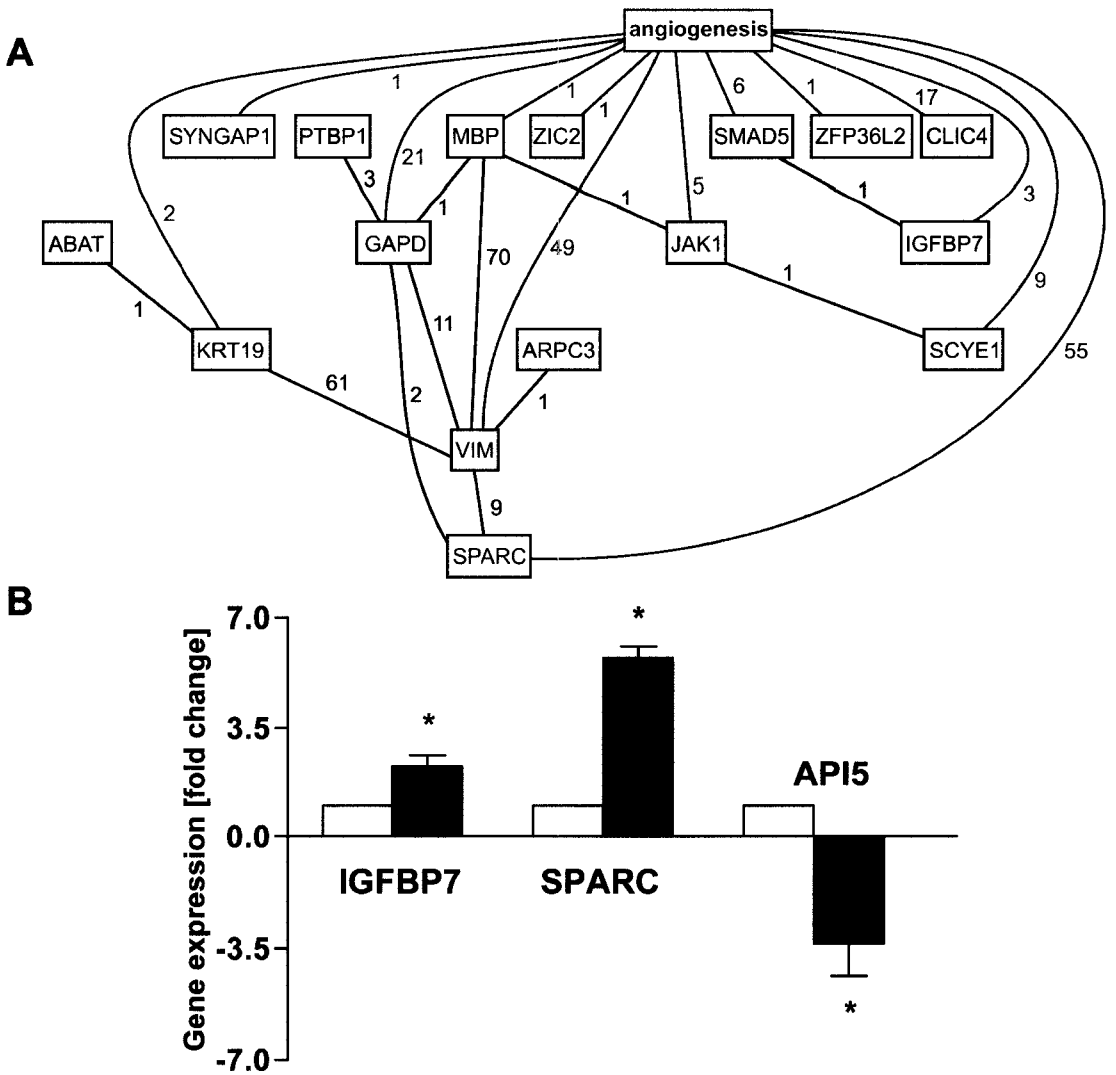
Indicated number of publications includes all primary links between the gene and the knowledge term. Publications can be accessed in Pubmed by clicking on the number in the Table.

	cancer* (1187453)	arter* (930581)	glioma (36244)	angiogenesis (25065)
IGFBP7 (109)	56	38	2	5
HLA-B (5059)	505	154	5	0
SPARC (1285)	277	184	19	70
ZFP36L2 (43)	11	0	0	1
VIM (11680)	5483	1487	297	57
KRT19 (1331)	759	66	3	5
ZIC2 (65)	6	0	1	1
SYNGAP1 (45)	2	8	0	2
FLNB (230)	7	7	0	0
NBEAL1 (1)	0	0	1	0
GAPD (4568)	300	168	10	8
TMEPAI (16)	11	0	0	0
ARPC3 (180)	0	0	0	0
PTBP1 (1999)	44	7	5	0
JMJD1C (5)	2	0	0	0
RSAD2 (7)	1	0	1	0
ZNF224 (3)	1	0	0	0
C20orf17 (0)	0	0	0	0
ATRNL1 (0)	0	0	0	0
CLIC4 (615)	8	1	0	1
SCYE1 (356)	47	91	4	16
API5 (70)	6	2	0	0
JAK1 (1348)	259	39	9	5
SEC5L1 (4)	0	0	0	0
C9orf52 (0)	0	0	0	0
GNB1L (20)	0	0	0	0
PNRC2 (3)	1	0	0	0
SETBP1 (22)	0	0	0	0
ABAT (1319)	7	20	4	0
USP40 (0)	0	0	0	0
RAI14 (2)	0	0	0	0
FLJ20366 (7)	0	0	0	0
SYN2 (118)	3	0	2	0
SCARA5 (1)	0	0	0	0
TRAF7 (3)	2	0	0	0
TRIM2 (5)	0	0	0	0
MBP (6365)	573	7	52	2
TRA2A (1)	0	0	0	0
SMAD5 (170)	35	21	1	8
LOC91526 (0)	0	0	0	0
PUM2 (11)	1	0	0	0
LONPL (1)	0	0	0	0

Figure 2

Validation of differentially expressed genes between control brain vessels and GBM vessels by literature mining using LitMiner™ (A) and by Q-PCR (B)

(A) Red lines represent direct links to knowledge term [angiogenesis], blue lines represent secondary links, and black lines represent tertiary links. Numbers beside the lines correspond to the number of published articles reporting the link. (B) Expression of IGFBP7, SPARC and API5 in LCM-captured vessels from non-malignant brain (open bars) and GBM tumors (black bar) determined by Q-PCR. The expression of each gene was normalized to that of β-actin in the same sample and fold change was calculated with respect to control vessels. Each bar is the mean ± s.e.m. of duplicate determinations in three separate tissues. Asterisks indicate a significant ($P < 0.05$; Student's t-test) difference between control and GBM vessels.



Validation of differentially expressed genes by Q-PCR

Three genes associated with angiogenesis through direct literature linkage, IGFBP7, SPARC, SCYE1, encoded secreted proteins. Q-PCR gene expression analysis was performed for these genes, as well as for the most down-regulated gene, API5, in three GBM and two non-malignant brain tissues using either non-amplified (10,000 LCM-captured cells) or amplified RNA. IGFBP7 (two spots on microarray, up-regulated 3.71- and 1.51-fold) and SPARC (two spots on microarray up-regulated 2.03-and 1.50-fold) showed three fold and six fold up-regulation, respectively, in GBM vessels by Q-PCR (Fig. 2B). In contrast, SCYE1 mRNA up-regulation observed by microarray analyses could not be confirmed in three GBM vessel extractions analysed (data not shown). API5 (three spots on microarray down-regulated 2.95-, 2.63-, and 2.50-fold) showed three fold reduced levels in GBM vessels by Q-PCR (Fig. 2B).

Validation of differentially expressed genes by immunohistochemistry

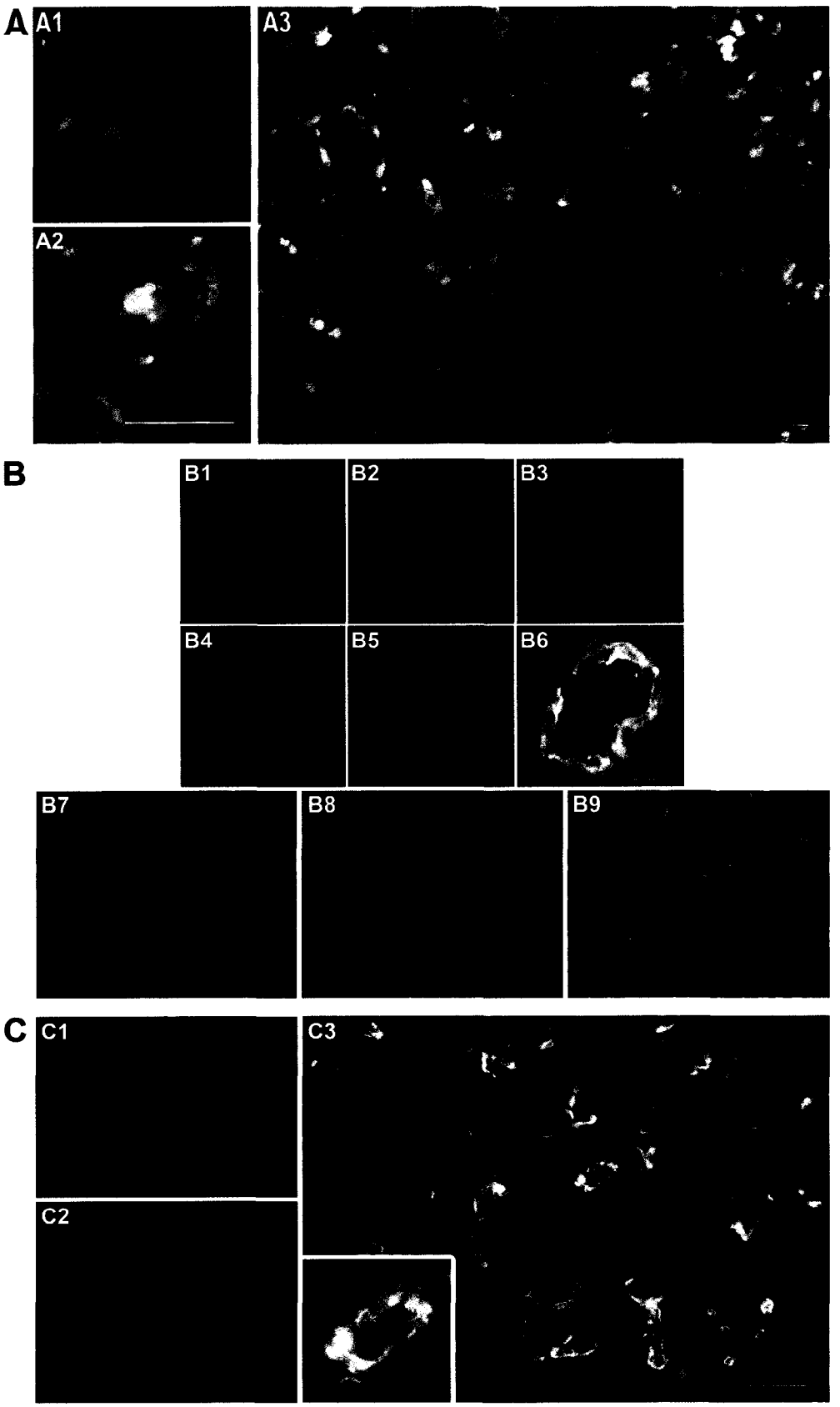
SPARC and IGFBP7 protein expression was further analysed by immunohistochemistry. SPARC immunoreactivity was present in tumor cells throughout GBM sections (Fig. 3A3), but was particularly intense in cells adjacent to and coming into a close contact with tumor vessels detected by laminin immunostaining (Fig. 3A2). SPARC immunoreactivity was absent from either vessels or perivascular cells in non-malignant brain sections (Fig. 3A1). To identify the nature of perivascular SPARC-expressing cells, double immunostaining was performed for SPARC and either the endothelial marker, Factor VIII-rAg, the astrocyte marker, GFAP, or the pericyte/smooth muscle cell marker, α-smooth muscle actin (α-SMA). SPARC staining did not co-

localize with either Factor VIII-rAg or with weak GFAP immunoreactivity observed in rare tumor cells (data not shown). α-SMA immunoreactive cells were also distinct from those immunopositive for SPARC (data not shown).

Figure 3

SPARC and IGFBP7 proteins expression in GBM tumor sections determined by immunohistochemistry

(A) Tissue sections derived from non-malignant brain tissue (1) and GBM tumor (2-3) were stained for laminin (green) and SPARC (red). No SPARC immunostaining was detected in normal brain tissue (1), strong SPARC expression was observed in cells in close proximity to GBM vessels (2-3), and less intense SPARC staining in some GBM parenchymal cells (3). Scale bar = 50 μm. (B) Tissues sections derived from non-malignant brain tissue (1-3) and GBM tumors (4-6; 7-9) were stained for laminin (green) and IGFBP7 (red) by immunohistochemistry. Overlay images show co-localization of laminin and IGFBP7 (6, 9). For 1-6, scale bar = 20 μm; for 7-9, scale bar = 50 μm. (C) Double immunohistochemistry against Factor VIII-rAg (1) and IGFBP7 (2) in GBM sections. Overlay image shows periendothelial deposition of IGFBP7, as well as intra-endothelial co-localization of both antigens (inset) (3). Cellular nuclei were stained with Hoechst in some sections. Scale bar = 50 μm.



In contrast to prominent SPARC expression in perivascular cells, IGFBP7 immunoreactivity was exclusively associated with laminin-stained GBM vessels (Figs. 3B4–B9), and was not observed in either vessels from non-malignant brain (Figs. 3B1-B3), or in tumor parenchyma (Figs. 3B7–B9). Virtually all GBM vessels were strongly immunopositive for IGFBP7 (Figs. 3B7–B9).

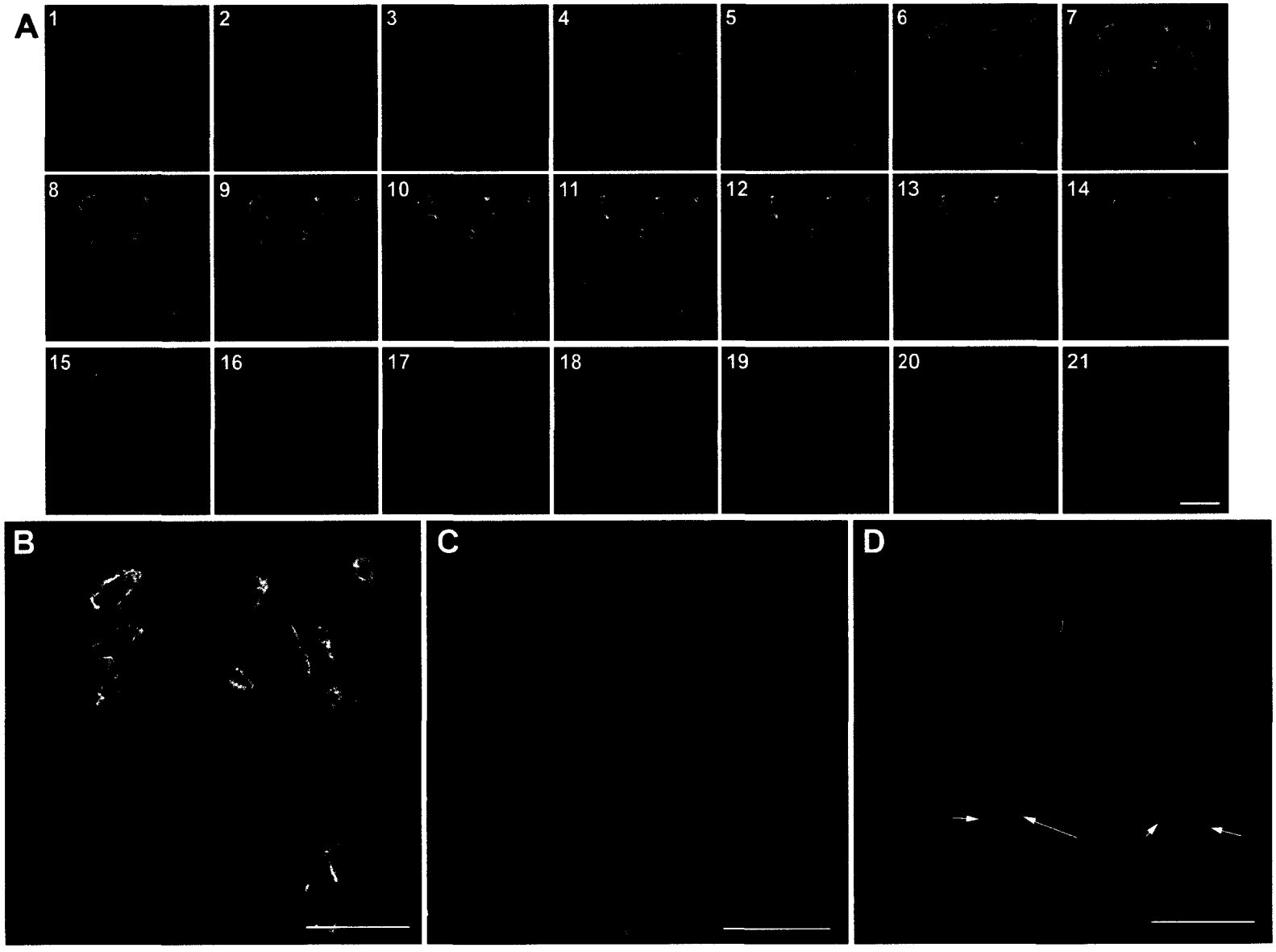
To investigate whether IGFBP7 is produced by endothelial cells of GBM vessels, double immunostaining was performed for IGFBP7 and the intracellular endothelial marker, Factor VIII-rAg (Figs. 3C1–C3). A significant intracellular co-localization of IGFBP7 and punctuate Factor VIII-rAg staining was observed (Fig. 3C3 and inset) in all GBM vessels, suggesting that endothelial cells are most likely the source of IGFBP7.

The localization of IGFBP7 in GBM vessels was analysed by confocal serial z-sections of vessels triple-labeled with IGFBP7 (red), laminin (blue), and UEA 1 (green) (Figs. 4A1–A21). UEA 1 binds to fucose residues highly expressed on the luminal surface of human brain endothelial cells, whereas the vascular basement membrane protein, laminin, is located on the abluminal side of the endothelial cells. The reconstruction of serial images shows that IGFBP7 immunoreactivity exhibits a discrete localization in GBM vessel basal lamina interposed between the laminin and UEA 1 throughout the thickness of the vessel (Fig. 4B). IGFBP7 immunoreactivity was absent from brain vessels in control tissues (Fig. 4C). Further analyses showed that IGFBP7 immunoreactivity (red) often forms 'a cuff' that separates laminin (green)-stained vascular and parenchymal strands of the GBM vessel basement membrane, which are normally fused (Fig. 4D).

Figure 4

IGFBP7 localization in GBM vessels using confocal microscopy

(A) Serial z-sections of GBM vessels stained for IGFBP7 (red), laminin (blue) and UEA 1 (green). Higher magnification of one GBM z-section (B) and of one non-malignant brain z-section (C) shows that IGFBP7 is accumulated between laminin and UEA 1 in GBM vessels. Arrows in D point to strands of the vascular (closer to the lumen) and parenchymal (further from the lumen) basement membranes of GBM vessels stained with laminin. Scale bar = 50 μm.



DISCUSSION

This study demonstrates the utility of vessel-selective gene profiling approaches in identifying vascular tumor markers that can be used for diagnosis or therapeutic targeting. Several differentially expressed genes identified in GBM vessels have been implicated in extracellular matrix remodeling and angiogenesis-related signaling pathways. SPARC was highly expressed in perivascular cells establishing close contacts with GBM vessels, whereas IGFBP7 was strongly up-regulated in tumor endothelial cells and deposited into the vicinity of the vascular basement membrane.

Several microarray profiling studies performed in various grades of gliomas (Nutt et al. 2003; Rich et al. 2005) identified groups or patterns of differentially expressed genes as diagnostic or prognostic classifiers. Bioinformatics analyses of these data sets discovered genes with predictive value when assessing survival risks from GBM (Rich et al. 2005). Because these studies examined gene expression in whole GBM tissues, known for their high heterogeneity (Mischel et al. 2003), markers with diagnostic or prognostic value expressed in rare cell populations such as tumor stem cells or tumor vasculature were likely under represented. This is underscored by the fact that only two up-regulated genes, SPARC and vimentin, were commonly identified in our study and microarray studies in whole glioma tissues (Nutt et al. 2003; Rich et al. 2005). Interestingly, SPARC was one of the three genes with the highest predictive value in assessing survival risks for GBM (Rich et al. 2005), with two others, doublecortex and semaphorin3B, also playing key roles in cellular migration.

Neovasculature remains a promising therapeutic target for reducing the growth or spread of GBM (Cheng et al. 1996). In GBM, tumor vasculature, unlike tumor tissue,

could be easily targeted systemically for molecular imaging or therapeutic ablation provided that selective 'markers' of tumor vessels to guide such applications are discovered and characterized. Therefore, the goal of this study was to identify selective biomarkers of GBM vessels using a combination of selective, LCM-assisted vessel extraction, RNA amplification, and cDNA microarray analyses.

The current study is the first comprehensive differential transcriptome analysis of GBM vessels *in situ*, showing 42 differentially expressed genes between GBM and brain vessels from non-malignant brain. Discovery of gene relations to each other and to various relevant 'knowledge or process terms' was performed by searches of a vast body of published literature using LitMiner^{TM} software. Close to 40% of identified differentially expressed genes have been previously reported in the literature in association with the term 'vessels,' thereby validating the high selectivity of LCM protocol used to procure brain vessels. The majority of identified differentially expressed genes had reported links to one or more processes affecting tumorigenesis including cell growth, adhesion, migration, transformation, transcription, translation, cell cycle control, and apoptosis, while 13 identified genes had a primary literature link to the process of angiogenesis. Several genes not previously reported in gliomas or other tumors had putative functions in a variety of cellular processes including apoptosis (TRAF7), differentiation (TRA2A, PUM2, ZNF224), and neuronal activity (TRIM2). The remaining genes were either deposited as multiple clusters or had no *Homo sapiens* transcribed sequences.

ECM remodeling is essential for endothelial cell activation and migration which contribute to angiogenesis (Wang et al. 2005). Interestingly, three genes (SPARC,

IGFBP7, and SCYE1) up-regulated in GBM vessels, and linked to angiogenesis by literature associations, encoded proteins secreted into the extracellular space. Whereas the up-regulation of SPARC and IGFBP7 in GBM vessels was also observed by Q-PCR analyses, changes in SCYE1 mRNA could not be confirmed by Q-PCR. In microarray data sets, false discovery rate is typically reduced, but cannot be eliminated, by combining, as in this study, statistical significance and magnitude of change criteria (Norris and Kahn 2006). Therefore, SCYE1 could be a true false positive in microarray data, or a 'real' change that requires higher number of GBM samples to be confirmed statistically by Q-PCR.

Immunohistochemical analysis in GBM sections identified high SPARC expression in perivascular cells adjacent to and often coming into direct contact with the vascular basement membrane protein laminin, but not in the endothelial cells of GBM vessels. These observations suggested that, in addition to endothelial cells, closely associated perivascular cells extracted during LCM-assisted vessel dissection could account for some observed changes in gene expression, including that of SPARC. A less intense immunostaining for SPARC was also observed in tumor cells distant from vessels, similar to what was reported in diffuse astrocytomas (Huang et al. 2000). SPARC is a Ca^{2+}-binding glycoprotein that interacts with a number of the ECM components including vitronectin, thrombospondin 1, entactin/nidogen, fibrillar collagens, and collagen type IV and has been implicated in tissue healing, remodeling, and angiogenesis (Brekken and Sage 2001). SPARC has been shown to facilitate cell migration by increasing the expression of several ECM proteolytic enzymes, including collagenase, stromelysin, and MMP-2 (Brekken and Sage 2001; Gilles et al. 1998), and to

regulate ECM organization through the modulation of integrin-linked kinase activity (Barker et al. 2005). SPARC is preferentially cleaved by MMP-3 into three major fragments with different biological activities; at least one of these fragments stimulated endothelial cell proliferation and angiogenesis (Sage et al. 2003). In experimental models of gliomas, SPARC has been described as a marker of invasive glioma phenotype, proliferative endothelial cells, and reactive astrocytes in the normal brain adjacent to tumor cells (Rempel et al. 1998; Schultz et al. 2002). In a xenograft model of C6 glioma, SPARC expression correlated with glioma-induced angiogenesis (Vajkoczy et al. 2000). Our study is the first immunohistochemical evidence of high SPARC expression in a subset of perivascular cells in human GBM; the nature of these perivascular cells remains unknown–they are Factor VIII-rAg, GFAP-, and α-SMA-negative, suggesting that they are not differentiated endothelial cells, perivascular astrocytes, or pericytes. Since SPARC is cleaved by proteases into products that are not always recognized by various anti-SPARC antibodies, it is possible that the up-regulation of SPARC mRNA observed in microarray studies originated from both endothelial cells and perivascular cells, while immunohistochemical detection was limited to intact SPARC protein predominating in perivascular cells. Based on the postulated role of SPARC in endothelial and tumor cell proliferation and migration, we suggest that these perivascular cells secrete SPARC into the perivascular space where the full length protein or its proteolytic fragments could facilitate endothelial cell migration and angiogenesis.

The current study identified IGFBP7 as a selective marker of GBM vessels. IGFBP7 was strongly up-regulated in GBM vessels in both microarray and Q-PCR analyses and selectively expressed in virtually all GBM vessels by immunochemistry.

IGFBP7 immunoreactivity co-localized with the endothelial marker, Factor VIIIrAg, and formed thick deposits in periendothelial space. The immunohistochemical analyses suggested that IGFBP7 is produced by GBM endothelial cells and secreted into the ECM where it likely interacts with other components of the basal lamina and forms protein deposits that often separate vascular and parenchymal strands of the basement membrane. Interestingly, an increased expression of vascular IGFBP7 was also observed during adaptive angiogenesis in brains of mice exposed to chronic hypoxia (unpublished observations), suggesting that IGFBP7 may be a marker of brain vessels undergoing remodeling in various pathologies.

IGFBP7 is a cell-adhesive glycoprotein with structural homology to insulin-like growth factor binding proteins but with lower binding affinity to IGF than other IGFBPs (Kim et al. 1997). IGFBP7 interacts with various ECM components, including collagen IV (Akaogi et al. 1996) as well as with cell surface heparin sulfate glycosaminoglycans (Kishibe et al. 2000). Interestingly, IGFBP7 is not expressed in normal brain vessels (Akaogi et al. 1996; Degeorges et al. 2000), but accumulates in capillary-like tubes of vascular endothelial cells *in vitro* (Akaogi et al. 1996) and in blood vessels of various human cancers (van Beijnum et al. 2006) including GBM. Biological roles of IGFBP7 remain poorly defined. Proteolytic cleavage of IGFBP7 by the membrane-bound serine protease matriptase produces fragments with different biological activities (Ahmed et al. 2006). IGFBP7 has also been shown to stimulate endothelial production of the potent vasodilator PGI2 (Hata et al. 2000), which may contribute to the increased permeability of GBM vessels. Tumor suppressor-like functions of IGFBP7 have been described in various cancer cells (Landberg et al. 2001; Mutaguchi et al. 2003). Given its high

accumulation in vascular basal lamina observed in this study, IGFBP7 could feasibly play a role in neovessel stabilization in GBM.

In summary, this study demonstrates the utility of transcriptional profiling in discovery of selective vascular 'address molecules'. GBM vessels express several genes/proteins implicated in various aspects of ECM remodeling, cell migration, angiogenesis, and apoptosis. Interestingly, the anti-apoptotic gene, API5, thought to contribute to tumorigenicity by promoting cell survival (Sasaki et al. 2001), was significantly down-regulated in GBM vessels. Therefore, the balance of pro-angiogenic and pro-apoptotic signals in GBM vessels likely determines the overall rate of GBM angiogenesis and/or tumor invasion. Address molecules selectively expressed/deposited in tumor vasculature or those associated with vascular remodeling could be exploited for selective vascular targeting of diagnostic imaging agents or cytotoxic drugs to tumors.

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REFERENCES

Abe M, Sato Y. 2001. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. Angiogenesis 4(4):289-98.

Ahmed S, Jin X, Yagi M, Yasuda C, Sato Y, Higashi S, Lin CY, Dickson RB, Miyazaki K. 2006. Identification of membrane-bound serine proteinase matriptase as processing enzyme of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/angiomodulin/mac25). Febs J 273(3):615-27.

Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K, Miyazaki K. 1996. Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. Proc Natl Acad Sci USA 93(16):8384-9.

Barker TH, Baneyx G, Cardo-Vila M, Workman GA, Weaver M, Menon PM, Dedhar S, Rempel SA, Arap W, Pasqualini R and others. 2005. SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity. J Biol Chem 280(43):36483-93.

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. 1999. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103(2):159-65.

Brekken RA, Sage EH. 2001. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol 19(8):816-27.

Cheng SY, Huang HJ, Nagane M, Ji XD, Wang D, Shih CC, Arap W, Huang CM, Cavenee WK. 1996. Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. Proc Natl Acad Sci U S A 93(16):8502-7.

Degeorges A, Wang F, Frierson HF, Jr., Seth A, Sikes RA. 2000. Distribution of IGFBP-rP1 in normal human tissues. J Histochem Cytochem 48(6):747-54.

Ge AZ, Butcher EC. 1994. Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF-beta ligand. Gene 138(1-2):201-6.

Gilles C, Bassuk JA, Pulyaeva H, Sage EH, Foidart JM, Thompson EW. 1998. SPARC/osteonectin induces matrix metalloproteinase 2 activation in human breast cancer cell lines. Cancer Res 58(23):5529-36.

Gladson CL. 1996. Expression of integrin alpha v beta 3 in small blood vessels of glioblastoma tumors. J Neuropathol Exp Neurol 55(11):1143-9.

Hamid SA, Daly C, Campbell S. 2003. Visualization of live endothelial cells ex vivo and in vitro. Microvasc Res 66(2):159-63.

Hata Y, Clermont A, Yamauchi T, Pierce EA, Suzuma I, Kagokawa H, Yoshikawa H, Robinson GS, Ishibashi T, Hashimoto T and others. 2000. Retinal expression, regulation, and functional bioactivity of prostacyclin-stimulating factor. J Clin Invest 106(4):541-50.

Huang H, Colella S, Kurrer M, Yonekawa Y, Kleihues P, Ohgaki H. 2000. Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays. Cancer Res 60(24):6868-74.

Iscove NN, Barbara M, Gu M, Gibson M, Modi C, Winegarden N. 2002. Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. Nat Biotechnol 20(9):940-3.

Jansen M, de Witt Hamer PC, Witmer AN, Troost D, van Noorden CJ. 2004. Current perspectives on antiangiogenesis strategies in the treatment of malignant gliomas. Brain Res Brain Res Rev 45(3):143-63.

Kim HS, Nagalla SR, Oh Y, Wilson E, Roberts CT, Jr., Rosenfeld RG. 1997. Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc Natl Acad Sci U S A 94(24):12981-6.

Kishibe J, Yamada S, Okada Y, Sato J, Ito A, Miyazaki K, Sugahara K. 2000. Structural requirements of heparan sulfate for the binding to the tumor-derived adhesion factor/angiomodulin that induces cord-like structures to ECV-304 human carcinoma cells. J Biol Chem 275(20):15321-9.

Kleihues P, Soylemezoglu F, Schauble B, Scheithauer BW, Burger PC. 1995. Histopathology, classification, and grading of gliomas. Glia 15(3):211-21.

Konig R, Baldessari D, Pollet N, Niehrs C, Eils R. 2004. Reliability of gene expression ratios for cDNA microarrays in multiconditional experiments with a reference design. Nucleic Acids Res 32(3):e29.

Landberg G, Ostlund H, Nielsen NH, Roos G, Emdin S, Burger AM, Seth A. 2001. Downregulation of the potential suppressor gene IGFBP-rP1 in human breast cancer is associated with inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation in estrogen receptor negative tumors. Oncogene 20(27):3497-505.

Leon SP, Folkerth RD, Black PM. 1996. Microvessel density is a prognostic indicator for patients with astroglial brain tumors. Cancer 77(2):362-72.

MacDonald TJ, Taga T, Shimada H, Tabrizi P, Zlokovic BV, Cheresh DA, Laug WE. 2001. Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery 48(1):151-7.

Mischel PS, Nelson SF, Cloughesy TF. 2003. Molecular analysis of glioblastoma: pathway profiling and its implications for patient therapy. Cancer Biol Ther 2(3):242-7.
Miyagami M, Tazoe M, Nakamura S. 1998. Expression of vascular endothelial growth factor and p53 protein in association with neovascularization in human malignant gliomas. Brain Tumor Pathol 15(2):95-100.

Mojsilovic-Petrovic J, Nesic M, Pen A, Zhang W, Stanimirovic D. 2004. Development of rapid staining protocols for laser-capture microdissection of brain vessels from human and rat coupled to gene expression analyses. J Neurosci Methods 133(1-2):39-48.

Moreno MJ, Ball M, Andrade MF, McDermid A, Stanimirovic DB. 2006. Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells. Glia 53(8):845-57.

Mutaguchi K, Yasumoto H, Mita K, Matsubara A, Shiina H, Igawa M, Dahiya R, Usui T. 2003. Restoration of insulin-like growth factor binding protein-related protein 1 has a tumor-suppressive activity through induction of apoptosis in human prostate cancer. Cancer Res 63(22):7717-23.

Norris AW, Kahn CR. 2006. Analysis of gene expression in pathophysiological states: balancing false discovery and false negative rates. Proc Natl Acad Sci U S A 103(3):649-53.

Nutt CL, Mani DR, Betensky RA, Tamayo P, Cairncross JG, Ladd C, Pohl U, Hartmann C, McLaughlin ME, Batchelor TT and others. 2003. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. Cancer Res 63(7):1602-7.

Rempel SA, Golembieski WA, Ge S, Lemke N, Elisevich K, Mikkelsen T, Gutierrez JA. 1998. SPARC: a signal of astrocytic neoplastic transformation and reactive response in human primary and xenograft gliomas. J Neuropathol Exp Neurol 57(12):1112-21.

Rich JN, Hans C, Jones B, Iversen ES, McLendon RE, Rasheed BK, Dobra A, Dressman HK, Bigner DD, Nevins JR and others. 2005. Gene expression profiling and genetic markers in glioblastoma survival. Cancer Res 65(10):4051-8.

Sage EH, Reed M, Funk SE, Truong T, Steadele M, Puolakkainen P, Maurice DH, Bassuk JA. 2003. Cleavage of the matricellular protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis. J Biol Chem 278(39):37849-57.

Saleh M, Stacker SA, Wilks AF. 1996. Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial growth factor sequence. Cancer Res 56(2):393-401.

Sallinen SL, Sallinen PK, Haapasalo HK, Helin HJ, Helen PT, Schraml P, Kallioniemi OP, Kononen J. 2000. Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. Cancer Res 60(23):6617-22.

Sasaki H, Moriyama S, Yukiue H, Kobayashi Y, Nakashima Y, Kaji M, Fukai I, Kiriyama M, Yamakawa Y, Fujii Y. 2001. Expression of the antiapoptosis gene, AAC-11, as a prognosis marker in non-small cell lung cancer. Lung Cancer 34(1):53-7.

Schlageter KE, Molnar P, Lapin GD, Groothuis DR. 1999. Microvessel organization and structure in experimental brain tumors: microvessel populations with distinctive structural and functional properties. Microvasc Res 58(3):312-28.

Schultz C, Lemke N, Ge S, Golembieski WA, Rempel SA. 2002. Secreted protein acidic and rich in cysteine promotes glioma invasion and delays tumor growth in vivo. Cancer Res 62(21):6270-7.

St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B and others. 2000. Genes expressed in human tumor endothelium. Science 289(5482):1197-202.

Vajkoczy P, Menger MD. 2000. Vascular microenvironment in gliomas. J Neurooncol 50(1-2):99-108.

Vajkoczy P, Menger MD, Goldbrunner R, Ge S, Fong TA, Vollmar B, Schilling L, Ullrich A, Hirth KP, Tonn JC and others. 2000. Targeting angiogenesis inhibits tumor infiltration and expression of the pro-invasive protein SPARC. Int J Cancer 87(2):261-8.

van Beijnum JR, Dings RP, van der Linden E, Zwaans BM, Ramaekers FC, Mayo KH, Griffioen AW. 2006. Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. Blood 108(7):2339-48.

Wang D, Anderson JC, Gladson CL. 2005. The role of the extracellular matrix in angiogenesis in malignant glioma tumors. Brain Pathol 15(4):318-26.
Wang JL, Liu YH, Lee MC, Nguyen TM, Lee C, Kim A, Nguyen M. 2000. Identification of tumor angiogenesis-related genes by subtractive hybridization. Microvasc Res 59(3):394-7.

Zhang W, Mojsilovic-Petrovic J, Andrade MF, Zhang H, Ball M, Stanimirovic DB. 2003. The expression and functional characterization of ABCG2 in brain endothelial cells and vessels. Faseb J 17(14):2085-7.

Zhao H, Hastie T, Whitfield ML, Borresen-Dale AL, Jeffrey SS. 2002. Optimization and evaluation of T7 based RNA linear amplification protocols for cDNA microarray analysis. BMC Genomics 3(1):31.

PREFACE TO CHAPTER 3

Chapter 2 described identification and validation of genes differentially expressed between GBM and non-malignant brain vessels. Transcriptional profiling using a combination of laser capture microdissection of vessels and microarray analyses resulted in identification of 42 genes selectively regulated in GBM vessels. One of these genes, IGFBP7 was subsequently validated as a selective biomarker of GBM vessels by Q-PCR and immunohistochemistry. Since the function of IGFBP7 in endothelial cells is unknown, the studies described in Chapter 3 will focus on deciphering mechanisms of IGFBP7 induction in tumor vessels and endothelial cells and its potential role in angiogenesis. These studies will test the hypothesis that specific secreted mediators present in the GBM microenvironment are responsible for IGFBP7 induction in tumor vessels. In studies described in Chapter 3, TGF-β family members released by GBM cells were identified as key inducers of IGFBP7 in brain endothelial cells. Signaling pathways leading to this induction and the effects of IGFBP7 on endothelial capillary-like tube formation *in vitro* characterized in these studies suggested that IGFBP7 might be involved in late phase angiogenesis.

CHAPTER 3

Glioblastoma secreted factors induce IGFBP7 and angiogenesis by modulating Smad-2-dependent TGF-β signaling

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Running title: Tumor cells induce IGFBP7 in endothelium

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Contribution of the co-authors:

- Dr. Danica Stanimirovic and Dr. Maria Moreno provided scientific guidance and supervised this research project. Dr. Stanimirovic has edited and Dr. Moreno revised this manuscript.
- Dr. Yves Durocher designed the IGFBP7 plasmid construct and allowed me the access to his facility to produce IGFBP7 recombinant protein.
- Dr. Paromita Deb-Rinker designed IGFBP7 primer sets A, B and C and trained me in the PCR bisulfite method to analyze the methylation status of IGFBP7.

ABSTRACT

Insulin-like growth factor binding protein 7 (IGFBP7) is a selective biomarker of glioblastoma (GBM) vessels, strongly expressed in tumor endothelial cells and vascular basement membrane. IGFBP7 gene regulation and its potential role in tumor angiogenesis remain unclear. Mechanisms of IGFBP7 induction and its angiogenic capacity were examined in human brain endothelial cells (HBEC) exposed to tumor-like conditions. HBEC treated with GBM cell (U87MG)-conditioned media (-CM) exhibited 4-fold up-regulation of IGFBP7 mRNA and protein compared to control cells. IGFBP7 gene regulation in HBEC was methylation-independent. U87MG-CM analysed by ELISA contained ~5 pM TGFβ-1, a concentration sufficient to stimulate IGFBP7 in HBEC to similar levels as U87MG-CM. Both pan-TGF-βs neutralizing antibody (1D11) and the TGF-β1 receptor (ALK5) antagonist, SB431542, blocked U87MG-CM-induced IGFBP7 expression in HBEC, indicating that TGF-β1 is an important tumor-secreted effector capable of IGFBP7 induction in endothelial cells. HBEC exposed to either U87MG-CM or IGFBP7 protein exhibited increased CLT formation in Matrigel™. Both TGF-β1- and U87MG-CM- induced Smad-2 phosphorylation and U87MG-CM-induced CLT formation in HBEC were inhibited by the ALK5 antagonist, SB431542. These data suggest that pro-angiogenic IGFBP7 may be induced in brain endothelial cells by TGF-βs secreted by GBM, most likely through TGF-β1/ALK5/Smad-2 pathway.

INTRODUCTION

Gliomas are the most common tumors of the central nervous system (CNS) derived from glial cells and classified into four clinical grades (World Health Organization – WHO grades I-IV) (Dai and Holland, 2001; Kleihues *et al.*, 1995). Glioblastoma multiforme (GBM; WHO grade IV) is the most malignant type with a mean survival time of approximately 9-12 months (Vajkoczy and Menger, 2000). Common features of GBMs include fast cell proliferation, invasion into normal brain parenchyma, intratumoral necrosis, hypoxia, and high angiogenic activity (Louis, 2006; Vajkoczy *et al.*, 1999).

The microenvironment of the CNS is maintained by the blood brain barrier (BBB) formed by specialized endothelial cells, which are distinguished from those in the periphery by high mitochondrial content (Oldendorf *et al.*, 1977), lack of fenestrations, minimal pinocytic activity, and the presence of tight junctions that restrict paracellular permeability of hydrophilic molecules (Kniesel and Wolburg, 2000). GBM vessels loose many BBB properties, leading to clinical signs of brain edema due to increased vessel permeability (Long, 1970). Morphologically, GBM vessels are characterized by fenestrations, increased number of caveolae, wide intercellular junctions, abnormal pericytes and a discontinuous basement membrane (Dinda *et al.*, 1993; Hirano and Matsui, 1975). GBM vessels express unique gene and protein biomarkers (Pen *et al.*, 2007), often associated with angiogenesis and/or increased permeability, including aquaporin 4 (Davies, 2002), plasmalemmal vesicle associated protein-1 (PV-1) (Madden *et al.*, 2004), and TEM7 (PLXDC1) (Beaty *et al.*, 2007). Recent transcriptomic analysis of laser-capture microdissected vessels from GBM tumors identified insulin-like growth

factor binding protein 7 (IGFBP7) as a highly selective biomarker of tumor vessels (Pen *et al.*, 2007). IGFBP7 was expressed by GBM endothelial cells and extensively deposited into the vascular basal lamina (Pen *et al.*, 2007).

IGFBP7 is a related member of the IGFBP family (Hwa *et al.*, 1999), cloned by several groups (Akaogi *et al.*, 1994; Murphy *et al.*, 1993; Yamauchi *et al.*, 1994), that, unlike the other family members (IGFBP1-6), exhibits a low affinity for IGF but a high and specific affinity for insulin (Oh *et al.*, 1996). IGFBP7 is a cell adhesive glycoprotein of about 30 kDa (Akaogi *et al.*, 1994), which is regulated by proteolytic cleavage into a two-chain form by a membrane-bound serine protease matriptase (Ahmed *et al.*, 2006). Varied IGFBP7 expression patterns have been reported in different tumor types (Ruan *et al.*, 2007). IGFBP7 transcription in tumor cells is modulated by DNA methylation (Komatsu *et al.*, 2000), retinoic acid and TGF-β1 (Hwa *et al.*, 1999). IGFBP7 has been implicated in tumor growth suppression (Burger *et al.*, 1998; Sprenger *et al.*, 1999) through induction of apoptosis or cellular senescence (Mutaguchi *et al.*, 2003; Wilson *et al.*, 2002). Recently, Wajapeyee et al. (Wajapeyee *et al.*, 2008) identified a mechanism by which BRAF proto-oncogene triggers cellular senescence in melanocytes through induction of IGFBP7.

In contrast to the tumor suppressor role of IGFBP7 in cancer cells, IGFBP7 is highly up-regulated in vessels of several tumors, including GBM (Akaogi *et al.*, 1996; Pen *et al.*, 2007), and interacts *in vitro* with various extracellular matrix proteins to stimulate adhesion and migration/invasion of vascular endothelial cells on plastic substrates (Kishibe *et al.*, 2000; Sato *et al.*, 1999); hence, the original name 'angiomodulin' (Akaogi *et al.*, 1996). Although literature evidence suggests that IGFBP7

might exhibit angiogenesis-modulating properties (Akaogi *et al.*, 1996; van Beijnum *et al.*, 2006), the role of IGFBP7 in tumor angiogenesis remains poorly understood.

Since IGFBP7 is selectively expressed in GBM vessels, the goal of this study was to identify the component(s) of the glial tumor microenvironment that induce(s) IGFBP7 expression in brain endothelial cells and to determine the potential role of IGFBP7 in angiogenesis. IGFBP7 expression in brain endothelial cells was found to be un-responsive to hypoxia, but up-regulated by secreted factors from glioblastoma cells through TGF-β1/ALK5/Smad-2 signaling pathway.

RESULTS

Glioblastoma conditioned media induces IGFBP7 in human brain endothelial cells

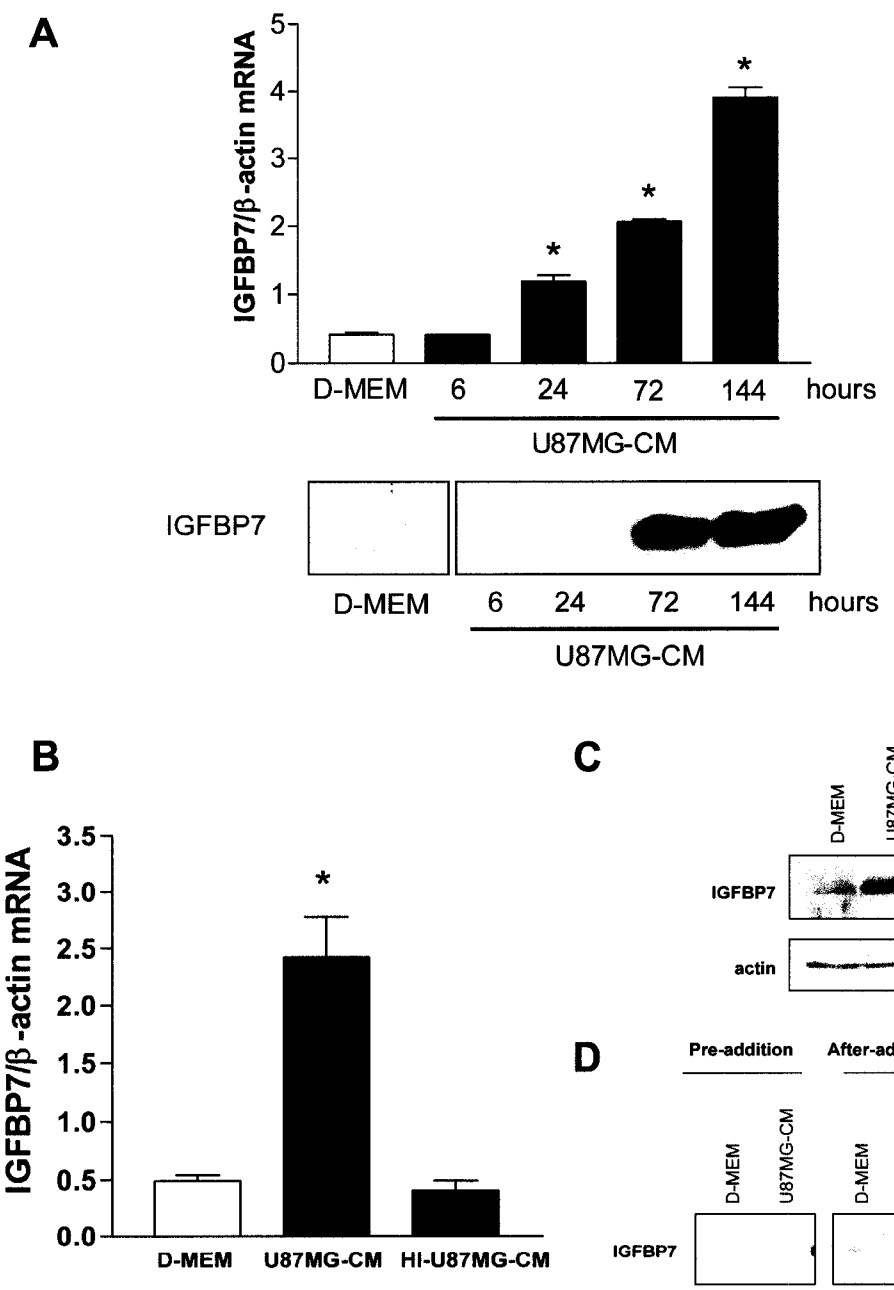
To examine whether tumor-like microenvironment affects the expression of IGFBP7 in brain endothelial cells, cultured HBEC were exposed to either hypoxia (Vaupel *et al.*, 1998) or media conditioned by the human GBM cell line, U87MG (U87MG-CM). Under control conditions, HBEC exhibited low abundance of IGFBP7 transcripts (2-3 fold lower than β-actin in the same cells) (Fig. 1A; upper panel) and protein (Fig. 1A; lower panel, C, D), in agreement with the observed absence of IGFBP7 protein immunoreactivity in non-malignant brain vessels *in situ* (Pen et al., 2007). Whereas hypoxia failed to induce IGFBP7 (A.P., unpublished), HBEC exposed to U87MG-CM responded with a time-dependent up-regulation of IGFBP7 mRNA (Fig. 1A; upper panel) and IGFBP7 protein (Fig 1A; lower panel), reaching maximal levels after 3-6 days of incubation in U87MG-CM. The pronounced up-regulation of IGFBP7 protein at 6 days of exposure to U87MG-CM was detected by Western Blot in both

HBEC lysates (Fig. 1C) and in the secreted fractions (Fig. 1A; lower panel &D). Conditioned media from another human glioblastoma cell line, T98G, induced IGFBP7 mRNA and protein in HBEC in a similar fashion as that observed with U87MG-CM (Fig. S1). IGFBP7 protein was not detected in either U87MG-CM (Fig. 1D) or T98G-CM (Fig. S1; lower panel).

Figure 1

IGFBP7 expression in human brain endothelial cells (HBEC) exposed to glioblastoma cell-conditioned-media (U87MG-CM)

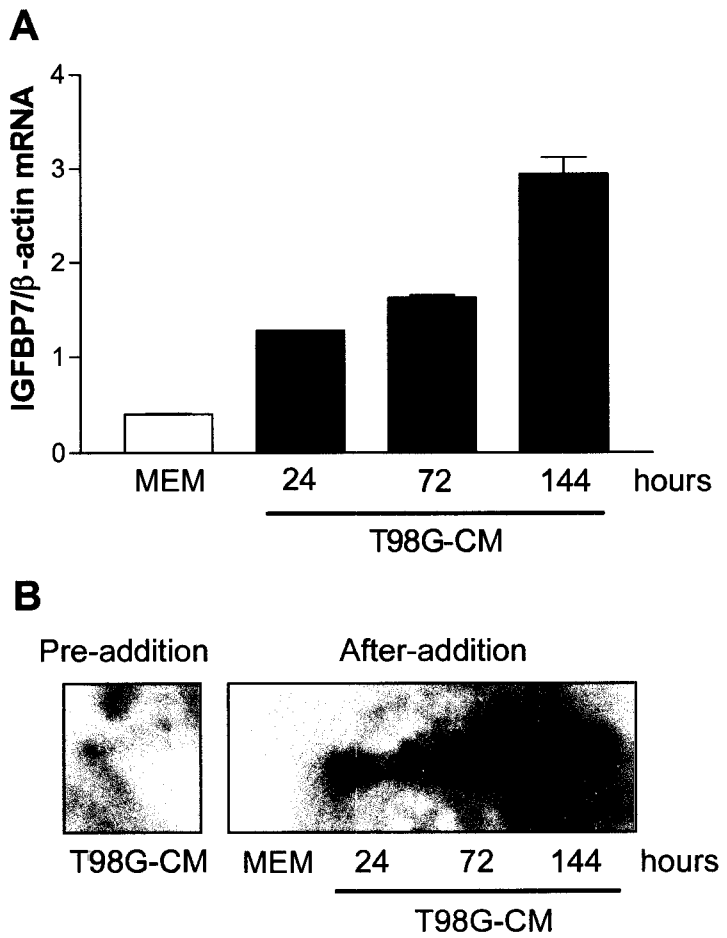
(A) IGFBP7 mRNA (upper panel) and protein (lower panel) expression, determined by Q-PCR and Western Blot, respectively, in HBEC exposed to serum-free D-MEM or U87MG-CM for indicated periods. (B) IGFBP7 mRNA expression in HBEC exposed to serum-free D-MEM, U87MG-CM, or heat-inactivated U87MG-CM (HI-U87MG-CM) for 6 days. (C) IGFBP7 protein expression in total HBEC lysates and (D) in the HBEC media prior to and 6 days after the addition of U87MG-CM or HI-U87MG-CM. Relative expression of IGFBP7 mRNA was normalized to that of β-actin in same cell extracts. Each bar represents the mean ± s.e.m. of 4-11 experiments. Asterisk indicates a significant ($P < 0.05$; ANOVA followed by Newman Keuls' post-test) difference between D-MEM- and U87MG-CM treated HBEC. Actin was used as a loading control for the Western Blot bands in cell lysates. Gels are representative of at least three separate experiments showing similar results.



Supplementary Figure 1

IGFBP7 expression in human brain endothelial cells (HBEC) exposed to glioblastoma cell-T98G-conditioned-media (T98G-CM)

IGFBP7 mRNA (A) and protein (B) expression (cell media), determined by Q-PCR and Western Blot, respectively, in HBEC exposed to D-MEM or T98G-CM for indicated periods. Relative expression of IGFBP7 mRNA was normalized to that of β-actin in same cell extracts. Each bar represents the mean ± s.e.m. of two experiments performed in duplicate. Gel is representative of two separate experiments showing similar results.



Heat-inactivated U87MG-CM lost the ability to induce IGFBP7 mRNA (Fig. 1B) and protein (Fig. 1C&D) expression in HBEC, suggesting that heat-liable mediator(s), most likely proteins, produced by GBM cells were responsible for IGFBP7 induction.

IGFBP7 gene methylation in HBEC is not affected by glioblastoma-conditioned media

To investigate whether IGFBP7 induction in HBEC treated with U87MG-CM was the result of DNA demethylation, the methylation status of IGFBP7 gene in HBEC treated with either D-MEM (control) or U87MG-CM was analysed (as described in Supplementary Materials and Methods) using primer sets (Supplementary Table 1) designed to amplify CpG sites present in the promoter region (primer sets A and B) or exon 1 and exon 1/intron 1 (primer sets C and D, respectively) of IGFBP7 gene (Chen *et al.*, 2007). The methylation status of D-MEM- and U87MG-CM-treated HBEC in the exon 1 region (+73 to +472; primer pair IGFBP7-C) of IGFBP7 spanning 45 CpG sites could not be determined, possibly due to the high density of CpG islands present in this region resulting in unspecific PCR products. The PCR amplified fragment spanning 19 CpG sites (+464 to +649; primer pair IGFBP7-D) showed a higher (but not statistically significant) number of methylation sites in control HBEC DNA compared to U87MG-CM-treated HBEC DNA (Fig. S2; graph). No differences in methylation state were detected within the 5 CpG (-941 to -766; primer pair IGFBP7-A) and 6 CpG (-789 to -577; primer pair IGFBP7-B) sites of IGFBP7 promoter regions (Fig. S2). Since no specific CpG sites were consistently and significantly methylated in control and demethylated in U87MG-CM-treated HBEC, we concluded that IGFBP7 induction in

U87MG-CM exposed HBEC was most likely regulated by mechanisms other than IGFBP7 DNA methylation.

Supplementary Table 1

Primers designed to amplify selected sequences for bisulfite PCR

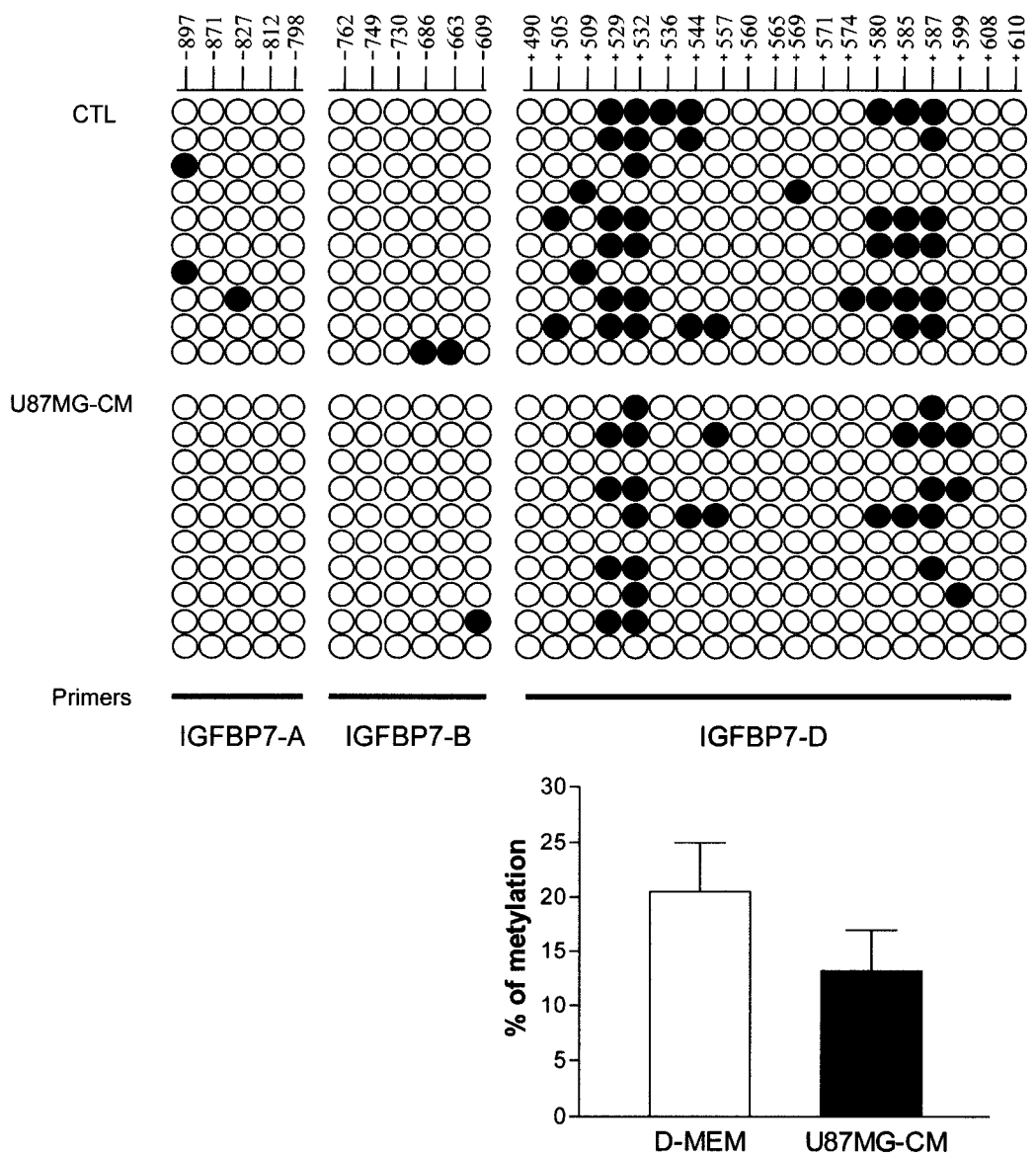
Name	Sense primers	Antisense primers
IGFBP7-A	5'-GTTTTTGTTTTTGTTTTTTTT-3'	5'-CTAAATAATAACCTTCTCCCC-3'
IGFBP7-B	5'-GTTTTTGGGAAGAGAGGTTTTTAAG-3'	5'-CTAAAAAAAACAAAAATCCCAACTC-3'
IGFBP7-C	5'-AGTTGGGATTTTTGTTTTTTTTAGTT-3'	5'-AAAACCTCTTAACTACCTACACCTCC-3'
IGFBP7-D	5'-GGGGAGAAGGTTATTATTTAGGTTAGTAA-3'	5'-CCCTCCCATCTAACTCCTAAAATAC-3'

Each set of forward and reverse primers was designed using Methprimer (http://www.urogene.org/methprimer), except for IGFBP7-D, which is identical as the primer designed by Chen et al., 2007.

Supplementary Figure 2

DNA methylation profile of the 5'-upstream region of the IGFBP7 gene

CpG methylation in the IGFBP7 promoter/exon 1/intron 1 regions in HBEC treated with D-MEM (CTL) and with U87MG-CM for 6 days. Positions of the CpG sites in IGFBP7 5'-upstream region are indicated. The 10 clones sequenced for each site are shown for both CTL and U87MG-CM treated HBEC. The positions of primers (except for primer pair IGFBP7-C) indicate the CpG sites that are part of the same PCR product. Open circles represent unmethylated cytosines and black circles represent methylated CpG sites. Comparison of the percentage of methylation sites in the region of exon 1/intron 1 spanning 19 CpG sites (primer pair IGFBP7-D) between CTL and U87MG-CM treated HBEC is represented in the graph. Each bar represents the mean ± s.e.m. of 10 colonies per group. Unpaired Student's t-test showed no significance difference between D-MEM and U87MG-CM treated HBEC.



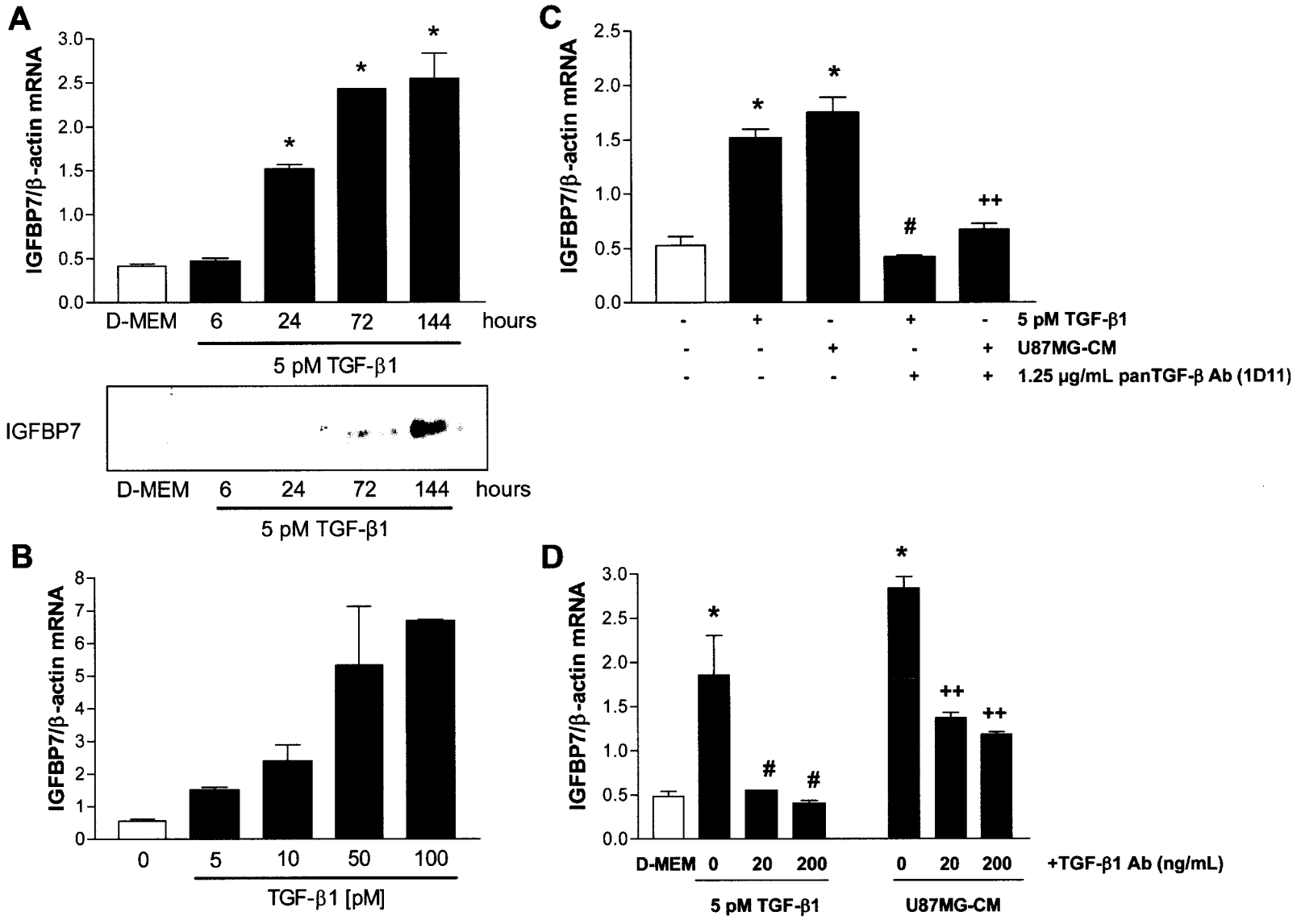
TGF-β1 induces IGFBP7 expression in HBEC

The nature of IGFBP7-inducing mediators present in U87MG-conditioned media was examined next. From our previous studies (Moreno *et al.*, 2006) analyzing U87MG cell transcriptome, we selected and examined two secreted angiogenic proteins identified by ELISA in U87MG-CM, VEGF-A and SPARC, for their ability to induce IGFBP7 expression in HBEC. 20 ng/mL of either VEGF-A or SPARC demonstrated minimal effects on IGFBP7 expression in HBEC (A.P., unpublished). Based on previous studies showing that TGF-β1 induces IGFBP7 expression in bovine retinal capillary endothelial cells (Hata *et al.*, 2000), we examined whether TGF-β1 is released in U87MG-CM. The average amount of TGF-β1 secreted in U87MG-CM determined by ELISA was ~5 pM. HBEC exposed to 5 pM TGF-β1 responded with a time-dependent up-regulation of IGFBP7 mRNA (Fig. 2A; upper panel) and protein (Fig 2A; lower panel), reaching maximal levels 3-6 days after IGFBP7 addition. In HBEC exposed to TGF-β1 concentrations ranging from 5-100 pM for 6 days, IGFBP7 mRNA was up-regulated in a dose-dependent manner, with EC_{50} = 12 pM (Fig. 2B). The IGFBP7 induction with 5 pM TGF-β1 was similar (2.5-4 fold) to that observed with U87MG-CM (Fig. 1 & 2).

Figure 2

TGF-β1 induces IGFBP7 expression in HBEC

(A) IGFBP7 mRNA (upper panel) and protein (lower panel) expression, determined by Q-PCR and Western Blot, respectively, in HBEC exposed to D-MEM or 5 pM TGFβ-1 for indicated periods. (B) The expression of IGFBP7 mRNA in HBEC exposed to either D-MEM or the indicated concentrations of TGF-β1 for 6 days. (C) IGFBP7 mRNA expression in HBEC exposed to 5 pM TGF-β1 or U87MG-CM in the absence or presence of 1.25 μg/mL of the pan-TGF-β neutralizing antibody 1D11 (R&D System), (D) IGFBP7 mRNA expression in HBEC exposed to 5 pM TGF-β1 or U87MG-CM in the absence or presence of 20 ng/mL or 200 ng/mL of TGF-β1-specific neutralizing antibody (R&D System). Relative expression of IGFBP7 mRNA was normalized to that of β-actin in same cell extracts. Each bar represents the mean ± s.e.m. of at least three experiments. Asterisks indicate significance between D-MEM-treated and TGF-β1- or U87MG-CM-treated HBEC; # and ++ indicate significance between 5 pM TGF-β1- or U87MG-CM-treated HBEC, respectively, in the absence or presence of the neutralizing antibodies. Significance ($P < 0.001$) was determined by ANOVA followed by Newman-Keuls' post-test comparison among means.

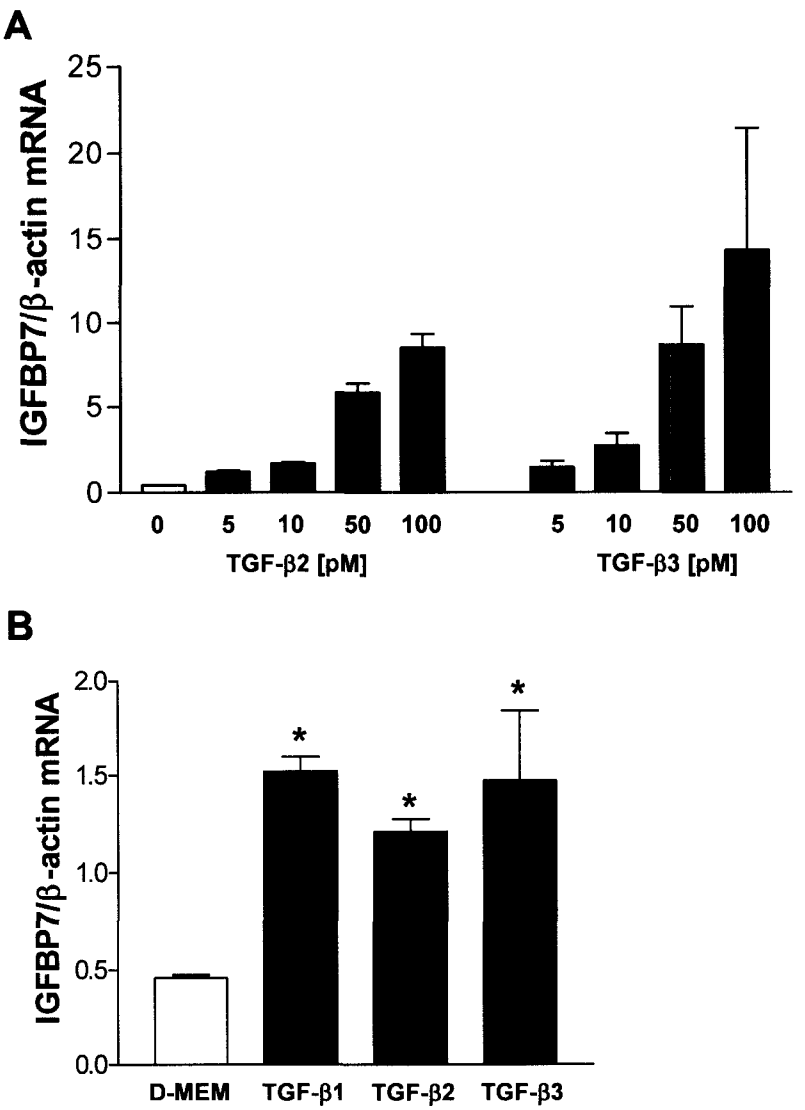


To confirm that secreted TGF-β1 is responsible for the IGFBP7 up-regulation, U87MG-CM were pre-treated with an antibody that neutralizes the activities of TGF-β1, TGF-β2 and TGF-β3 (pan-TGF-β antibody 1D11); the 1D11 antibody-treated U87MG-CM failed to induce IGFBP7 in HBEC (Fig. 2C). In contrast, an antibody specific for TGF-β1 ligand (R&D System) only partially (~50%) inhibited the IGFBP7-inducing activity of U87MG-CM (Fig. 2D), suggesting that other TGF-β isoforms participate in IGFBP7 induction by U87MG-CM. Two other TGF-β isoforms, TGF-β2 and TGF-β3, also induced IGFBP7 expression in HBEC in a concentration-dependent manner – the IGFBP7 induction was similar in magnitude for all three TGF-β isoforms (Fig. S3). However, only TGF-β2 isoform was detected in U87MG-CM by ELISA (~2-5 pM) suggesting that TGF-β2, in addition to TGFβ-1, may participate in U87MG-CM-mediated induction of IGFBP7 expression in HBEC.

Supplementary Figure 3

IGFBP7 induction by TGF-β2 and -β3 in HBEC

(A) The expression of IGFBP7 mRNA was evaluated by real-time Q-PCR in HBEC exposed to either serum-free D-MEM or to indicated concentrations of TGF-β2 or TGF-β3 for 6 days. (B) IGFBP7 expression in HBEC stimulated with 5 pM of TGF-β1, TGF-β2 or TGF-β3. Each bar represents the mean ± s.e.m. of duplicate experiments. Asterisks indicate significance between D-MEM and TGF-β1, TGF-β2 or TGF-β3 treated HBEC. Significance ($P < 0.05$) was determined by ANOVA followed by Newman-Keuls' post-test comparison among means.



TGF-β1 modulates IGFBP7 expression through ALK5 receptor and the signal transduction adapter proteins Smad-2 and Smad-1/-5

TGF-β1 has been reported to regulate angiogenesis in endothelial cells through two Smad pathways: the canonical Smad-2/-3/-4 pathway via its predominant type I receptor ALK5, and the Smad-1/-5/-4 pathway via ALK1 (Lebrin *et al.*, 2005). To investigate which signaling cascade triggers TGF-β1-mediated induction of IGFBP7, HBEC were exposed to TGF-β1 or U87MG-CM in the presence of SB431542, a selective antagonist of ALK5, but not of ALK1 (Inman *et al.*, 2002). Both IGFBP7 mRNA (Fig. 3A) and protein induction and release (Fig. 3B) stimulated by TGF-β1 or U87MG-CM were completely inhibited in the presence of 10 μM SB431542 (Fig. 3A, B). The levels of phosphorylated Smad-2 and Smad-1/-5 were examined in nuclear and cytosolic extracts of cells treated with TGF-β1 or U87MG-CM in the absence or presence of SB431542; whereas the levels of Smad-2 and Smad-5 in the cytosolic extracts remained unchanged in differently treated cells (Fig. 4; lower panel), a strong Smad-2 phosphorylation and a weaker Smad-1/-5 phosphorylation in response to both TGF-β1 and U87MG-CM was observed in the nuclear extracts of HBEC (Fig 4; upper panel). TGF-β1 was previously shown to induce both Smad-2 and Smad-1/-5 phosphorylation in wild-type mouse embryonic endothelial cells and bovine aortic endothelial cells (Goumans *et al.*, 2002). Both TGF-β1- and U87MG-CM-induced Smad-2 and Smad-1/5 phosphorylation were inhibited in the presence of SB431542. Since SB431542 has no effect on ALK1/Smad phosphorylation (Goumans *et al.*, 2003; Watabe *et al.*, 2003), these observations suggest that ALK1-mediated phosphorylation of Smad1/5 in HBEC cells is probably induced secondary to the activation of ALK5. Findings by Goumans et

al. (Goumans *et al.*, 2003) demonstrating that ALK5 kinase activity is required for ALK1 activation by TGF-β support this conclusion. These results suggest that U87MG-CM induces IGFBP7 in HBEC preferentially through ALK5 pathway leading to Smad-2 protein phosphorylation.

Figure 3

IGFBP7 induction by U87MG-CM in HBEC is inhibited by ALK5 antagonist

HBEC were exposed to either a serum-free D-MEM, 5 pM TGF-β1, U87MG-CM in the absence or presence of 10 μM ALK5 antagonist (SB431542) for 6 days. (A) IGFBP7 mRNA expression was determined by real-time Q-PCR. The expression of IGFBP7 was normalized to that of β-actin in same cell extracts. Each bar represents the mean ± s.e.m. of at least 3 experiments. Asterisks indicate significance between D-MEM treated and 5 pM TGF-β1 or U87MG-CM treated HBEC; # and ++ indicate significance between 5 pM TGF-β1 or U87MG-CM treated HBEC without or with ALK5 antagonist respectively. Significance ($P < 0.05$) was determined by ANOVA followed by Newman-Keuls' post-test comparison among means. (B) The expression of IGFBP7 protein in cell lysates or secreted into the media was evaluated by Western Blot. Actin was used as a loading control. Gels shown are representative of at least three separate experiments showing similar results.

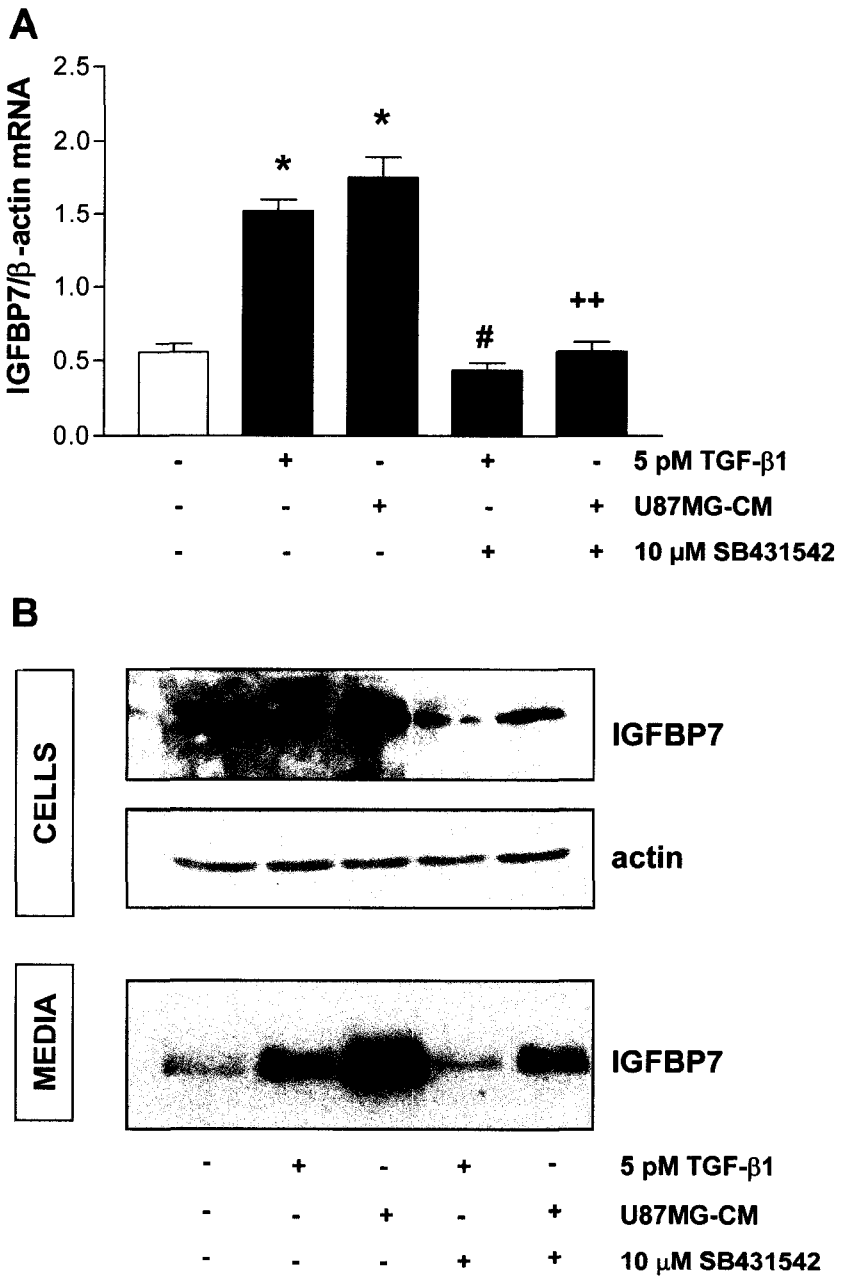
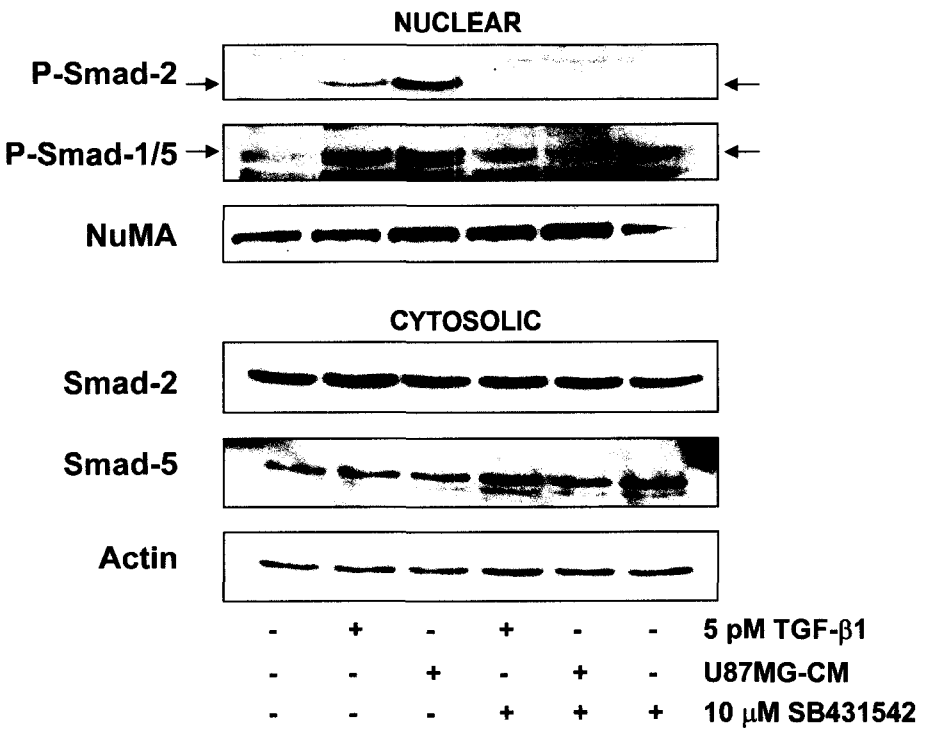


Figure 4

TGF-β1 induces IGFBP7 through ALK5/Smad 2 pathway

HBEC were treated with D-MEM, 5 pM TGF-β1 or U87MG-CM alone or in the presence of 10 μM of the ALK5 antagonist SB431542 for 30 minutes at 37°C before lysis. SB431542 was added to HBEC 20 minutes prior to TGF-β1 or U87MG-CM. Nuclear and cytosolic extracts were isolated as described in Materials and Methods. The phosphorylation of Smad-2 and Smad-1/-5 was analysed by Western Blot using respective anti-phospho-Smad antibodies. Cytosolic fraction was probed by respective anti-Smad antibodies. NuMA and actin were used as controls for nuclear and cytosolic protein loading, respectively. Gels shown are representative of three separate experiments showing similar results.



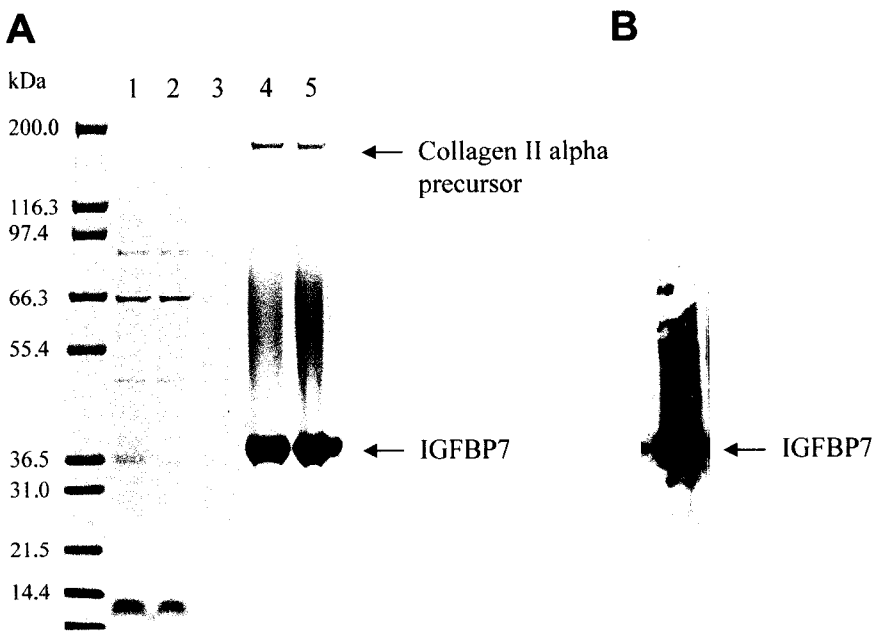
IGFBP7-induced capillary formation

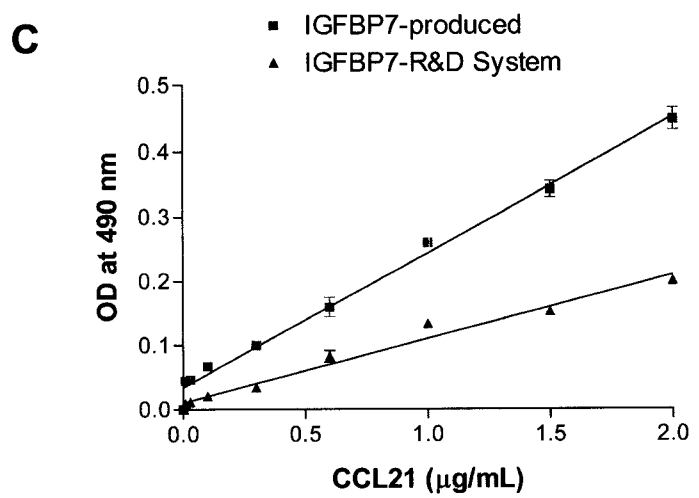
To evaluate the effect of IGFBP7 protein in endothelial cells, a recombinant IGFBP7 was produced and purified (as described in Supplementary Materials and Methods). Recombinant IGFBP7 was detected by SDS-PAGE-Coomassie blue staining (Fig. S4A) and by Western Blot using a penta-His HRP antibody (Fig. S4B) as a band of approximately 37 kDa. Mass spectrometry analysis indicated that IGFBP7 purity was ~90-95% and that the contaminant band observed at ~200 kDa in SDS-PAGE gel was collagen II alpha precursor (A.P., unpublished). IGFBP7 functionality/activity was determined by its ability to bind CCL21 (Nagakubo *et al.*, 2003) (Fig. S4C).

Supplementary Figure 4

Production of recombinant IGFBP7 protein

IGFBP7 was purified using Fractogel EMD chelate gravity flow column as described in Materials and Methods. SDS-PAGE Coomassie blue stained gel (A) and Western Blot (B) of purified IGFBP7. (C) Bioactivity of purified IGFBP7 was confirmed by its ability to bind CCL21 and compared to that of commercially available IGFBP7 (R&D System).



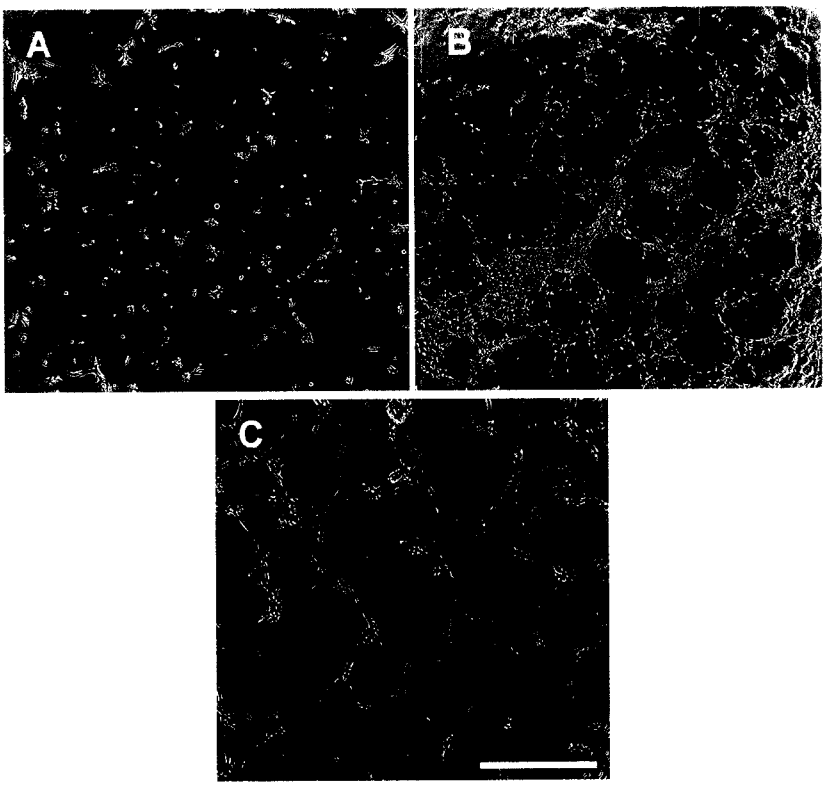


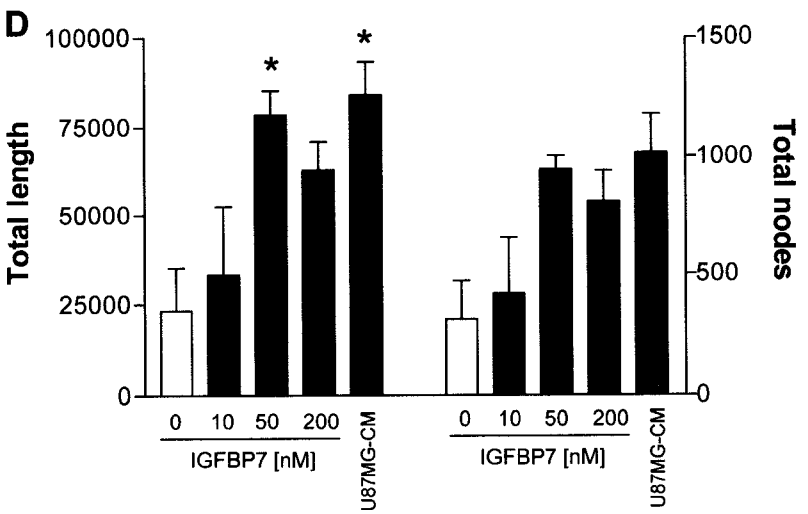
To assess whether IGFBP7 has angiogenic activity, HBEC grown in Matrigel™, a biological basement membrane matrix, were exposed to D-MEM or U87MG-CM or to different concentrations of IGFBP7. HBEC exposed to U87MG-CM for 24 h (Fig. 5C) formed an extensive network of capillary-like tubes (CLTs) compared to cells cultured in D-MEM alone (Fig. 5A), quantified as significant increases in both total length of capillary cords and the number of branching nodes (Fig. 5D). IGFBP7 also induced CLT formation in HBEC (Fig 5B) with a maximal induction observed at 50 nM (Fig. 5D), suggesting a pro-angiogenic activity of IGFBP7 in the examined concentration range.

Figure 5

Effect of IGFBP7 on capillary-like tube formation by HBEC grown in Matrigel™

Representative photomicrographs of capillary-like tubes formed by HBEC grown in Matrigel™ and exposed to D-MEM (A), 50 nM IGFBP7 (B) and U87MG-CM (C) for 24 h are shown. Scale bar = 500 μm. D) The total length of capillary-like tubes and the number of branching points were quantified using Northern Eclipse v.5.0. Each bar represents the mean ± s.e.m. of triplicate experiments. Asterisk indicates a significant ($P < 0.05$; ANOVA followed by Newman Keuls' post-test) difference between HBEC treated with D-MEM and IGFBP7 or U87MG-CM.



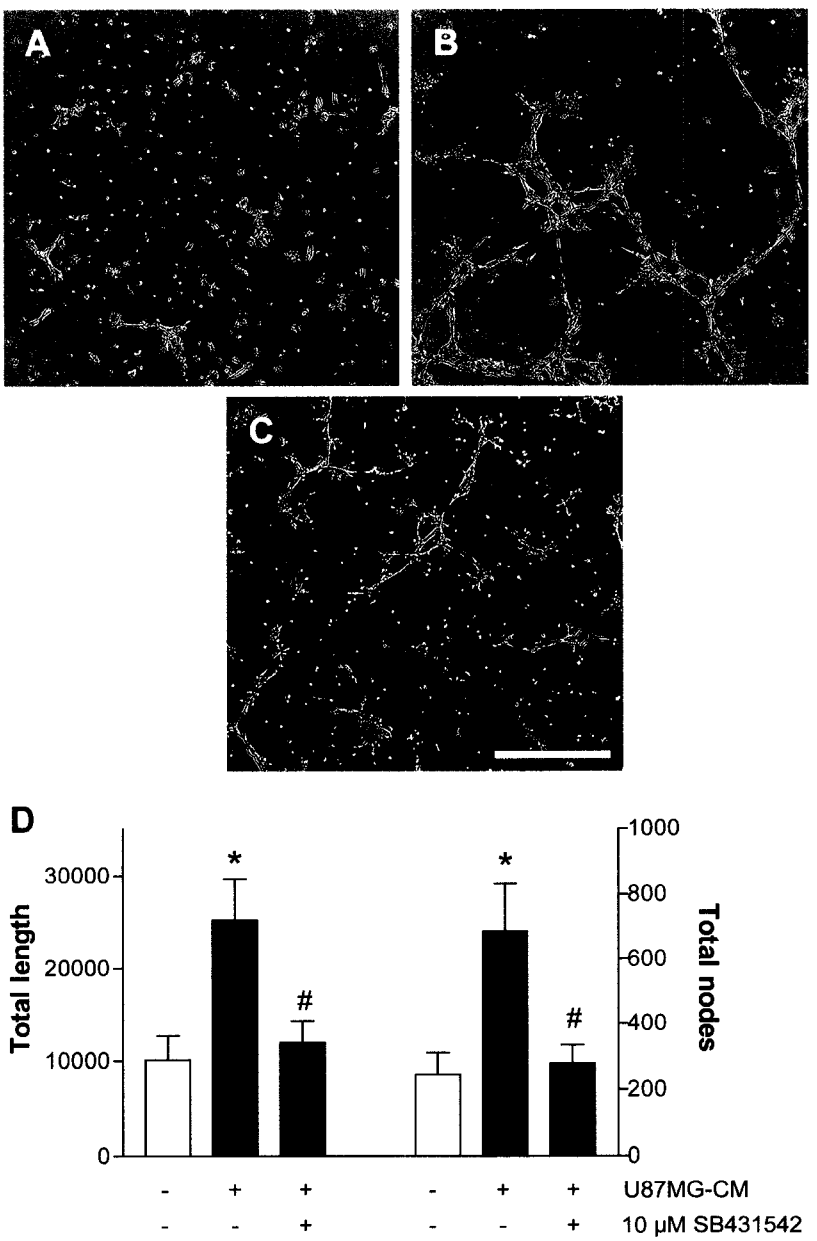


To examine if the pro-angiogenic activity of U87MG-CM is associated with TGF-β signaling pathway, HBEC were exposed to U87MG-CM in the presence of 10 μM SB431542. U87MG-CM-induced CLT formation in HBEC (Fig 6 A&B) was reduced to control levels in the presence of 10 μM SB431542 (Fig. 6C&D). The same concentration of SB431542 solvent, DMSO, did not affect CLT formation (A.P., unpublished). These results imply that TGF-β signaling through ALK5 is necessary for the pro-angiogenic activity of U87MG-CM.

Figure 6

TGF-β1 antagonist inhibits U87MG-CM-induced capillary-like tube formation

HBEC were grown in Matrigel™ in D-MEM (A), U87MG-CM alone (B) or supplemented with 10 μM SB431542 (C) for 24 h. Scale bar = 500 μm. D) The total length of capillary-like tubes and the number of branching points were quantified using Northern Eclipse v.5.0. Each bar represents the mean ± s.e.m. of eleven experiments. Asterisks indicate significance between D-MEM treated and U87MG-CM treated HBEC; # indicates significance between U87MG-CM in the absence or presence of 10 μM SB431542. Significance ($P < 0.05$) was determined by ANOVA followed by Newman-Keuls' post-test comparison among means.



DISCUSSION

The selective up-regulation of IGFBP7 in glioblastoma vessels compared to non-malignant brain vessels (Pen *et al.*, 2007) suggests a role for IGFBP7 in tumor vascular function. However, the mechanisms of IGFBP7 induction and its role in tumor angiogenesis are poorly understood. The present study provides the first evidence that IGFBP7 is induced in human brain endothelial cells by glioblastoma-cell secreted mediators, including TGF-β1, through TGF-β1/ALK5/Smad-2 pathway and by a DNA methylation-independent mechanism. Furthermore, IGFBP7 is shown to stimulate HBEC angiogenic phenotype and may play a role in angiogenesis induced by tumor microenvironment.

IGFBP7 stimulation by tumor microenvironment in brain endothelial cells was investigated by probing both potential mechanisms of IGFBP7 gene regulation in endothelial cells and the nature and signaling pathways induced by secreted mediators (proteins in conditioned media) of glioblastoma cells.

Compelling literature evidence indicates that hypermethylation of CpG islands of IGFBP7 is responsible for down-regulation of IGFBP7 in tumor cells contributing to tumor progression (Chen *et al.*, 2007; Komatsu *et al.*, 2000; Lin *et al.*, 2007). In contrast to colon (Lin *et al.*, 2007) and lung (Chen *et al.*, 2007) IGFBP7-negative cancer cell lines in which sites +490 to +610 of the 5' CpG island of IGFBP7 gene were all methylated, neither these sites (+490 to +610) nor promoter regions of IGFBP7 were highly methylated in either control or U87MG-CM–treated HBEC. These results suggest that IGFBP7 gene regulation by DNA methylation is likely different between cancer cells and

endothelial cells, and that DNA demethylation was not involved in regulating IGFBP7 gene induction in HBEC.

IGFBP7 expression in peripheral endothelial cells can be modulated by cell confluence, hypoxia, and cytokines including VEGF, bFGF, and TGF-β1 (Hata *et al.*, 2000). In this study, IGFBP7 expression in HBEC was potently induced by U87MG-CM and TGF-β1 and was not affected by VEGF or hypoxia. TGF-β is a 25 kDa homodimeric protein present in three isoforms: TGF-β1, -β2 and -β3 (Lebrin *et al.*, 2005); the three isoforms of TGF-β interact with the same cell surface serine/threonine kinase receptor type I (TβR-I) and type II (TβR-II) (de Caestecker, 2004; ten Dijke *et al.*, 1988) to induce several common but also distinct responses (Lebrin *et al.*, 2005). Glioblastoma tumors express all three TGF-β isoforms (Constam *et al.*, 1992; Olofsson *et al.*, 1992; Weller *et al.*, 1995; Wick *et al.*, 2001; Yamada *et al.*, 1995). Although all three TGFβ isoforms can induce IGFBP7 expression in HBEC, only TGF-β1 and -β2 were detected in U87MG-CM. Since the specific TGF-β1 – neutralizing antibody only partially blocked U87MG-CM-induced IGFBP7 expression, in contrast to the complete inhibition observed with the pan-TGF-βs antibody (1D11), it is suggested that TGF-β1 and -β2 isoforms are key U87MG-released mediators that stimulate IGFBP7 in HBEC.

TGF-β has been implicated in various biological functions including cell cycle control, carcinogenesis, invasion, metastasis (Massague, 1998), and vascular remodeling (Pepper, 1997), acting as either an inhibitor or stimulator of angiogenesis depending on experimental conditions (Goumans *et al.*, 2002). TGF-β signaling is initiated via binding of the ligand to its constitutively active cell surface receptor, TβR-II. Upon binding,

TβR-II activates and phosphorylates TβR-I, also known as activin receptor-like kinase (ALK) (de Caestecker, 2004). Subsequently, the signal is propagated to the nucleus by phosphorylated Smad proteins where they participate in transcriptional regulation of target genes (Derynck *et al.*, 1998). While activated ALK5 induces phosphorylation of Smad-2/-3, ALK1 induces the phosphorylation of Smad-1/-5 (Chen and Massague, 1999). Preferential induction of ALK1 or ALK5 signaling activity (Goumans *et al.*, 2002) is dependent on TGF-β concentrations: Smad-2 phosphorylation is initiated at 1 pM and is maximal at 10 pM TGF-β, whereas Smad-5 phosphorylation is triggered by 10-20 pM of TGF-β (Goumans *et al.*, 2002). We propose that TGF-βs (-1 and -2) secreted by glioblastoma cells mediate IGFBP7 induction in HBEC by preferentially activating ALK5/Smad-2 signaling pathway based on the following observations: a) measured levels of TGF-β1 in U87MG-CM were ~5 pM, b) the U87MG-CM induction of IGFBP7 was completely blocked by a selective inhibitor of ALK5, SB431542, and c) increases in phosho-Smad-2 detected in nuclear extracts of HBEC in response to both TGF-β1 and U87MG-CM were also inhibited by SB431542. The observed weak increases in phosphorylated Smad-1/-5 after TGF-β1 or U87MG-CM addition indicate that IGFBP7 expression can also be induced by activation of ALK1/Smad-1/-5 pathway. However, since SB431542 partially blocked these responses, the phosphorylation of Smad-1/-5 appears to be in part secondary to the activation of ALK5. TGF-β1 can also act through receptor-dependent, but Smad-independent pathways, including the activation of mitogen-activated protein (MAP) kinase (Blanchette *et al.*, 2001), c-Jun N-terminal kinase (JNK) (Engel *et al.*, 1999) and protein kinase C (PKC) (Yakymovych *et al.*, 2001).

In this study, the IGFBP7 induction was observed as early as 24 h after HBEC treatment with either TGF-β1 or U87MG-CM, reaching maximal levels of mRNA and/or protein up-regulation after 6 days. Given this prolonged time-course, autocrine stimulation by other TGF-β1-induced mediators might in part be responsible for IGFBP7 induction.

The study further provides evidence of pro-angiogenic function of IGFBP7 in HBEC. Whereas IGFBP7 failed to stimulate HBEC proliferation (A.P., unpublished), it induced a dose-dependent elaboration of capillary like tube networks in HBEC grown in Matrigel™. In agreement with this observation, a recent study demonstrated that IGFBP7 antibody inhibits endothelial tube formation in an *in vitro* collagen gel-based sprout formation assay (van Beijnum *et al.*, 2006). HBEC capillary-like tube formation induced by U87MG-CM was inhibited by the ALK5 antagonist, SB431542, implicating TGF-β/ALK5 signaling pathway in U87MG-CM-induced angiogenic responses. Since the same pathway was responsible for IGFBP7 induction in HBEC, it is tempting to speculate that autocrine actions of IGFBP7 could participate in U87MG-CM-induced angiogenic responses in HBEC. TGF-β mediates the transcription of several genes related to the extracellular matrix (ECM) remodeling, including SPARC and collagen I (Reed *et al.*, 1994), MMP-2, TIMP-1 (Kwak *et al.*, 2006), plasminogen activator inhibitor-1 (Keeton *et al.*, 1991) and collagen IV (Poncelet and Schnaper, 1998). Secreted IGFBP7 is known to interact with ECM components (Akaogi *et al.*, 1996; St Croix *et al.*, 2000), and could thus participate in the TGF-β1-induced ECM turnover and angiogenesis. Whereas inhibition of CLT formation was observed by TGF-β1 alone, in combination with VEGF and angiopoietin 1, CLT formation is rescued and stabilized,

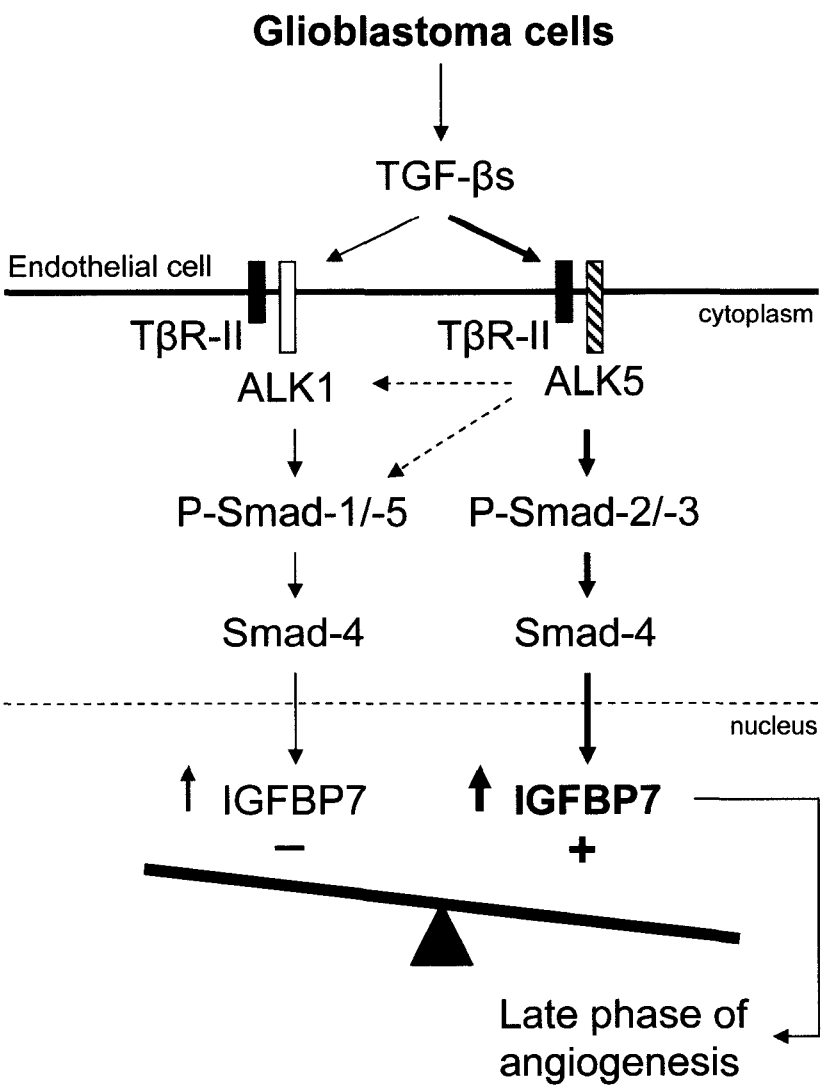
suggesting that the process is regulated by the interaction of angiogenic and anti-angiogenic growth factors (Ramsauer and D'Amore, 2007).

The process of angiogenesis is characterized by two different phases (Lebrin *et al.*, 2005): an activation phase accompanied by increased vascular permeability, degradation of the basement membrane, endothelial cell proliferation and migration, and the late phase typified by the inhibition of endothelial cell proliferation and migration, reforming of the basement membrane and stabilization and maturation of the vessel. A recent model proposed that the activation phase of angiogenesis is promoted by TGF-β1/ALK1/Smad-1/-5 signaling pathway, whereas the late phase is induced by TGF-β1/ALK5/Smad-2/-3 pathway (Goumans *et al.*, 2002); given that both Smad-2 and Smad-1/-5 can be activated by TGF-β in endothelial cells, the overall angiogenesis outcome is likely dependent on the balance between TGF-β-induced ALK1 or ALK5 activation (Goumans *et al.*, 2002). Since a) endothelial IGFBP7 induction by tumor microenvironment is predominantly mediated by ALK5/Smad-2 pathway, b) IGFBP7 accumulates in the basal lamina of glioblastoma vessels *in vivo* (Pen *et al.*, 2007) and c) IGFBP7 does not stimulate endothelial cell proliferation, we propose that IGFBP7 might play a role in the late phase of angiogenesis in the context of glioblastoma tumors. Proposed mechanisms of glioblastoma- and TGF-β-mediated induction of IGFBP7 in HBEC are shown schematically in Fig. 7.

Figure 7

Proposed mechanism of U87MG-CM and TGF-β – mediated IGFBP7 induction in HBEC

U87MG-CM secretes several factors including TGF-βs. TGF-βs bind to TGF-β type II receptor (TβR-II) at the surface of endothelial cells, which in turn recruits TGF-β type I receptor (TβR-I) ALK1 or ALK5. Activated TβR-II and TβR-I complexes stimulate both Smad-1/-5 and Smad-2/-3 phosphorylation; IGFBP7 transcription in this system appears to be preferentially induced via Smad-2/-3 complexing with Smad-4 (bold arrows). ALK1-mediated phosphorylation of Smad-1/-5 could be secondary to the activation of ALK5 (dash arrows). IGFBP7, in concert with other TGF-β-induced genes/proteins, participates in promoting late phase angiogenesis.



The proposed angiogenic role of IGFBP7 may appear contradictory to literature evidence of tumor-suppressor actions of IGFBP7 (Mutaguchi *et al.*, 2003; Sprenger *et al.*, 1999; Wajapeyee *et al.*, 2008; Wilson *et al.*, 2002). The first line of explanation is that IGFBP7 could trigger differential signaling pathways in tumor and endothelial cells – for example, in melanocytes, BRAF oncogene induces IGFBP7 which causes cellular senescence through inhibition of BRAF-MEK-ERK signaling (Wajapeyee *et al.*, 2008), whereas in endothelial cells IGFBP7 is induced through TGF-β/ALK5/Smad-2 and participates in late-stage angiogenesis. Since there is currently no clear understanding whether late stage angiogenesis contributes to tumor progression or tumor growth stabilization, the overall effect of IGFBP7 in tumor milieu cannot be easily generalized. Further understanding of the regulation and functions of this highly selective biomarker of tumor vessels will be important in developing appropriate therapies for GBM tumors as well as other tumors characterized by high vascular expression of IGFBP7 such as colon cancer (Umeda *et al.*, 1998; van Beijnum *et al.*, 2006).

MATERIALS AND METHODS

Reagents and antibodies

TGF-β1 recombinant protein, TGF-β1 polyclonal antibody (AF-101-NA) and pan-TGF-β neutralizing monoclonal antibody (1D11) were purchased from R&D System (Minneapolis, MN, USA). The TGF-β type I receptor (ALK5) inhibitor, SB431542 was purchased from Sigma (Saint-Louis, Missouri, USA). Primary antibodies for Western Blot included mouse monoclonal antibodies to Smad-2 (BD Biosciences, Bedford, MA, USA) and to actin (Chemicon, Temecula, CA, USA), polyclonal antibody to P-Smad-2,

P-Smad-1/-5 and Smad-5 (Cell Signaling, Beverly, MA, USA), and polyclonal antibody to IGFBP7 (R&D System).

Cell cultures

Human brain endothelial cells (HBEC) were obtained from small intracortical microvessels (20-112 μm) harvested from temporal cortex from patients treated surgically for idiopathic epilepsy as previously described (Stanimirovic *et al.*, 1996). Tissues were obtained with approval from the Office of Research Ethics Boards. HBEC were grown at 37°C in media containing Earle's salts, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4.35 g/L sodium bicarbonate, and 3 mM L-glutamine, 10% fetal bovine serum (FBS), 5% human serum, 20% of media conditioned by murine melanoma cells, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, and 10 μg/mL endothelial cell growth supplement. HBEC cultures were routinely characterized for purity and the expression of endothelial cell markers (Stanimirovic *et al.*, 1996). Passages 4-6 were used in this study.

The human glioma cell lines U87MG and T98G were established from surgically removed type III glioma/GBM and GBM, respectively, and were both obtained from the American Type Culture Collection. U87MG cells were grown at 37°C in Dulbecco's Modified Eagle's medium (D-MEM) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B with 0.085 g/L NaCl and 10% heat-inactivated FBS (HyClone, Logan, Utah) in humidified atmosphere of 5% CO_2/95% air. T98G cells were cultured in Minimum Essential Medium Eagle (MEM) (Sigma) supplemented with 10% FBS. Media for both cell lines were changed every 2-3 days.

Preparation of U87MG- and T98G- conditioned media

U87MG cells and T98G cells ($5x10^4$cells/mL) were plated in poly-L-lysine (25 μg/mL) pre-coated and non-coated 75 cm^2 Falcon tissue culture flasks, respectively (Becton Dickinson Labware, Franklin Lakes, NJ, USA), and incubated at 37°C in growth medium. Four days after plating, U87MG and T98G growth media were replaced with serum-free D-MEM and MEM, respectively. Serum-free media were collected after 3 days and filtered (Millex-GV sterilizing filter membrane, 0.22 μm).

ELISA

The levels of secreted human TGF-β1, TGF-β2 and TGF-β3 in serum-free CM from U87MG cells were determined using 'DuoSet® ELISA Development System' kits (R&D System) according to manufacturer's protocol.

Cell treatments

HBEC grown in 35 mm dishes were washed with D-MEM and growth medium was replaced by D-MEM alone or supplemented with different concentration of TGF-β1 recombinant protein, U87MG-CM either inactivated by boiling at 95°C for 10 minutes or fresh, in the absence or presence of a pan-TGF-β neutralizing antibody (1D11), an ALK5 inhibitor (SB431542) or a TGF-β1 polyclonal antibody for various periods of time. The treatments were refreshed at day 3. The expression of IGFBP7 was evaluated by real-time Q-PCR and Western Blot analyses.

Quantitative real-time Q-PCR

Total RNA from either untreated or treated HBEC was extracted using TRIzol Reagent (Gibco BRL, Gaithersburg, MD) and reverse-transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada). Quantitative Q-PCR reactions were performed with iTaq™ SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada) using an ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Primers used for real-time Q-PCR were designed according to sequences in GenBank (Table 1). The thermal PCR conditions were 15 seconds denaturation at 95°C and 1 minute annealing-extension at 60°C for 40 cycles. Fluorescence was detected at the end of each 60°C phase. The transcript of IGFBP7 was normalized to the transcript levels of the housekeeping gene β-actin.

Table 1

Primers designed to amplify selected genes by Q-PCR

Gene	Sense primers for Q-PCR	Antisense primers for Q-PCR
IGFBP7	5'-GCGAGCAAGGTCCTTCCATA-3'	5'-GGGATTCCGATGACCTCACA-3'
β-actin	5'-TGTCCACCTTCCAGCAGATGT-3'	5'-AGTCCGCCTAGAAGCATTTGC-3'

Each set of forward and reverse primers was designed from NCBI published sequences using the Primer Express Software v2.0.

Western Blot

HBEC were lysed in RIPA buffer [1% IGEPAL, 0.5% deoxycholic acid, 0.1% SDS, protease inhibitor capsule (Sigma)] and conditioned media (CM) were collected. The cell lysates were centrifuged at 20,000 X g for 10 minutes at 4°C and protein content in the supernatant was measured using BCA protein assay (Pierce, Rockford, USA). For detection of Smad proteins, nuclear extracts were isolated using a Nuclear Extraction Kit (Panomics, Redwood City, CA, USA) as described by manufacturer's protocol. 10-20 μg of total protein/sample or equal volumes of CM were subjected to 12% SDS-PAGE; proteins were then transferred to Immobilon membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in TBST [20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, and 0.1% Tween 20] for 1 h at room temperature (RT) and incubated with the primary antibodies overnight at 4°C. After washing with TBST, blots were probed with horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG, anti-rabbit IgG or anti-goat IgG) (Sigma) for 1 h at RT. Immunoblots were visualized using Amersham Biosciences enhanced chemiluminescence detection system (Piscataway, New Jersey, USA).

Capillary-like tube (CLT) formation

In vitro angiogenesis was assessed by endothelial tube formation in growth factor reduced Matrigel™ (BD Biosciences). 24-well plates were coated with 300 μl of diluted Matrigel™ solution and incubated at 37°C for at least an hour to allow the Matrigel™ solution to polymerize. HBEC (40,000 cells) were harvested and suspended in one of the following: 500 μL D-MEM alone, D-MEM supplemented with 10 nM, 50 nM or 200 nM

of recombinant IGFBP7, serum-free U87MG-CM alone or supplemented with 10 μM of SB431542. HBEC were then plated on top of the solidified Matrigel™ solution and maintained at 37°C. Endothelial tube formation was digitally photographed under a phase contrast Olympus 1X50 microscope (Olympus U-CMT) at 4x magnification, 24 h after treatment. Captured images were analysed using Northern Eclipse v.5.0 software. Images were thresholded, converted to binary images, and skeletonized. The total length of the CLT networks and the number of nodes (branching points) formed by HBEC were quantified. Experiments were performed with at least three different HBEC isolations.

Statistical analysis

Results are expressed as mean ± s.e.m. The unpaired t test was used for single comparisons of groups with equal variance and normal distribution. ANOVA followed by Newman-Keuls post-test was used to compare multiple data to each group.

SUPPLEMENTARY MATERIALS AND METHODS

DNA methylation

DNA methylation was assessed using the bisulfite PCR method (Deb-Rinker *et al.*, 2005). HBEC were exposed to D-MEM or U87MG-CM for 6 days. Total genomic DNA was isolated from each sample using an adapted protocol from Miller et al. (Miller *et al.*, 1988). The bisulfite modification was then carried out with the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to manufacturer's protocol to detect cytosine methylation. Primer pairs IGFBP7-A to D (Supplementary Table 1) were designed using Methprimer (http://www.urogene.org/methprimer) (Chen *et*

al., 2007). Bisulfite-modified DNA was then subjected to PCR amplification, resulting in PCR products of 176 bp (-941 to -766; primer pair IGFBP7-A), 213 bp (-789 to -577; primer pair IGFBP7-B), 400 bp (+73 to +472; primer pair IGFBP7-C) and 186 bp (+464 to +649; primer pair IGFBP7-D) covering 5, 6, 45 and 19 CpG sites within the promoter and/or exon 1/intro 1 of IGFBP7, respectively. The thermal PCR conditions were: 95°C for 10 minutes, 35 cycles of 95°C for 1 minute, 55-58°C for 1 minute and 72°C for 1 minute, followed by an extension at 72°C for 10 minutes. The PCR products were then cloned using the TA Cloning Kit (Invitrogen). The plasmid DNA isolated from ten clones of each PCR product was sequenced and the CpG sites were analysed for methylation.

Production of recombinant IGFBP7

The full length IGFBP7 cDNA (GeneBank BC017201) in the vector pOTB7 was purchased from ATCC. Using the plasmid IGFBP7/pOTB7 as template, IGFBP7 cDNA fragment was amplified by PCR using forward primer 5'-TCGAATTCCCGCCATGGAGCGGCCGTCG-3' and reverse primer 5'-TAGGGATCCTAGCTCGGCACCTTCACCT-3'. The PCR product was then digested with EcoR1 and BamH1 and inserted into a vector pTT5SH8Q2 (in frame with Streptag-II and polyhistidine epitopes) to yield IGFBP7/pTT5SH8Q2 plasmid and produced by transient transfection into human embryonic kidney 293 cell line stably expressing Epstein-Barr virus Nuclear Antigen-1 (EBNA1) as previously described (Durocher *et al.*, 2002). Briefly, 500 mL of 293-EBNA1 cells (clone 6E; Y.D., unpublished) at a density of 1 million cells/mL were transfected with 475 μg of IGFBP7/pTT5SH8Q2 and 25 μg of

GFP/pTT (pTT vector encoding the Green Fluorescent Protein) control plasmid using 1.5 mg of 25 kDa linear polyethylenimine (PEI) (ratio plasmid/PEI of 1:3). The culture was harvested 5 days after transfection, and the medium was clarified by centrifugation at 3500 x g for 10 minutes and filtered through 0.22-μm membrane. Clarified culture medium was loaded on a cobalt-loaded Fractogel EMD chelate gravity flow column. The column was washed with 10 volumes of Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl and 25 mM imidazole, pH 7.0) and bound IGFBP7 was eluted by using Wash Buffer containing 300 mM imidazole. A buffer exchange for phosphate-buffered saline was performed by gel filtration on EconoPak columns (BioRad, Hercules, CA, USA), and the final purified material was sterile-filtered, aliquoted, and stored at –80 °C. Recombinant IGFBP7 was analyzed by SDS-PAGE (4-12% Bis-Tris NuPAGE gradient gel) and visualized by Coomassie blue staining. The activity of IGFBP7 was evaluated based on its ability to bind rh6Ckine/CCL21 chemokine as described by Nagakubo et al. (Nagakubo *et al.*, 2003) and shown to be similar to the activity of available IGFBP7 (R&D System).

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REFERENCES

Ahmed S, Jin X, Yagi M, Yasuda C, Sato Y, Higashi S *et al.* (2006). Identification of membrane-bound serine proteinase matriptase as processing enzyme of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/angiomodulin/mac25). *Febs J* **273:** 615-27.

Akaogi K, Okabe Y, Funahashi K, Yoshitake Y, Nishikawa K, Yasumitsu H *et al.* (1994). Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. *Biochem Biophys Res Commun* **198:** 1046-53.

Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K *et al.* (1996). Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. *Proc Natl Acad Sci USA* **93:** 8384-9.

Beaty RM, Edwards JB, Boon K, Siu IM, Conway JE, Riggins GJ. (2007). PLXDC1 (TEM7) is identified in a genome-wide expression screen of glioblastoma endothelium. *J Neurooncol* **81:** 241-8.

Blanchette F, Rivard N, Rudd P, Grondin F, Attisano L, Dubois CM. (2001). Cross-talk between the p42/p44 MAP kinase and Smad pathways in transforming growth factor beta 1-induced furin gene transactivation. *J Biol Chem* **276:** 33986-94.

Burger AM, Zhang X, Li H, Ostrowski JL, Beatty B, Venanzoni M *et al.* (1998). Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. *Oncogene* **16:** 2459-67.

Chen Y, Pacyna-Gengelbach M, Ye F, Knosel T, Lund P, Deutschmann N *et al.* (2007). Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) has potential tumour-suppressive activity in human lung cancer. *J Pathol* **211:** 431-8.

Chen YG, Massague J. (1999). Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. *J Biol Chem* **274:** 3672-7.

Constam DB, Philipp J, Malipiero UV, ten Dijke P, Schachner M, Fontana A. (1992). Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. *J Immunol* **148:** 1404-10.

Dai C, Holland EC. (2001). Glioma models. *Biochim Biophys Acta* **1551:** M19-27.

Davies DC. (2002). Blood-brain barrier breakdown in septic encephalopathy and brain tumours. *J Anat* **200:** 639-46.

de Caestecker M. (2004). The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev* **15:** 1-11.

Deb-Rinker P, Ly D, Jezierski A, Sikorska M, Walker PR. (2005). Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. *J Biol Chem* **280:** 6257-60.

Derynck R, Zhang Y, Feng XH. (1998). Smads: transcriptional activators of TGF-beta responses. *Cell* **95:** 737-40.

Dinda AK, Sarkar C, Roy S, Kharbanda K, Mathur M, Khosla AK *et al.* (1993). A transmission and scanning electron microscopic study of tumoral and peritumoral microblood vessels in human gliomas. *J Neurooncol* **16:** 149-58.

Durocher Y, Perret S, Kamen A. (2002). High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* **30:** E9.

Engel ME, McDonnell MA, Law BK, Moses HL. (1999). Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* **274:** 37413-20.

Goumans MJ, Lebrin F, Valdimarsdottir G. (2003). Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. *Trends Cardiovasc Med* **13:** 301-7.

Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo J* **21:** 1743-53.

Hata Y, Clermont A, Yamauchi T, Pierce EA, Suzuma I, Kagokawa H *et al.* (2000). Retinal expression, regulation, and functional bioactivity of prostacyclin-stimulating factor. *J Clin Invest* **106:** 541-50.

Hirano A, Matsui T. (1975). Vascular structures in brain tumors. *Hum Pathol* **6:** 611-21.

Hwa V, Oh Y, Rosenfeld RG. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev* **20:** 761-87.

Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD *et al.* (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **62:** 65-74.

Keeton MR, Curriden SA, van Zonneveld AJ, Loskutoff DJ. (1991). Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. *J Biol Chem* **266:** 23048-52.

Kishibe J, Yamada S, Okada Y, Sato J, Ito A, Miyazaki K *et al.* (2000). Structural requirements of heparan sulfate for the binding to the tumor-derived adhesion factor/angiomodulin that induces cord-like structures to ECV-304 human carcinoma cells. *J Biol Chem* **275:** 15321-9.
Kleihues P, Soylemezoglu F, Schauble B, Scheithauer BW, Burger PC. (1995). Histopathology, classification, and grading of gliomas. *Glia* **15:** 211-21.

Kniesel U, Wolburg H. (2000). Tight junctions of the blood-brain barrier. *Cell Mol Neurobiol* **20:** 57-76.

Komatsu S, Okazaki Y, Tateno M, Kawai J, Konno H, Kusakabe M *et al.* (2000). Methylation and downregulated expression of mac25/insulin-like growth factor binding protein-7 is associated with liver tumorigenesis in SV40T/t antigen transgenic mice, screened by restriction landmark genomic scanning for methylation (RLGS-M). *Biochem Biophys Res Commun* **267:** 109-17.

Kwak HJ, Park MJ, Cho H, Park CM, Moon SI, Lee HC *et al.* (2006). Transforming growth factor-beta1 induces tissue inhibitor of metalloproteinase-1 expression via activation of extracellular signal-regulated kinase and Sp1 in human fibrosarcoma cells. *Mol Cancer Res* **4:** 209-20.

Lebrin F, Deckers M, Bertolino P, Ten Dijke P. (2005). TGF-beta receptor function in the endothelium. *Cardiovasc Res* **65:** 599-608.

Lin J, Lai M, Huang Q, Ma Y, Cui J, Ruan W. (2007). Methylation patterns of IGFBP7 in colon cancer cell lines are associated with levels of gene expression. *J Pathol* **212:** 83-90.

Long DM. (1970). Capillary ultrastructure and the blood-brain barrier in human malignant brain tumors. *J Neurosurg* **32:** 127-44.

Louis DN. (2006). Molecular pathology of malignant gliomas. *Annu Rev Pathol* **1:** 97-117.

Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y *et al.* (2004). Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* **165:** 601-8.

Massague J. (1998). TGF-beta signal transduction. *Annu Rev Biochem* **67:** 753-91.

Miller SA, Dykes DD, Polesky HF. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16:** 1215.

Moreno MJ, Ball M, Andrade MF, McDermid A, Stanimirovic DB. (2006). Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells. *Glia* **53:** 845-57.

Murphy M, Pykett MJ, Harnish P, Zang KD, George DL. (1993). Identification and characterization of genes differentially expressed in meningiomas. *Cell Growth Differ* **4:** 715-22.

Mutaguchi K, Yasumoto H, Mita K, Matsubara A, Shiina H, Igawa M *et al.* (2003). Restoration of insulin-like growth factor binding protein-related protein 1 has a tumor-suppressive activity through induction of apoptosis in human prostate cancer. *Cancer Res* **63:** 7717-23.

Nagakubo D, Murai T, Tanaka T, Usui T, Matsumoto M, Sekiguchi K *et al.* (2003). A high endothelial venule secretory protein, mac25/angiomodulin, interacts with multiple high endothelial venule-associated molecules including chemokines. *J Immunol* **171:** 553-61.

Oh Y, Nagalla SR, Yamanaka Y, Kim HS, Wilson E, Rosenfeld RG. (1996). Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II. *J Biol Chem* **271:** 30322-5.

Oldendorf WH, Cornford ME, Brown WJ. (1977). The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Ann Neurol* **1:** 409-17.

Olofsson A, Miyazono K, Kanzaki T, Colosetti P, Engstrom U, Heldin CH. (1992). Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. *J Biol Chem* **267:** 19482-8.

Pen A, Moreno MJ, Martin J, Stanimirovic DB. (2007). Molecular markers of extracellular matrix remodeling in glioblastoma vessels: microarray study of laser-captured glioblastoma vessels. *Glia* **55:** 559-72.

Pepper MS. (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* **8:** 21-43.

Poncelet AC, Schnaper HW. (1998). Regulation of human mesangial cell collagen expression by transforming growth factor-beta1. *Am J Physiol* **275:** F458-66.

Ramsauer M, D'Amore PA. (2007). Contextual role for angiopoietins and TGFbeta1 in blood vessel stabilization. *J Cell Sci* **120:** 1810-7.

Reed MJ, Vernon RB, Abrass IB, Sage EH. (1994). TGF-beta 1 induces the expression of type I collagen and SPARC, and enhances contraction of collagen gels, by fibroblasts from young and aged donors. *J Cell Physiol* **158:** 169-79.

Ruan W, Xu E, Xu F, Ma Y, Deng H, Huang Q *et al.* (2007). IGFBP7 plays a potential tumor suppressor role in colorectal carcinogenesis. *Cancer Biol Ther* **6:** 354-9.

Sato J, Hasegawa S, Akaogi K, Yasumitsu H, Yamada S, Sugahara K *et al.* (1999). Identification of cell-binding site of angiomodulin (AGM/TAF/Mac25) that interacts with heparan sulfates on cell surface. *J Cell Biochem* **75:** 187-95.

Sprenger CC, Damon SE, Hwa V, Rosenfeld RG, Plymate SR. (1999). Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. *Cancer Res* **59:** 2370-5.

St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E *et al.* (2000). Genes expressed in human tumor endothelium. *Science* **289:** 1197-202.

Stanimirovic D, Morley P, Ball R, Hamel E, Mealing G, Durkin JP. (1996). Angiotensin II-induced fluid phase endocytosis in human cerebromicrovascular endothelial cells is regulated by the inositol-phosphate signaling pathway. *J Cell Physiol* **169:** 455-67.

ten Dijke P, Hansen P, Iwata KK, Pieler C, Foulkes JG. (1988). Identification of another member of the transforming growth factor type beta gene family. *Proc Natl Acad Sci USA* **85:** 4715-9.

Umeda F, Ono Y, Sekiguchi N, Hashimoto T, Masakado M, Nakamura K *et al.* (1998). Increased mRNA expression of a novel prostacyclin-stimulating factor in human colon cancer. *J Gastroenterol* **33:** 213-7.

Vajkoczy P, Goldbrunner R, Farhadi M, Vince G, Schilling L, Tonn JC *et al.* (1999). Glioma cell migration is associated with glioma-induced angiogenesis in vivo. *Int J Dev Neurosci* **17:** 557-63.

Vajkoczy P, Menger MD. (2000). Vascular microenvironment in gliomas. *J Neurooncol* **50:** 99-108.

van Beijnum JR, Dings RP, van der Linden E, Zwaans BM, Ramaekers FC, Mayo KH *et al.* (2006). Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood* **108:** 2339-48.

Vaupel P, Thews O, Kelleher DK, Hoeckel M. (1998). Oxygenation of human tumors: the Mainz experience. *Strahlenther Onkol* **174 Suppl 4:** 6-12.

Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. (2008). Oncogenic BRAF Induces Senescence and Apoptosis through Pathways Mediated by the Secreted Protein IGFBP7. *Cell* **132:** 363-74.

Watabe T, Nishihara A, Mishima K, Yamashita J, Shimizu K, Miyazawa K *et al.* (2003). TGF-beta receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. *J Cell Biol* **163:** 1303-11.

Weller M, Malipiero U, Groscurth P, Fontana A. (1995). T cell apoptosis induced by interleukin-2 deprivation or transforming growth factor-beta 2: modulation by the phosphatase inhibitors okadaic acid and calyculin A. *Exp Cell Res* **221:** 395-403.

Wick W, Platten M, Weller M. (2001). Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta. *J Neurooncol* **53:** 177-85.

Wilson HM, Birnbaum RS, Poot M, Quinn LS, Swisshelm K. (2002). Insulin-like growth factor binding protein-related protein 1 inhibits proliferation of MCF-7 breast cancer cells via a senescence-like mechanism. *Cell Growth Differ* **13:** 205-13.

Yakymovych I, Ten Dijke P, Heldin CH, Souchelnytskyi S. (2001). Regulation of Smad signaling by protein kinase C. *Faseb J* **15:** 553-5.

Yamada N, Kato M, Yamashita H, Nister M, Miyazono K, Heldin CH *et al.* (1995). Enhanced expression of transforming growth factor-beta and its type-I and type-II receptors in human glioblastoma. *Int J Cancer* **62:** 386-92.

Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H. (1994). Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. *Biochem J* **303 (Pt 2):** 591-8.

PREFACE TO CHAPTER 4

In Chapter 3, evidence was provided indicating that IGFBP7 is induced in HBEC by GBM secreted TGF-βs through activation of ALK5/Smad-2 pathway. The same pathway was involved in GBM- and IGFBP7- stimulated angiogenic processes. The following chapter examines the effects of IGFBP7 on tumor cell growth. Several prior studies have suggested a tumor suppressive role for IGFBP7 (Chen *et al.*, 2007; Mutaguchi *et al.*, 2003; Ruan *et al.*, 2007; Sato *et al.*, 2007; Wajapeyee *et al.*, 2008; Wilson *et al.*, 2002). To examine the potential anti-tumorigenic effect of IGFBP7 in GBM, the ability of IGFBP7 protein to affect the growth of a GBM cell line, U87MG, was assessed *in vitro* using monolayer cell culture and soft agar assays, and *in vivo*, using an experimental glioma model consisting of U87MG cells grafted on chicken chorioallantoic membrane (CAM) of fertilized eggs. Histological and immunofluorescence analyses of glioma tumors grown on CAMs were performed. *In vivo* studies described in this chapter are preliminary data. To complete this study, additional CAM assays with higher number of eggs per group (untreated and IGFBP7-treated) will be performed at a later date.

CHAPTER 4

Insulin-like growth factor binding protein 7 exhibits tumor suppressive and pericyte-recruiting properties in glioblastoma tumors

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Running title: IGFBP7 has a tumor suppressor effect in GBM

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Contribution of the co-authors:

- Chick chorioallantoic membrane (CAM) tumor assays were conducted by Mrs. Jaqueline Slinn.
- Dr. Yves Durocher provided guidance in the production of the recombinant IGFBP7 protein that was used for the *in vitro* studies and produced the recombinant IGFBP7 protein for the CAM tumor assays.
- Dr. Maria Moreno and Dr. Danica Stanimirovic provided scientific guidance and supervision throughout this research work and edited this manuscript.

ABSTRACT

Insulin-like growth factor binding protein 7 (IGFBP7) is down-regulated in several solid cancers. A growing body of evidence suggests that IGFBP7 acts as a tumor suppressor gene through mechanisms involving senescence and apoptotic pathways. The tumor suppressor effect of IGFBP7 in glioblastoma multiforme (GBM) tumors was examined in this study using a human GBM cell line, U87MG. Exogenous IGFBP7 (1.8 nM-360 nM) moderately inhibited (~20%) U87MG cell proliferation in monolayer culture, whereas 20nM and 100 nM of IGFBP7 significantly reduced (~70% and ~ 75%, respectively) U87MG colony size in soft agar under anchorage-independent conditions. The inhibitory effect of IGFBP7 on U87MG cell growth was further assessed *in vivo* using human U87MG cells grafted on the chick chorioallantoic membrane (CAM). In this model, U87MG cells formed solid and highly vascularized tumors within 7 days. Tumors treated with 500 nM of IGFBP7 were reduced in size (~40%) compared to control tumors. Immunofluorescence analyses showed that IGFBP7-treated tumor vessels were clustered, unevenly distributed and associated with higher number of α-SMA positive cells compared to untreated tumors, suggesting that IGFBP7 may participate in mural cell (smooth muscle cell/pericyte) recruitment or differentiation, thereby contributing to vessel stabilization. This study suggests that IGFBP7 suppresses GBM tumor cell growth and promotes stabilization of tumor vessels.

INTRODUCTION

Glioblastoma multiforme (World Health Organization-WHO grade IV; GBM) is the most malignant tumor of the central nervous system with the mean survival time of approximately one year from diagnosis (Vajkoczy and Menger, 2000). GBM is a highly invasive tumor characterized by regional necrosis and extensive microvascular proliferation (Louis, 2006). Molecular and genetic studies have identified several genes implicated in glioma genesis including activation of epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGF), and inactivation of tumor suppressor genes including phosphatase/tensin homolog protein (PTEN) and p53 (Bansal *et al.*, 2006). Several growth factor-mediated signaling pathways, including those stimulated by the insulin-like growth factors (IGFs), have been shown to play important roles in glioma progression (Bansal *et al.*, 2006; Trojan *et al.*, 2007).

IGF axis is composed of IGF-I and -II, IGF receptor I and II, insulin-like growth factor binding proteins (IGFBP)-1 to -6, IGFBP proteases and IGFBP-related proteins (IGFBP-rP1 to IGFBP-rP10) (Hwa *et al.*, 1999). IGFs regulate several processes including cell growth, differentiation and apoptosis (Burger *et al.*, 2005). Their actions are modulated by IGFBPs, which have a high affinity for IGFs, and by IGFBP-rPs, which bind IGFs with low affinity (Hwa *et al.*, 1999). IGFBP-rP1, also named insulin-like growth factor binding protein 7 (IGFBP7) is a ~30 kDa secreted protein residing on chromosome 4q12 (Swisshelm *et al.*, 1995). IGFBP7 shares approximately 30% amino acids homology with other members of the IGFBP family, but its low affinity for IGF and high affinity for insulin (Oh *et al.*, 1996) suggest that its actions are IGF-independent.

IGFBP7 up-regulation has been reported in several cancers including colon (Umeda *et al.*, 1998) and acute lymphoblastic leukemia (How *et al.*, 1999). IGFBP7 accumulates in vessels of various tumors (Akaogi *et al.*, 1996; St Croix *et al.*, 2000) including GBM (Pen *et al.*, 2007), where it has been proposed to participate in late phase angiogenesis (Pen *et al.*, 2008). Whereas the early phase angiogenesis involves endothelial cell proliferation and migration, the late phase angiogenesis is characterized by reconstitution of vascular basement membrane, establishment of cell-cell junctions and pericyte recruitment and integration into the vessel wall (Jain, 2003; Pepper, 1997). In addition to its suggested role in angiogenesis, a growing body of evidence indicates that IGFBP7 may also act as a tumor suppressor gene. IGFBP7 down-regulation has been observed in some cancers, including prostate (Hwa *et al.*, 1998; Sprenger *et al.*, 1999), breast (Landberg *et al.*, 2001), liver (Komatsu *et al.*, 2000), meningioma (Murphy *et al.*, 1993), and osteosarcoma (Kato *et al.*, 1996). Down-regulation of IGFBP7 has been associated with favourable prognosis (Burger *et al.*, 1998) and over-expression of IGFBP7 with reduction of tumor cell growth (Landberg *et al.*, 2001; Wilson *et al.*, 2002) through apoptotic or cellular senescence-like mechanisms (Mutaguchi *et al.*, 2003; Sprenger *et al.*, 2002; Wajapeyee *et al.*, 2008; Wilson *et al.*, 2002). Hypermethylation of CpG islands in IGFBP7 promoter/exon1/intron1 regions has also been shown to suppress the transcription of IGFBP7, contributing to cancer progression (Chen *et al.*, 2007; Komatsu *et al.*, 2000; Lin *et al.*, 2007).

This study analysed the effects of IGFBP7 on GBM cell grown in culture, soft agar or chicken chorioallantoic membranes (CAMs). IGFBP7 was found to inhibit GBM

cell growth and tumor formation both *in vitro* and *in vivo*, and to affect tumor vessel organization by inducing SMC/pericyte recruitment and/or differentiation.

RESULTS

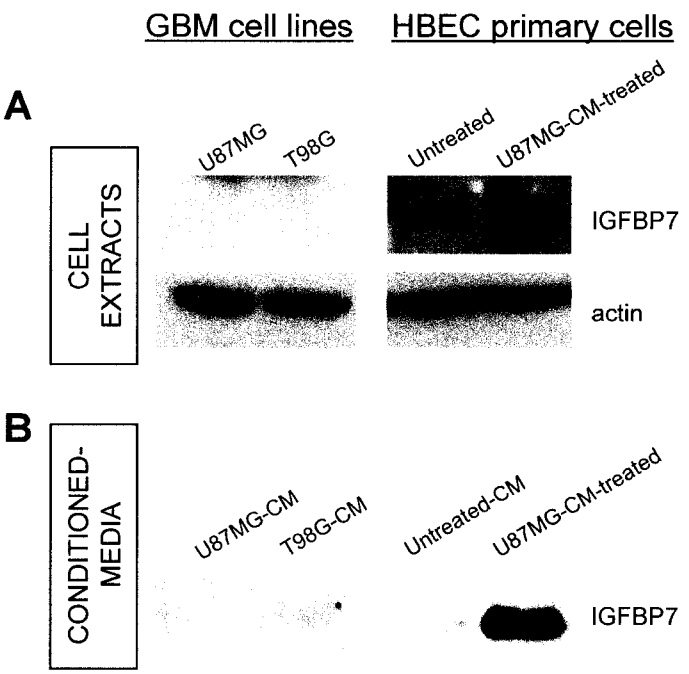
IGFBP7 expression in GBM cells

To examine whether GBM cells express IGFBP7, IGFBP7 protein levels were analysed in two GBM cell lines (U87MG and T98G) by Western Blot. IGFBP7 protein was not detected in either U87MG or T98G cell lysates (Fig. 1A; upper panel), whereas a weak IGFBP7 expression was observed in their respective secreted fractions (Fig. 1B). In contrast, high IGFBP7 protein expression was detected in HBEC exposed to U87MG-conditioned-media (-CM) in both cell lysate (Fig. 1A; upper panel) and secreted fraction (Fig. 1B) (Pen *et al.*, 2008). These results are consistent with the observed lack of IGFBP7 immunoreactivity in brain parenchymal cells and its high expression in vessels of GBM tumors *in vivo* (Pen *et al.*, 2007).

Figure 1

IGFBP7 expression in GBM cells

IGFBP7 protein expression was determined by Western Blot in two glioblastoma cell lines, U87MG and T98G, and in human brain endothelial cells (HBEC) exposed to U87MG-conditioned media (-CM) for 6 days, in both total cell lysates (A) and secreted media (B). Actin was used as loading control (A).



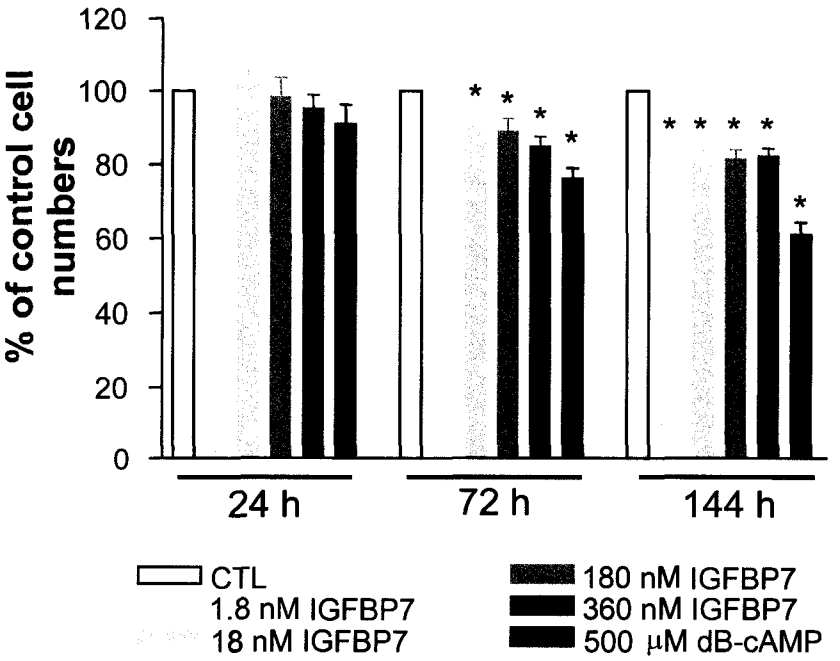
IGFBP7 inhibits U87MG cell proliferation

The effect of increasing concentrations of recombinant IGFBP7 on U87MG anchorage-dependent proliferation was measured using CyQuant Proliferation assay. Exposure of U87MG cells to IGFBP7 for 24 h did not significantly affect their proliferation (Fig. 2). The number of U87MG cells decreased (~10% and ~20%) after incubating with 18 nM-180 nM and 360 nM of IGFBP7, respectively, for 72 h. A similar ~20% reduction in U87MG cell numbers was observed when cells were incubated with either 1.8 nM or 360 nM of IGFBP7 for 144 h (Fig. 2). Consistent with previous data (Moreno *et al.*, 2006), U87MG cells exposed to 500 μM of dibutyryl cyclic adenosine monophosphate (dB-cAMP) for 144 h showed ~40% reduction in cell numbers (Fig. 2). These results suggest that IGFBP7 moderately decreases U87MG cell growth under anchorage-dependent conditions.

Figure 2

Effect of IGFBP7 on U87MG cell proliferation

U87MG cells were treated with D-MEM alone or with 1.8 nM-360 nM of IGFBP7 or 500 μM of dB-cAMP for 24 h, 72 h or 144 h. Cell numbers were counted using CyQuant proliferation assay kit as described in Materials and Methods. Results are expressed as percent of control cell numbers. Each bar represents the mean ± s.e.m. of three independent experiments done in triplicate. Asterisks indicate a significant difference ($P < 0.05$; ANOVA followed by Newman Keuls' post-test) between untreated and IGFBP7-treated cells.



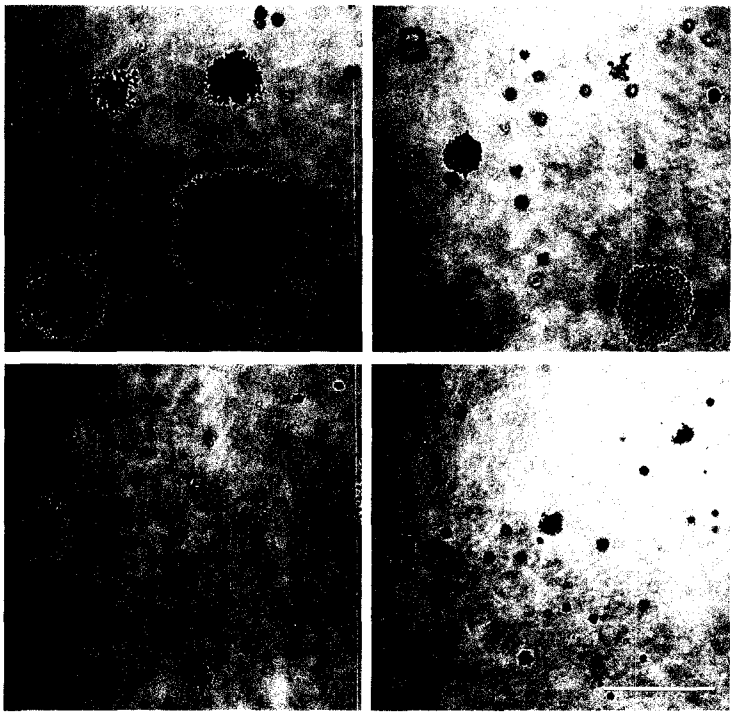
IGFBP7 suppresses anchorage-independent U87MG cell growth

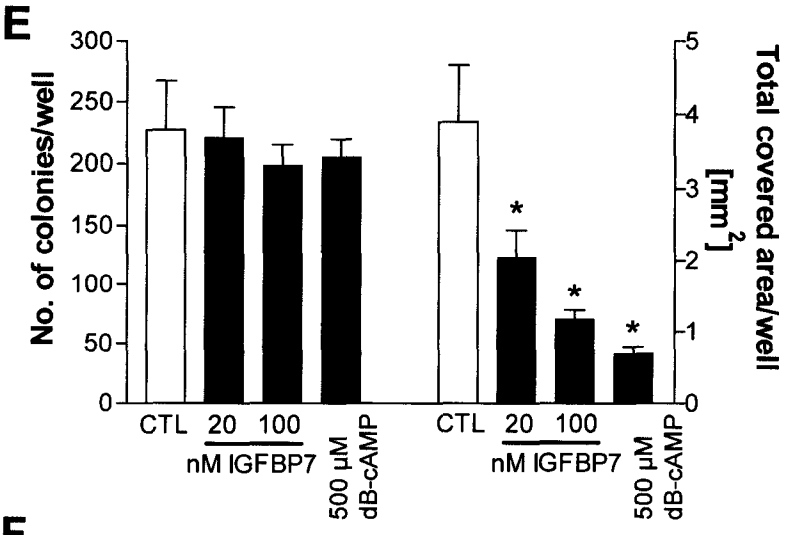
Anchorage-independent growth is a hallmark of cancer cell malignancy (Chiarugi and Fiaschi, 2007). The ability of U87MG cells to grow in an anchorage-independent fashion was demonstrated previously (Moreno *et al.*, 2006). The effect of IGFBP7 on U87MG cell anchorage-independent growth was determined using soft agar assays. The size of U87MG colonies grown in the presence of 20 nM and 100 nM of IGFBP7 or 500 μM of dB-cAMP was significantly reduced compared to untreated cells (Fig. 3A-D). Quantitative analyses of microphotograph images indicated that, although the total number of colonies per well was not significantly different among untreated and IGFBP7- or dB-cAMP- treated U87MG cells (Fig. 3E; left panel), U87MG colony size (total covered area) was markedly reduced after treatments with 20 nM (~50%) and 100 nM (~70%) of IGFBP7 as well as with 500 μM dB-cAMP (~80%) (Fig. 3E; right panel). Cell viability measured with Alamar Blue™ at the end of the experiment also showed a reduction in the number of viable U87MG cells after treatments with 20 nM (~70%) and 100 nM (~75%) of IGFBP7. These results indicate that IGFBP7 strongly suppresses the anchorage-independent growth of U87MG cells.

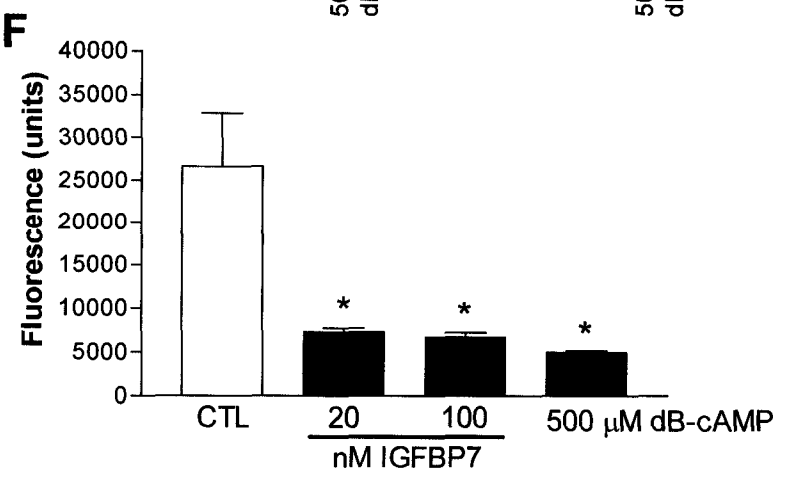
Figure 3

Effect of IGFBP7 on U87MG colony formation in semi-solid agar

U87MG cells were grown in semi-solid agar in the absence (A) or presence of 20 nM (B) or 100 nM of IGFBP7 (C) or 500 μM of dB-cAMP (D) for 21 days. Scale bar = 500 μm. The number and the total surface area covered by U87MG colonies were quantified from phase contrast images (6 fields/well) using Northern Eclipse v.5.0. software (E). Number of viable U87MG cells was assessed by Alamar Blue™ staining (F). Ear bar represents the mean ± s.e.m. of three experiments done in triplicates. Asterisks indicate a significant difference ($P < 0.05$; ANOVA followed by Newman Keuls' post-test, n=3) between untreated and IGFBP7- treated cells.







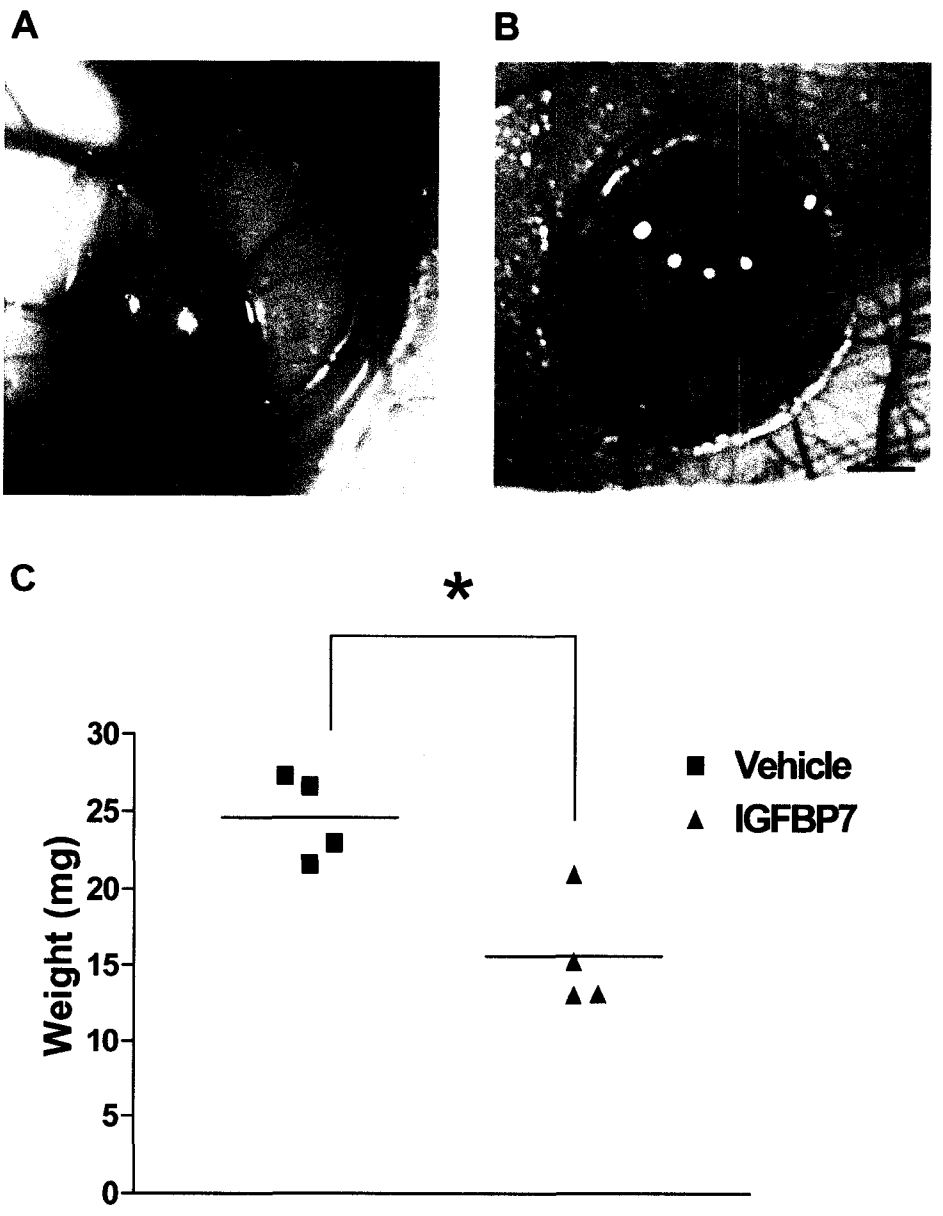
IGFBP7 reduces U87MG tumor growth in CAM

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane commonly used to study angiogenesis *in vivo* (Ribatti *et al.*, 2001). Tumor cells can also be grafted on the CAM to study tumor growth and angiogenesis (Knighton *et al.*, 1977). Several molecules have been demonstrated to be anti-angiogenic and/or anti-tumorigenic by their ability to reduce the formation of vessels and/or tumor masses using CAM assays (Pfeifer *et al.*, 2000; van der Schaft *et al.*, 2002). U87MG cells grafted on the CAM have been shown to be a reliable model to study GBM progression and growth because it recapitulates key features of human glioma (Hagedorn *et al.*, 2005). Hagedorn and colleagues (2005) have shown that 2 days after implantation of U87MG cells in CAM, a well-defined solid tumor is formed, which become highly vascularized and associated with hemorrhage, necrosis and oedema in the following days. In this study, CAM tumor model was used to assess the effect of IGFBP7 on U87MG cell growth *in vivo*. 7 days after U87MG cell seeding on CAMs, solid and highly vascularized tumors were formed in each egg (Fig. 4A). Tumors treated with 500 nM of IGFBP7 were visually smaller than control tumors (Fig. 4A-B). Treatment with 500 nM of IGFBP7 (n=4) reduced tumor weight by ~40% compared to DMSO-treated CAMs (n=4) (15.6±1.8 mg and 24.6±1.4 mg, respectively) (Fig. 4C). These results suggest that IGFBP7 reduces GBM tumor growth in experimental CAM model.

Figure 4

Effects of IGFBP7 on U87MG tumor growth in CAM assay

Microphotographs showing U87MG tumors growing on CAM 7 days after inoculation treated with either DMSO (vehicle) (A) or 500 nM of IGFBP7 (B). Graph representing the weight of individual tumors in control group (vehicle) (n=4) and in IGFBP7-treated group (n=4) (C). Scale bar = 2 mm. Asterisk indicates a significant difference ($P < 0.05$; Unpaired Student's t-test) between untreated and IGFBP7-treated group.



Vessel density and SMC/pericyte recruitment in IGFBP7-treated tumors

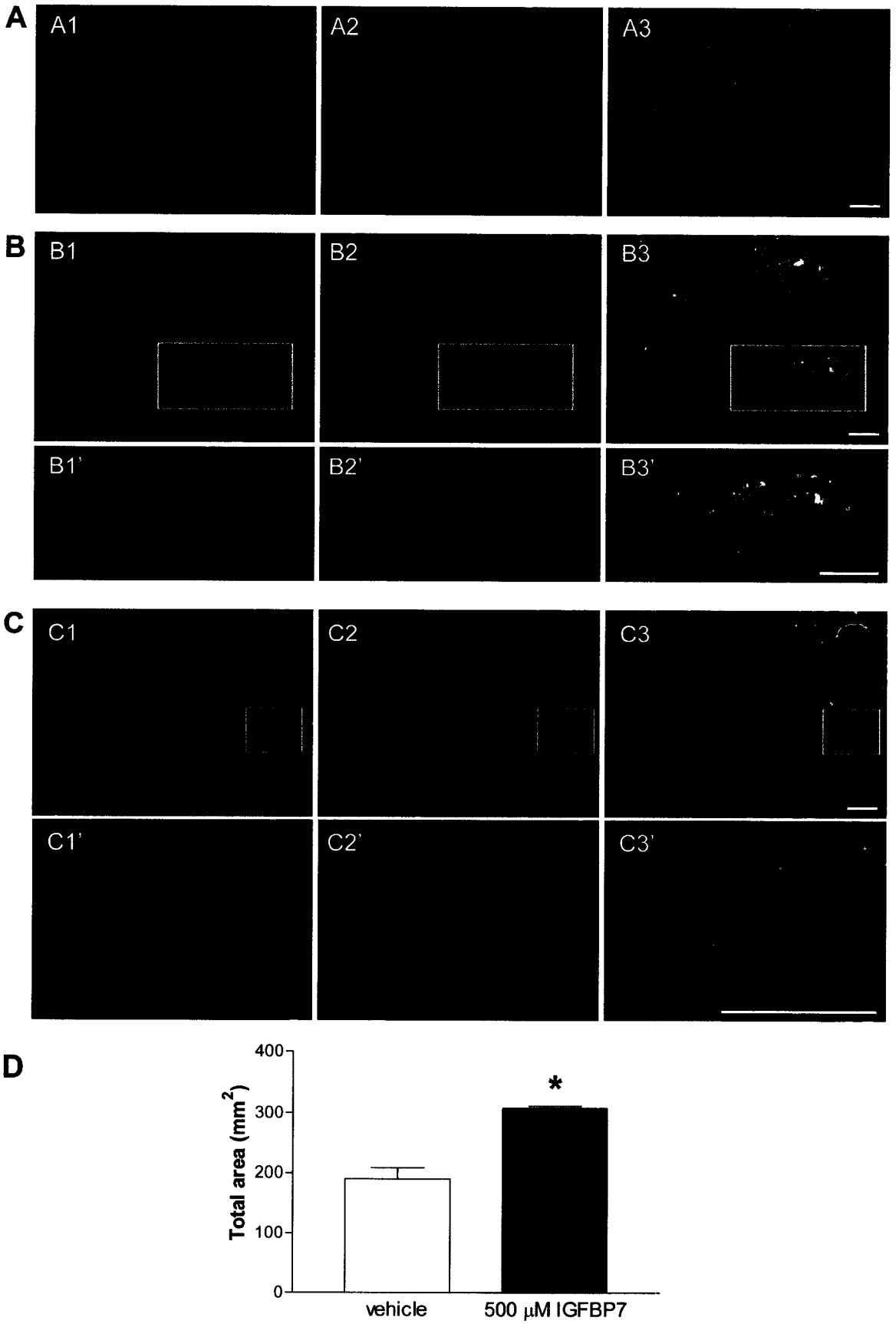
Immunofluorescence analyses of U87MG tumors grown on CAM showed that both untreated and IGFBP7-treated tumors were highly vascularized. Untreated tumors contained thin, tortuous and highly dense vessels that were evenly distributed throughout the tumor (Fig. 5A1), whereas in IGFBP7-treated tumors, vessels were thickened, and groups of lectin positive cells clustered forming a larger number of glomeruloid-like structures in the tumor than in the untreated group (Fig. 5B1-B1').

In this study, α-SMA-immunopositivity was used to identify cells referred to as mural cells (SMC and/or pericytes) in the CAM-grown tumors. Mural cells are thought to mediate vessel stabilization by increasing ECM protein deposition and by promoting endothelial cell differentiation and quiescence (Armulik *et al.*, 2005). A higher number of α-SMA positive cells (~40%) was detected in IGFBP7- treated compared to untreated tumors (Fig. 5A2-B2&C2, 5D). Since α-SMA is highly expressed in mural cells, these results suggest that IGFBP7 either recruits SMCs/pericytes into the tumor or differentiates cells into a pericyte-like phenotype. Co-localization of α-SMA and lectin staining was observed in few vessels of both untreated and IGFBP7-treated tumors (Fig. 5A3, B3-B3'). A number of vessels in IGFBP7-treated tumors were surrounded by extensive amount of SMCs/pericytes organized in multiple layers (Fig. 5C-5C').

Figure 5

Effects of IGFBP7 on tumor vasculature and α-SMA expression in U87MG tumors grown in CAM

Sections of U87MG tumors grown in CAM for 7 days in the absence (A) or presence (B-C) of 500 nM of IGFBP7 were immunostained with Sambucus Nigra lectin (green; 1) to visualize vessels and with α-SMA (red; 2) to localize mural cells (SMCs/pericytes). Overlay images are represented in A3, B3, and C3. Boxes in B1-3 and C1-3 outline areas magnified in B1-3' and C1-3'. Cellular nuclei were stained with Hoechst (blue). Scale bar = 100 μm. (D) Quantification of α-SMA-positive cells in untreated and IGFBP7-treated tumor sections. Each bar represents the mean ± s.e.m. of total area covered by α-SMA-positive cells in 3 fields/tumor section for a total of 4 tumors per group. Asterisk indicates a significant difference ($P < 0.05$; Unpaired Student's t-test) between untreated and IGFBP7-treated group.



DISCUSSION

The present study provides the first *in vitro* and *in vivo* evidence that IGFBP7 exhibits a tumor suppressive role in GBM tumors, and increases mural cell (SMCs/pericytes) recruitment/differentiation in tumor vessels.

A growing body of evidence indicates that IGFBP7 suppresses tumor growth in several cancers (Chen *et al.*, 2007; Mutaguchi *et al.*, 2003; Ruan *et al.*, 2007; Sato *et al.*, 2007; Wajapeyee *et al.*, 2008; Wilson *et al.*, 2002). While IGFBP7 has been shown to be widely distributed in normal tissues (Degeorges *et al.*, 2000; Oh *et al.*, 1996), its expression appears to be lost or reduced in cancer cells (Oh *et al.*, 1996). For instance, strong IGFBP7 expression has been described in glial cells (Degeorges *et al.*, 2000), whereas no IGFBP7 expression was found in GBM tumor parenchyma (Akaogi *et al.*, 1996; Pen *et al.*, 2007). In agreement with the lack of IGFBP7 expression in GBM cells *in situ*, this study shows that the GBM cell lines, U87MG and T98G, produce and secrete very low levels of IGFBP7 protein. However, variable expression of IGFBP7 has been reported in other GBM cell lines (Oh *et al.*, 1996; Zumkeller *et al.*, 1998).

Cell growth is regulated by dynamic interactions between the extracellular matrix (ECM), transmembrane adhesion receptors (i.e. integrins) and the actin cytoskeleton (Danen and Yamada, 2001). Cell growth on a solid substrate (integrin-mediated cell adhesion) is defined as anchorage-dependent growth. In contrast to normal cells, cancer cells acquire the capacity to grow without adhesion to a substrate, e.g. in an anchorage-independent manner (Chiarugi and Fiaschi, 2007). Study of the effects of IGFBP7 on U87MG growth under anchorage-dependent and -independent conditions indicate that IGFBP7 only moderately reduces (~20%) U87MG cell proliferation in monolayer

culture, but strongly inhibits (~70-75%) U87MG growth in soft agar. Previous literature reports describe either moderate increases (Mutaguchi *et al.*, 2003; Sprenger *et al.*, 1999; Wilson *et al.*, 2002) or no significant effect (Sato *et al.*, 2007) of IGFBP7 on cell proliferation in monolayer cultures. In agreement with our findings, several studies have demonstrated that IGFBP7 suppresses cancer cell growth in soft agar (Lin *et al.*, 2007; Mutaguchi *et al.*, 2003; Ruan *et al.*, 2007; Sato *et al.*, 2007). These results suggest that IGFBP7 may employ different signaling pathways to cause cell growth inhibition under anchorage-dependent and anchorage-independent conditions. Several signaling pathways have been implicated in IGFBP7-induced tumor growth suppression. For instance, in a BRAFV600E melanoma cell line, IGFBP7 suppresses cell growth by inducing cellular senescence and apoptosis via inhibition of MAPK/ERK and up-regulation of BNIP3L (Wajapeyee *et al.*, 2008). IGFBP7-mediated over-expression of caspase 3 (Mutaguchi *et al.*, 2003), Sox-9 (Drivdahl *et al.*, 2004), and SOD-2 (Plymate *et al.*, 2003) as well as the alteration of cycle kinetics involving cyclin A (Sprenger *et al.*, 2002) have all been shown to result in tumor cell growth inhibition.

The tumor suppressor effect of IGFBP7 on U87MG cells observed in this study was further confirmed *in vivo* using an experimental glioma model and it is consistent with other recent *in vivo* studies showing that IGFBP7 substantially suppresses melanoma, lung, bladder and colon xenograft tumor growth in mice (Chen *et al.*, 2007; Sato *et al.*, 2007; Wajapeyee *et al.*, 2008).

The anti-tumor activity of IGFBP7 *in vivo* may result from both its direct effect on GBM cells and on the tumor vasculature. Although both untreated and IGFBP7-treated tumors were highly vascularized, IGFBP7-treated tumors displayed an increased

number of lectin-positive cells forming glomeruloid-like structures that were irregularly distributed within the tumor. The morphology of these vascular structures resembles the hyperplastic microvascular glomeruloid-like vessels observed in surgically removed GBMs (Brat and Van Meir, 2001). Since we have previously shown that IGFBP7 does not induce endothelial cell proliferation, the observed hyperplastic vasculature in the experimental glioma model used in this study is likely not caused by the hypertrophic effect of IGFBP7 on endothelial cells. This phenotype may rather be triggered by the adhesive properties of IGFBP7 which may reduce the ability of endothelial cells to migrate and distribute within the tumor. This aberrant vessel architecture may partially contribute to IGFBP7-reduced tumor growth due to abnormal oxygen/nutrient delivery (Louis, 2006).

The most prominent observation in IGFBP7-treated tumors was the presence of an extensive number of α-SMA positive cells with some of them surrounding the vessels in multiple layers. Pericytes play a major role in maintaining the integrity of blood vessels (von Tell *et al.*, 2006). The interaction between endothelial cells and pericytes leading to vessel stabilization and maturation involves four major pathways: angiopoietin 1 and its receptor Tie 2, platelet-derived growth factor (PDGF)-B and its receptor PDGF beta (PDGF-β), sphingosine-1-phosphate (S1P) and its endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG-1), and transforming growth factor (TGF)-β and its receptor system (Chantrain *et al.*, 2006). These signaling pathways act on pericyte recruitment by promoting pericyte proliferation and migration toward endothelial cells (PDGF-B/PDGF-β, Ang-1/Tie-2) (von Tell et al., 2006), by differentiating mesenchymal cells into a pericyte-like phenotype (TGF-β/TGF-β

receptors) (Hirschi et al., 1998), and/or by mediating endothelial-pericyte adhesion (S1P/$S1P_1$) (Paik et al., 2004). These pathways are intimately linked via complex feedback autoregulatory loops to affect vessel stabilization (Nishishita and Lin, 2004). The high expression of α-SMA positive cells observed in IGFBP7-treated tumors suggests that IGFBP7 has the ability to recruit/differentiate SMCs/pericytes. Since TGF-β1 is known to stimulate SMC/pericyte differentiation (Hirschi et al., 1998), and we have previously demonstrated that TGF-β1 induces IGFBP7 expression in endothelial cells through TGF-β1/ALK5/Smad-2 pathway (Pen et al., 2008), IGFBP7 may be a downstream effector of TGF-β responsible for observed differentiation of cells into pericyte-like phenotype and consequent vessel stabilization. Given that other cell types such as myofibroblasts can expressed α-SMA (Ronnov-Jessen and Petersen, 1996), and that pericytes cannot be positively identified based on a single marker, additional markers including desmin, high-molecular weight melanoma-associated antigen (NG-2) or PDGF-β will need to be used to further confirm the identity of these cells (Bergers and Song, 2005).

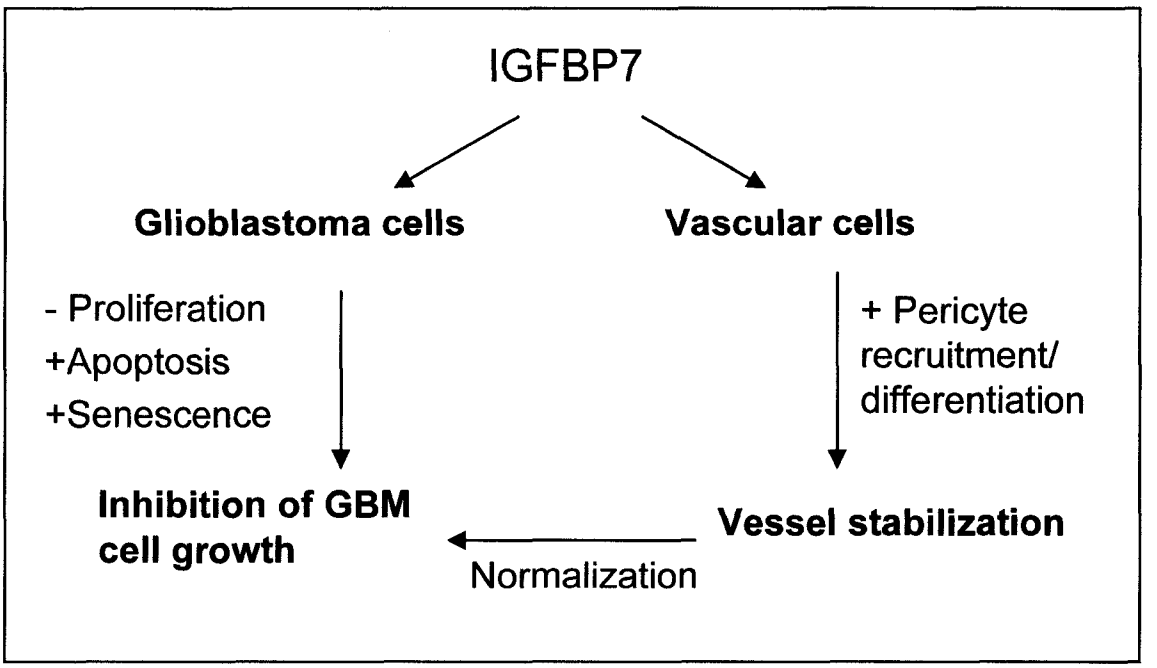
Although high expression levels of IGFBP7 in GBM vessels may imply that it promotes GBM vascularization, this study rather suggests that IGFBP7 participates in vessel stabilization and inhibits GBM tumor growth. We propose that IGFBP7 exhibits tumor suppressive effects in GBM by both reducing anchorage-independent GBM tumor cell growth and by stabilizing tumor vessels (Fig. 6). Previous studies have demonstrated that tumor vessel stabilization achieved by anti-angiogenic agents such as anti-VEGF and endostatin, can transiently 'normalize' tumor vessels (Ergun *et al.*, 2001; Tong *et al.*, 2004), resulting in improved efficacy of radiation and chemotherapy treatments

(Fukumura and Jain, 2007; Jain, 2005; Tong *et al.*, 2004). The dual effect of IGFBP7 in suppressing GBM cell growth and inducing vessel stabilization can therefore be exploited for therapeutic treatment of GBMs (Fig. 6).

Figure 6

Proposed mechanisms of IGFBP7-mediated tumor suppressor effects in GBM tumors

IGFBP7 exhibits tumor suppressive function by affecting GBM cells and vascular cells. IGFBP7 directly inhibits GBM cell growth most likely by inducing cellular apoptosis or senescence-like mechanisms and participates in SMC/pericyte recruitment/differentiation and vessel stabilization. This dual effect of IGFBP7 makes it an attractive molecule for treatment of GBMs.



MATERIALS AND METHODS

GBM cell culture

Human glioma cell lines U87MG and T98G were established from surgically removed type III glioma/GBM and GBM, respectively, and were both obtained from the American Type Culture Collection (ATCC). U87MG cells were grown at 37°C in Dulbecco's Modified Eagle's medium (D-MEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B with 0.085 g/L NaCl and 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, Utah) in humidified atmosphere of 5% CO_2/95% air. T98G cells were cultured in Minimum Essential Eagle medium (MEM) (Sigma, Oakville, ON, Canada) supplemented with 10% FBS.

Preparation of U87MG- and T98G- conditioned media

U87MG cells and T98G cells ($5x10^4$cells/ml) were plated in poly-L-lysine (25 μg/mL) pre-coated and un-coated 75 cm^2 Falcon tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ, USA), respectively, and were incubated at 37°C in growth medium. Four days after plating, U87MG and T98G growth media were replaced with serum-free D-MEM and MEM, respectively. Three days later, serum-free media were collected and filtered (Millex-GV sterilizing filter membrane, 0.22 μm).

Production of recombinant IGFBP7 protein

Recombinant IGFBP7 was produced as described previously (Pen *et al.*, 2008) and was dissolved in either PBS for *in vitro* studies or in DMSO for *in vivo* studies.

Anchorage-dependent proliferation assay

U87MG cell proliferation was assessed using CyQuant® Cell Proliferation Assay Kit (Molecular Probe, Eugene, OR, USA) as described by the manufacturer's protocol. Briefly, 3,000 U87MG cells were seeded in 96-well microplate in 150 µL of D-MEM alone or supplemented with different concentrations (1.8 nM to 360 nM) of recombinant IGFBP7 protein. D-MEM was replaced every 2 days and harvested at day 1, 3 and 6 by washing the micro-plates 3 times with Hank's buffered salt solution (HBSS). Plates were then quickly dried and stored at -80°C until analysis. Plates were thawed at room temperature (RT), 200 µL of CyQuant GR dye/lysis buffer was added to each well and plates were incubated for 5 min in the dark. Fluorescence measurements were detected at 485 nm excitation /530 nm emission using a cytofluorimeter plate reader (BioTek FL600). Fluorescence unit were converted into cell numbers using a cell reference standard curve.

Anchorage-independent growth in semi-solid agar

U87MG tumorigenicity was evaluated using growth and colony formation in semi-solid agar as previously described (Moreno *et al.*, 2006). Briefly, $1.8X10^3$ U87MG cells alone or supplemented with 20 nM or 100 nM of IGFBP7 or 500 µM of dB-cAMP were resuspended in medium containing 0.6% soft agar, and poured onto a 24-well plate previously layered with 0.6% agar. The solidified cell layer was covered by 50 µL of D-MEM alone or supplemented with 20 nM or 100 nM IGFBP7 or 500 µM of dB-cAMP. Plates were incubated at 37°C for 21 days and treatments were replaced every 3 days. Phase contrast images (6 fields/dish) were captured using a digital video camera

(Olympus U-CMT) and quantified using Northern Eclipse v.5.0 software. The number of viable cells was also measured by Alamar Blue™ as described by the manufacturer's protocol (Biosource, Invitrogen, Burlington, ON, Canada). Briefly, 40 μL of Alamar Blue™ was added to each well and fluorescence was measured at 530 nm excitation/590 nm emission at 5 min intervals over 3 hours using a cytofluorimeter plate reader (BioTek FL600). The time point at which fluorescence reached a plateau was selected for analysis and the data transposed on a bar graph.

Western Blot

For Western Blot, 20 μg of U87MG or T98G cell lysates or their respective conditioned-media (CM) were subjected to 12% SDS-PAGE. Cell lysate and CM from human brain endothelial cells (HBEC) exposed to D-MEM or U87MG-CM for 6 d were used as positive control. Samples were transferred to Immobilon membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in TBST [20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, and 0.1% Tween 20] for 1 h at RT and incubated with a goat anti-human IGFBP7 primary antibody (dilution 1:300) (R&D System, Minneapolis, MN, USA) overnight at 4°C or with a mouse anti-actin (dilution 1:10,000) (Chemicon, Temecula, CA, USA) for 1 h at RT. After washing with TBST, blots were probed with the horseradish peroxidase-conjugated secondary anti-goat IgG antibody (dilution 1:5000) and anti-mouse IgG antibody, respectively (Sigma) for 1 hour at RT. After washing with TBST, immunoblots were visualized using Amersham Biosciences enhanced chemiluminescence detection system (Piscataway, New Jersey, USA) according to the manufacturer's instructions.

Experimental glioma on the chick chorioallantoic membrane assay

To examine the effect of IGFBP7 on GBM tumor growth, U87MG cells were grown on CAMs in the absence or presence of 500 nM of IGFBP7. Fertilized chicken (gallus gallus) eggs were obtained from the Canadian Food Inspection Agency and placed into an egg incubator at 37°C and 67-70% humidity (day 0 of embryo development). At day 3, a small window was cut on the superior surface of the eggshell and then covered with protective surgical tape (Durapore^{TM}, VWR Canlab, Mississauga, ON, Canada) until day 10. Then, a sterile Thermanox plastic ring was placed onto the CAM and the surface of the CAM delimited by the ring, gently lacerated prior to inoculation of 10^6 U87MG cells pre-mixed with DMSO alone (vehicle) or 500 nM of recombinant IGFBP7. DMSO and IGFBP7 treatments were applied every day during four consecutive days. At day 17, tumors were photographed (Canon Powershot A720 IS digital camera), excised from the eggs and weighted. Tumors were then fixed with 4% paraformaldehyde for 2 h at RT prior incubation in 30% sucrose solution overnight at 4°C. Tumors were embedded in Tissue-Tek freezing medium (Miles Laboratories, Elkhart, IN), sectioned on a cryostat (Jung CM3000; Leica, Richmond Hill, ON, Canada) at 10 μm thickness, mounted on Superfrost Plus microscope slides (Fisher Scientific, Nepean, ON, Canada), and kept at -80°C until used.

Immunofluorescence

Tumor sections were rinsed in 0.2 M PBS (pH 7.3) and blocked with 10% normal goat serum in PBS supplemented with 0.1% Triton-X for 1 h at RT. Slides were then

incubated with a monoclonal mouse anti-α-smooth muscle actin (α-SMA) antibody (dilution 1:100) (R&D System) for 1 h at RT. After washing 3X in PBS, slides were incubated with a polyclonal goat anti-mouse Alexa 568 IgG (dilution 1:300) (Invitrogen) and Sambucus Nigra lectin for 30 min at RT. Sections were then rinsed in PBS and milli-Q water and cover slipped in Fluorescent Mounting medium (Dako Diagnostics, Mississauga, ON, Canada) spiked with Hoechst (dilution 1:1000) (DAPI; Sigma). Sections were visualized under the Olympus 1X2 UCB fluorescent microscope and images were captured using InVivo v.3.2.2 software (MediaCybernetics, Bethesda, MD, USA). Microphotographs were thresholded, converted into binary images and total area covered by α-SMA-positive cells in 3 fields/tumor section was quantified using Image-Pro Plus v.6.2 software (MediaCybernetics) for a total of 4 tumors/group.

Statistical analysis

Results are expressed as mean ± s.e.m. The unpaired t test was used for single comparisons of groups with equal variance and normal distribution. ANOVA followed by Newman-Keuls' post-test was used to compare multiple groups. A P value of less than 0.05 was considered statistically significant.

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REFERENCES

Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K *et al.* (1996). Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. *Proc Natl Acad Sci USA* **93:** 8384-9.

Armulik A, Abramsson A, Betsholtz C. (2005). Endothelial/pericyte interactions. *Circ Res* **97:** 512-23.

Bansal K, Liang ML, Rutka JT. (2006). Molecular biology of human gliomas. *Technol Cancer Res Treat* **5:** 185-94.

Bergers G, Song S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro-oncol* **7:** 452-64.

Brat DJ, Van Meir EG. (2001). Glomeruloid microvascular proliferation orchestrated by VPF/VEGF: a new world of angiogenesis research. *Am J Pathol* **158:** 789-96.

Burger AM, Spyropoulos DD, Kahn HJ, al. e. (2005). Development of an insulin-like growth factor binding protein-related protein 1 knockout mouse. *Proc Amer Assoc Cancer Res* **46:** 1080.

Burger AM, Zhang X, Li H, Ostrowski JL, Beatty B, Venanzoni M *et al.* (1998). Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. *Oncogene* **16:** 2459-67.

Chantrain CF, Henriet P, Jodele S, Emonard H, Feron O, Courtoy PJ *et al.* (2006). Mechanisms of pericyte recruitment in tumour angiogenesis: a new role for metalloproteinases. *Eur J Cancer* **42:** 310-8.

Chen Y, Pacyna-Gengelbach M, Ye F, Knosel T, Lund P, Deutschmann N *et al.* (2007). Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) has potential tumour-suppressive activity in human lung cancer. *J Pathol* **211:** 431-8.

Chiarugi P, Fiaschi T. (2007). Redox signalling in anchorage-dependent cell growth. *Cell Signal* **19:** 672-82.

Danen EH, Yamada KM. (2001). Fibronectin, integrins, and growth control. *J Cell Physiol* **189:** 1-13.

Degeorges A, Wang F, Frierson HF, Jr., Seth A, Sikes RA. (2000). Distribution of IGFBP-rP1 in normal human tissues. *J Histochem Cytochem* **48:** 747-54.

Drivdahl R, Haugk KH, Sprenger CC, Nelson PS, Tennant MK, Plymate SR. (2004). Suppression of growth and tumorigenicity in the prostate tumor cell line M12 by overexpression of the transcription factor SOX9. *Oncogene* **23:** 4584-93.

Ergun S, Kilic N, Wurmbach JH, Ebrahimnejad A, Fernando M, Sevinc S *et al.* (2001). Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes. *Angiogenesis* **4:** 193-206.

Fukumura D, Jain RK. (2007). Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. *Microvasc Res* **74:** 72-84.

Hagedorn M, Javerzat S, Gilges D, Meyre A, de Lafarge B, Eichmann A *et al.* (2005). Accessing key steps of human tumor progression in vivo by using an avian embryo model. *Proc Natl Acad Sci U S A* **102:** 1643-8.

How HK, Yeoh A, Quah TC, Oh Y, Rosenfeld RG, Lee KO. (1999). Insulin-like growth factor binding proteins (IGFBPs) and IGFBP-related protein 1-levels in cerebrospinal fluid of children with acute lymphoblastic leukemia. *J Clin Endocrinol Metab* **84:** 1283-7.

Hwa V, Oh Y, Rosenfeld RG. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev* **20:** 761-87.

Hwa V, Tomasini-Sprenger C, Bermejo AL, Rosenfeld RG, Plymate SR. (1998). Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells. *J Clin Endocrinol Metab* **83:** 4355-62.

Jain RK. (2003). Molecular regulation of vessel maturation. *Nat Med* **9:** 685-93.

Jain RK. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307:** 58-62.

Kato MV, Sato H, Tsukada T, Ikawa Y, Aizawa S, Nagayoshi M. (1996). A follistatin-like gene, mac25, may act as a growth suppressor of osteosarcoma cells. *Oncogene* **12:** 1361-4.

Knighton D, Ausprunk D, Tapper D, Folkman J. (1977). Avascular and vascular phases of tumour growth in the chick embryo. *Br J Cancer* **35:** 347-56.

Komatsu S, Okazaki Y, Tateno M, Kawai J, Konno H, Kusakabe M *et al.* (2000). Methylation and downregulated expression of mac25/insulin-like growth factor binding protein-7 is associated with liver tumorigenesis in SV40T/t antigen transgenic mice, screened by restriction landmark genomic scanning for methylation (RLGS-M). *Biochem Biophys Res Commun* **267:** 109-17.

Landberg G, Ostlund H, Nielsen NH, Roos G, Emdin S, Burger AM *et al.* (2001). Downregulation of the potential suppressor gene IGFBP-rP1 in human breast cancer is associated with inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation in estrogen receptor negative tumors. *Oncogene* **20:** 3497-505.

Lin J, Lai M, Huang Q, Ma Y, Cui J, Ruan W. (2007). Methylation patterns of IGFBP7 in colon cancer cell lines are associated with levels of gene expression. *J Pathol* **212:** 83-90.

Louis DN. (2006). Molecular pathology of malignant gliomas. *Annu Rev Pathol* **1:** 97-117.

Moreno MJ, Ball M, Andrade MF, McDermid A, Stanimirovic DB. (2006). Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells. *Glia* **53:** 845-57.

Murphy M, Pykett MJ, Harnish P, Zang KD, George DL. (1993). Identification and characterization of genes differentially expressed in meningiomas. *Cell Growth Differ* **4:** 715-22.

Mutaguchi K, Yasumoto H, Mita K, Matsubara A, Shiina H, Igawa M *et al.* (2003). Restoration of insulin-like growth factor binding protein-related protein 1 has a tumor-suppressive activity through induction of apoptosis in human prostate cancer. *Cancer Res* **63:** 7717-23.

Nishishita T, Lin PC. (2004). Angiopoietin 1, PDGF-B, and TGF-beta gene regulation in endothelial cell and smooth muscle cell interaction. *J Cell Biochem* **91:** 584-93.

Oh Y, Nagalla SR, Yamanaka Y, Kim HS, Wilson E, Rosenfeld RG. (1996). Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II. *J Biol Chem* **271:** 30322-5.

Pen A, Moreno MJ, Durocher Y, Deb-Rinker P, Stanimirovic DB. (2008). Glioblastoma-secreted factors induce IGFBP7 and angiogenesis by modulating Smad-2-dependent TGF-beta signaling. *Oncogene.*

Pen A, Moreno MJ, Martin J, Stanimirovic DB. (2007). Molecular markers of extracellular matrix remodeling in glioblastoma vessels: microarray study of laser-captured glioblastoma vessels. *Glia* **55:** 559-72.

Pepper MS. (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* **8:** 21-43.

Pfeifer A, Kessler T, Silletti S, Cheresh DA, Verma IM. (2000). Suppression of angiogenesis by lentiviral delivery of PEX, a noncatalytic fragment of matrix metalloproteinase 2. *Proc Natl Acad Sci U S A* **97:** 12227-32.

Plymate SR, Haugk KH, Sprenger CC, Nelson PS, Tennant MK, Zhang Y *et al.* (2003). Increased manganese superoxide dismutase (SOD-2) is part of the mechanism for prostate tumor suppression by Mac25/insulin-like growth factor binding-protein-related protein-1. *Oncogene* **22:** 1024-34.

Ribatti D, Nico B, Vacca A, Roncali L, Burri PH, Djonov V. (2001). Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis and anti-angiogenesis in vivo. *Anat Rec* **264:** 317-24.

Ronnov-Jessen L, Petersen OW. (1996). A function for filamentous alpha-smooth muscle actin: retardation of motility in fibroblasts. *J Cell Biol* **134:** 67-80.

Ruan W, Xu E, Xu F, Ma Y, Deng H, Huang Q *et al.* (2007). IGFBP7 plays a potential tumor suppressor role in colorectal carcinogenesis. *Cancer Biol Ther* **6:** 354-9.

Sato Y, Chen Z, Miyazaki K. (2007). Strong suppression of tumor growth by insulin-like growth factor-binding protein-related protein 1/tumor-derived cell adhesion factor/mac25. *Cancer Sci* **98:** 1055-63.

Sprenger CC, Damon SE, Hwa V, Rosenfeld RG, Plymate SR. (1999). Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. *Cancer Res* **59:** 2370-5.

Sprenger CC, Vail ME, Evans K, Simurdak J, Plymate SR. (2002). Over-expression of insulin-like growth factor binding protein-related protein-1(IGFBP-rP1/mac25) in the M12 prostate cancer cell line alters tumor growth by a delay in G1 and cyclin A associated apoptosis. *Oncogene* **21:** 140-7.

St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E *et al.* (2000). Genes expressed in human tumor endothelium. *Science* **289:** 1197-202.

Swisshelm K, Ryan K, Tsuchiya K, Sager R. (1995). Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. *Proc Natl Acad Sci U S A* **92:** 4472-6.

Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK. (2004). Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res* **64:** 3731-6.

Trojan J, Cloix JF, Ardourel MY, Chatel M, Anthony DD. (2007). Insulin-like growth factor type I biology and targeting in malignant gliomas. *Neuroscience* **145:** 795-811.

Umeda F, Ono Y, Sekiguchi N, Hashimoto T, Masakado M, Nakamura K *et al.* (1998). Increased mRNA expression of a novel prostacyclin-stimulating factor in human colon cancer. *J Gastroenterol* **33:** 213-7.

Vajkoczy P, Menger MD. (2000). Vascular microenvironment in gliomas. *J Neurooncol* **50:** 99-108.

van der Schaft DW, Dings RP, de Lussanet QG, van Eijk LI, Nap AW, Beets-Tan RG *et al.* (2002). The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *Faseb J* **16:** 1991-3.

von Tell D, Armulik A, Betsholtz C. (2006). Pericytes and vascular stability. *Exp Cell Res* **312:** 623-9.

Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. (2008). Oncogenic BRAF Induces Senescence and Apoptosis through Pathways Mediated by the Secreted Protein IGFBP7. *Cell* **132:** 363-74.

Wilson HM, Birnbaum RS, Poot M, Quinn LS, Swisshelm K. (2002). Insulin-like growth factor binding protein-related protein 1 inhibits proliferation of MCF-7 breast cancer cells via a senescence-like mechanism. *Cell Growth Differ* **13:** 205-13.

Zumkeller W, Muller D, Muller S, Gunther C, Westphal M. (1998). Expression and synthesis of insulin-like growth factor-binding proteins in human glioma cell lines. *Int J Oncol* **12:** 129-35.

CHAPTER 5: GENERAL DISCUSSION

Dr. Judah Folkman pioneered the field of tumor angiogenesis in the early 1970's by postulating that solid tumors cannot grow more than 1-2 mm in diameter without blood vessel supply and that strategies to inhibit angiogenesis might be exploited for the development of novel therapeutics to treat cancers (Folkman, 1971). It is now widely accepted that tumor growth is angiogenesis-dependent and that the degree of angiogenesis has prognostic significance (Weidner, 1995). Research into tumor angiogenesis and the development of anti-angiogenic drugs for cancer treatment has exploded over the past two decades, driven by the discovery of families of pro-angiogenic factors and their tyrosine kinase receptors (see Introduction), as well as the development of drugs which interfere with these receptors and their signaling pathways. Several anti-angiogenic drugs have been approved by the FDA as adjuvant therapies and others are currently undergoing clinical trials.

Although numerous advances in the field of glioma biology and genetics have been made, the heterogeneity and complexity of GBM biology have impeded significant improvements in their clinical outcomes. Therapeutic targeting of angiogenesis in GBMs is particularly attractive given the dense vascularization of these tumors. However, anti-angiogenic treatments attempted in GBMs have so far produced only marginal improvements (see Introduction). This underscored the need for a better understanding of molecular make-up of GBM vessels and mediators involved in cross-talk between vascular and tumor compartments that determine the complex processes of neo-vascularization and growth of these tumors.

To address this knowledge gap, the primary objective of this thesis was to characterize the transcriptome of GBM vessels to identify selective and novel biomarkers of tumor vasculature. The approach of selectively extracting vessels from GBM tumors using LCM microscopy for subsequent RNA amplification and cDNA microarray analyses used here was one of the first attempts to selectively analyse the transcriptome of vascular compartment in GBMs. This approach resulted in identification of several novel differentially expressed genes that are potential biomarkers of GBM vascular endothelium. A subset of these genes, including IGFBP7, has been further validated as GBM vascular biomarkers at the protein level.

The exploitation of validated biomarkers in disease diagnosis, prognosis and treatment monitoring has received great attention as an important element of translational research and the FDA-supported 'critical path' towards personalized medicine. Exploitation of vascular biomarkers for GBM tumors for these purposes has several potential advantages over similar molecular/genetic biomarkers discovered in GBM cells, because vascularization of GBMs is a prognostic indicator and endothelial biomarkers are easily accessible/targetable from the systemic circulation, whereas the access to parenchymal biomarkers is hindered by the blood-tumor barrier. Molecular imaging of proteins expressed in tumor vasculature, such as receptors and transporters, can be used for molecular diagnosis and for predicting the response to anti-angiogenic agents (Glunde *et al.*, 2007). For example, integrin $\alpha v\beta 3$, highly expressed on activated ECs, has been used to image and characterize vascular function during tumor angiogenesis (Beer and Schwaiger, 2008; Haubner *et al.*, 2005). The highly selective expression of IGFBP7 in

GBM vessels discovered in this study makes it a potential candidate for developing *in vivo* molecular imaging agents for non-invasively assessing GBM tumor angiogenesis.

Our preliminary assessment of IGFBP7 vascular expression levels in histologically graded gliomas (presented in Appendix 2), suggest that vascular IGFBP7 expression positively correlates with glioma grades, and may potentially be used as molecular marker to aid histological tumor classification. More accurate molecular tumor classification is expected to facilitate the choice of treatment regimens and to increase survival outcome in patients with gliomas. Gliomas are currently classified (WHO grade I to IV) based on histological analyses including necrosis and microvascular proliferation (see Introduction). However, histological diagnosis is often subjective and high variability in response to therapeutics and survival still occurs among patients with tumors presenting similar histological profiles (Nakamura *et al.*, 2007). Therefore, new biomarkers that add incremental value to the WHO grading system are still needed. GBM transcriptional profiling studies identified gene signatures that could be used to classify GBM subgroups with different overall survival (Liang *et al.*, 2005). IQGAP1, IGFBP2, and RhoA were identified as biomarkers that could assist the WHO grading system to allow for more precise staging of aggressive astrocytoma subgroups (McDonald *et al.*, 2007; Yan *et al.*, 2006). Although measurements of microvessel density (MVD) are also used as a prognostic tool to evaluate GBM patient outcomes (Leon *et al.*, 1996; Sharma *et al.*, 2006), MVD is not sufficient to evaluate the functional or angiogenic status of tumor vasculature (Nico *et al.*, 2008). Transcriptional profiling performed in this thesis, focused selectively on the tumor vascular compartment,

identified novel tumor vascular fingerprint(s) that may aid in the classification/staging of vascular function and malignancy of gliomas.

This thesis further contributed mechanistic and functional insights into the complex role of IGFBP7 in angiogenesis and tumor growth in the context of GBM. GBMs are heterogeneous tumors composed of several cell types (see Introduction) facing a hostile metabolic microenvironment characterized by hypoxia and acidosis (Helmlinger *et al.*, 1997; Tatum *et al.*, 2006). Under these conditions, intercellular cross-talk is mediated by the release of factors that contribute to the abnormal organization, structure and function of blood vessels and foster GBM growth and invasion (Jung *et al.*, 2002). Factors specifically expressed and released by the tumor microenvironment (e.g. HGF and TGF-β) represent promising candidates for therapeutic targeting. TGF-β regulates different phases of angiogenesis depending on how the environmental cues direct the activation of ALK1 or ALK5 signaling pathways. The ALK1 pathway is primarily involved in early phase angiogenesis (EC proliferation and migration), whereas the ALK5 pathway contributes to late phase angiogenesis (pericyte recruitment, basement membrane assembly and formation of cell junctions) (Goumans *et al.*, 2003; Lebrin *et al.*, 2005). We provided the first experimental evidence that TGF-βs secreted by GBM cells induce IGFBP7 in brain ECs through the activation of the ALK5-Smad2 pathway. TGF-β-induced IGFBP7 was then shown to deposit in the vascular basement membranes of GBM vessels and suggested to participate in late phase angiogenesis.

For many years, research on angiogenesis and anti-angiogenic strategies has focused on the early steps of angiogenesis characterized by EC proliferation and migration. Late phase angiogenesis, involving vessel stabilization by pericyte

recruitment and re-assembly of basement-membrane and cell-cell junctions, recently gained attention as an important component of tumor angiogenesis (Jain, 2003). The mechanisms of vessel stabilization and their consequences on tumor growth are still very controversial. The degree of vessel stabilization is principally assessed by microvessel pericyte coverage index. In GBM, some studies reported low microvessel pericyte coverage index (Bergers and Song, 2005; Eberhard *et al.*, 2000), whereas others described abundant α-SMA-positive pericytes (Stratmann *et al.*, 1998; Wesseling *et al.*, 1995). These discrepancies are likely caused by the use of inadequate markers for definitive pericyte identification and/or by the apparent heterogeneity of pericyte populations (Morikawa *et al.*, 2002). Regardless of the number of pericytes detected in tumors, pericytes found in tumor vessels are abnormal and loosely associated with ECs (Morikawa *et al.*, 2002). Pericytes have been shown to regulate vessel integrity, maintenance and function through induction of EC differentiation, growth arrest and survival (Bergers and Song, 2005; Gerhardt and Betsholtz, 2003).

Attempts to modulate vessel stabilization as a strategy to attenuate tumor growth have also resulted in divergent outcomes. Some studies demonstrated that strategies leading to vessel destabilization result in vessel regression and thereby, restrict tumor growth. For example, the induction of pericyte detachment from tumor vessels by blocking PDGFR signaling with a tyrosine kinase inhibitor SU6888 restricted tumor growth in a transgenic mouse model of pancreatic islet carcinogenesis (Rip1Tag2) (Bergers *et al.*, 2003). On the other hand, the vasculature of low malignant tumors generally exhibits high pericyte coverage suggesting that vessel stability is associated with lower rates of growth (Kilic *et al.*, 1999) and that vascular stabilization may be

beneficial in controlling tumor progression. The tumor vessel normalization strategy is based on observations that, while in the normal tissue the balance between pro- and anti-angiogenic factors is tightly controlled, an excess of pro-angiogenic factors and/or depletion of anti-angiogenic factors causes abnormalities in tumor vessels and the tumor microenvironment, facilitating constant growth of new tumor vessels (Bergers and Benjamin, 2003). Hence, down-regulation of pro-angiogenic factors or up-regulation of anti-angiogenic factors could revert the tumor vasculature to a more 'normal' state and limit tumor growth (Fukumura and Jain, 2007; Jain, 2005). Experimental support for this hypothesis was furnished by data showing that anti-angiogenic agents (e.g. anti-VEGFR2 or endostatin) stabilized tumor vessels by promoting a more morphologically and functionally 'normal' vasculature. This 'normalized' vascular phenotype is characterized by less leaky, tortuous and dilated vessels with a normal basement membrane and higher perivascular cell coverage (Ergun *et al.*, 2001; Jain, 2005; Tong *et al.*, 2004). 'Normalization' of tumor vessels also resulted in the normalization of tumor microenvironment including decreased IFP, increased tumor oxygenation and neutralized pH (Tong *et al.*, 2004; Winkler *et al.*, 2004). As a result, better drug penetration and increased efficacy of radiation therapy were achieved (Jain, 2005; Tong *et al.*, 2004; Winkler *et al.*, 2004).

This thesis contributed to this emerging field a discovery of an important new mediator of late phase angiogenesis, IGFBP7. GBM tumor-secreted TGF-βs were found to stimulate production and secretion of IGFBP7 from brain ECs through the ALK5-Smad2 pathway, also known to participate in TGF-β-induced pericyte recruitment through differentiation of cells into a pericyte-like phenotype. Secreted IGFBP7

accumulated in perivascular spaces closely associated with basement membranes in GBM vessels. The addition of exogenous IGFBP7 into CAM-grown GBM tumors resulted in pronounced increases in pericyte-like α-SMA-positive cells in perivascular regions, suggesting a role for IGFBP7 in pericyte recruitment, most likely through pericyte differentiation; this was accompanied by the reduction in tumor size, supporting the hypothesis that stabilization and normalization of tumor vessels may inhibit tumor growth. Based on these observations, this thesis further submits that the exogenous IGFBP7 or the induction of signaling pathways (e.g., those mediated by TGF-βs) leading to endogenous IGFBP7 production, could tip the balance of pro- and anti-angiogenic stimuli towards processes resulting in vessel stabilization and 'normalization'.

In addition to its role in vessel stabilization, IGFBP7-induced inhibition of tumor growth could also be attributed to the direct anti-proliferative and tumor-suppressing properties on GBM cells. This thesis demonstrated that IGFBP7 directly suppresses GBM cell proliferation and tumor growth, suggesting that IGFBP7 may be considered a potential anti-tumor therapeutic. Although the mechanisms leading to the inhibition of GBM cell proliferation are unknown, based on recent studies, IGFBP7 appears to affect tumor suppression in GBM cells through apoptosis and cellular senescence-like mechanisms (Wajapeyee *et al.*, 2008). Given that TGF-β is a major inducer of IGFBP7, it may also be considered as potential anti-tumor agent. However, since TGF-β can act as both tumor suppressor and tumor promoter, depending on the microenvironment (Luwor *et al.*, 2008), targeting its downstream effectors, such as IGFBP7, may circumvent the adverse effects arising from the multiple functionalities of TGF-βs.

Taken together, the studies presented in this thesis provide evidence of the dual tumor-suppressive and tumor vessel stabilizing properties of IGFBP7, both important elements of IGFBP7-mediated tumor growth inhibition.

Abnormal GBM cell proliferation caused by aberration of signaling elements in the growth and survival pathways are characteristics of GBM progression and growth (Sathornsumetee *et al.*, 2007). Growth factor receptor and signal transduction effector blockers such as EGFR kinase inhibitors (gefitinib and erlotinib) and mTOR inhibitor (temsirolimus) respectively, used as monotherapy in clinical trials failed to demonstrate survival benefits for GBM patients (Sathornsumetee *et al.*, 2007). Combination therapies using different agents to target the same pathway in tumor cells or to target different pathways in different cell types (e.g., tumor cells and ECs) have shown increased treatment efficacy. Preclinical studies using a combination of EGFR kinase inhibitors and mTOR demonstrated encouraging anti-tumor activity in GBM cells (Goudar *et al.*, 2005; Wang *et al.*, 2006). Bevacizumab (an anti-VEGF antibody that targets ECs) is more effective in combination with standard chemotherapeutic agents (Pope *et al.*, 2006; Vredenburgh *et al.*, 2007). Similarly, given the complexity of the angiogenic process, inhibiting a single pathway is unlikely to block pathogenic angiogenesis, due to redundant functions of other angiogenic molecules released by the tumor microenvironment (Folkman, 2004). The currently preferred anti-angiogenic approach is to exploit pleiotropic, multifunctional effectors to obtain either a total vessel regression or to promote complete vessel stabilization. The dual effect of IGFBP7 in inhibiting tumor cell growth and in inducing late phase angiogenesis and vessel stabilization suggests that

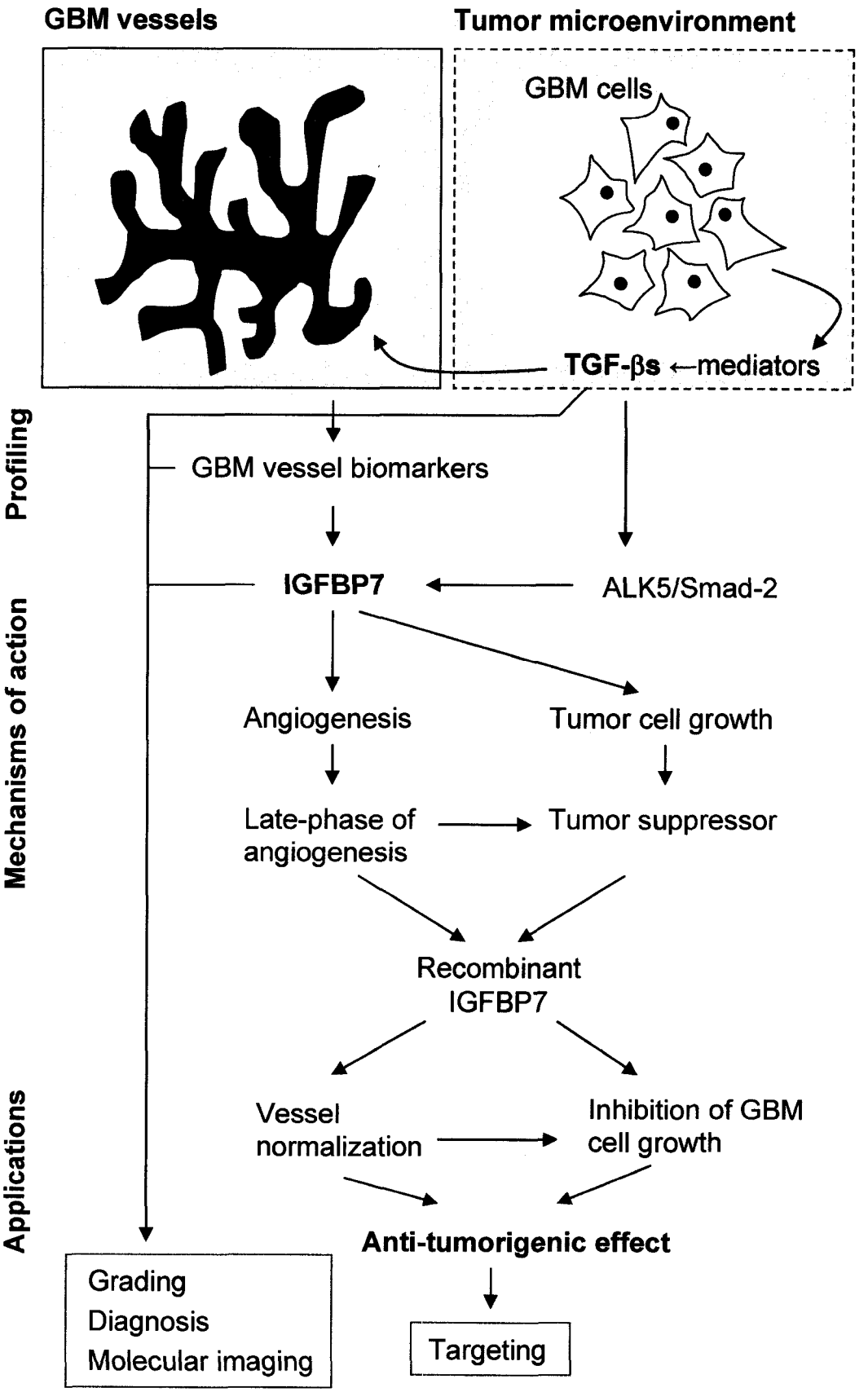
IGFBP7 may be an attractive molecule to develop as potential multifunctional anti-cancer agent.

Despite the advancement in glioma research, GBM remains an incurable disease. This thesis highlights the importance of GBM tumor microenvironment in regulating angiogenesis and underscores the significance of late-phase angiogenesis in the field of tumor angiogenesis. Publications derived from this thesis have contributed to the identification of novel biomarkers of GBM vessels/angiogenesis, and to a better understanding of mechanisms of IGFBP7 induction and its roles in late phase angiogenesis and tumor growth. These findings have significant implications in the field of glioma grading, diagnosis, molecular imaging, and therapeutic development. Based on this thesis and other recent studies examining the role of IGFBP7 in tumor biology, it is apparent that this secreted molecule may be a promising candidate for developing anti-angiogenic or anti-tumor approaches for GBM treatment. A summary of key findings presented in this thesis is shown in Fig. 1. We trust that this work, together with other advances made in glioma biology and treatment, will eventually lead to a better management of this devastating disease.

Figure 1

Summary of the findings presented in this doctoral thesis

The expression of genes in GBM vasculature is controlled by the tumor microenvironment. In this thesis, several potential GBM vessel biomarkers, including IGFBP7, have been identified using a combination of LCM coupled with microarray analyses (profiling phase- pink box). Further mechanistic and functional studies showed that IGFBP7 is induced by tumor-secreted TGF-βs through activation of the ALK5-Smad-2 pathway. IGFBP7 was further shown to participate in late phase angiogenesis and to inhibit GBM cell and tumor growth (mechanism of actions-blue box). These functions make IGFBP7 an attractive molecule for therapeutic approaches since it can simultaneously act in ECs by 'normalizing' tumor vessels and suppressing GBM cell growth (application-yellow box). In addition, the discovery of GBM biomarkers and tumor microenvironment mediators such as TGF-βs could have major implications for glioma grading, diagnosis and molecular imaging (application-yellow box).



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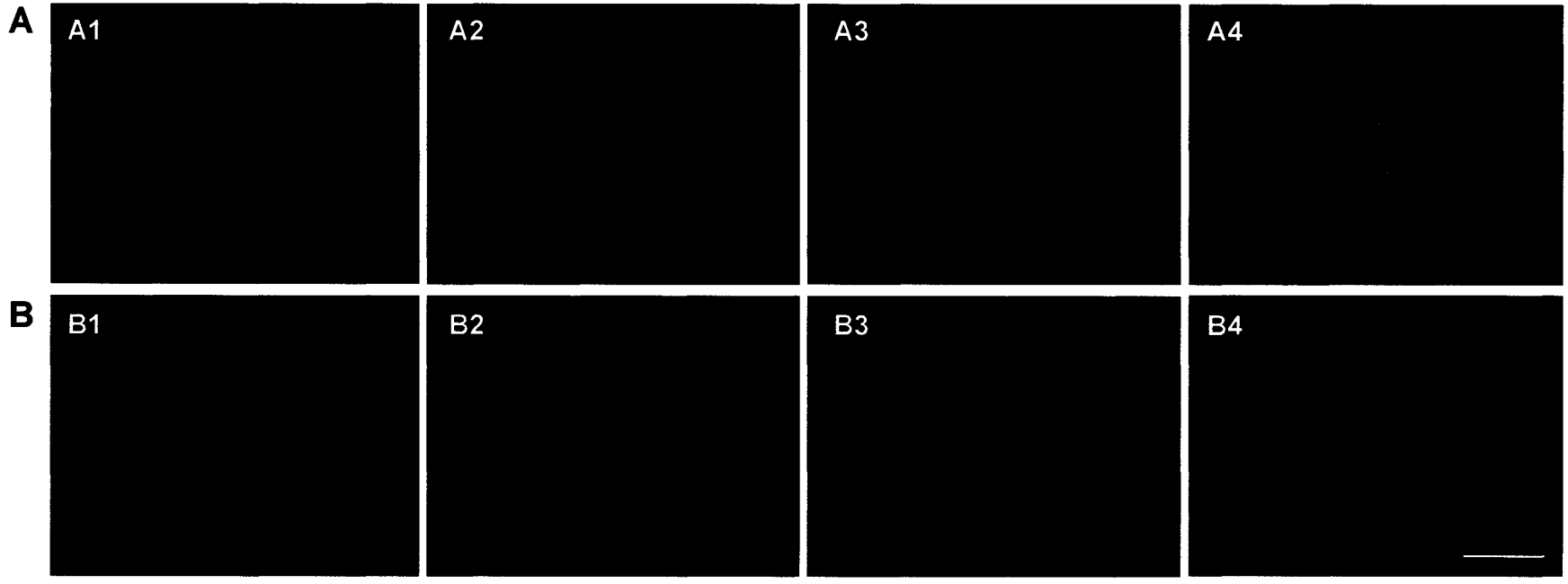
APPENDIX 2

IGFBP7 expression in low and high grade astrocytoma sections determined by immunofluorescence

Tissue sections derived from low grade (A) and high grade (B) astrocytomas were stained for IGFBP7 (red) and for UEA 1 (green) and laminin (blue) to visualize vessels. A strong expression of IGFBP7 was observed in high grade glioma tissue (A3) compared to low grade glioma section (B3). Overlay images show co-localization of IGFBP7 with UEA 1 and laminin (A4, B4). Scale bar = 100 μm.



REFERENCES

Abe, M. and Y. Sato. 2001. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. Angiogenesis. 4(4), 289-98.

Adachi, Y., F. Itoh, H. Yamamoto, Y. Arimura, Y. Kikkawa-Okabe, K. Miyazaki, D. P. Carbone and K. Imai. 2001. Expression of angiomodulin (tumor-derived adhesion factor/mac25) in invading tumor cells correlates with poor prognosis in human colorectal cancer. Int. J. Cancer. 95(4), 216-22.

Ahmed, S., X. Jin, M. Yagi, C. Yasuda, Y. Sato, S. Higashi, C. Y. Lin, R. B. Dickson and K. Miyazaki. 2006. Identification of membrane-bound serine proteinase matriptase as processing enzyme of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/angiomodulin/mac25). FEBS J. 273(3), 615-27.

Ahmed, S., K. Yamamoto, Y. Sato, T. Ogawa, A. Herrmann, S. Higashi and K. Miyazaki. 2003. Proteolytic processing of IGFBP-related protein-1 (TAF/angiomodulin/mac25) modulates its biological activity. Biochem. Biophys. Res. Commun. 310(2), 612-8.

Akaogi, K., Y. Okabe, K. Funahashi, Y. Yoshitake, K. Nishikawa, H. Yasumitsu, M. Umeda and K. Miyazaki. 1994. Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. Biochem. Biophys. Res. Commun. 198(3), 1046-53.

Akaogi, K., Y. Okabe, J. Sato, Y. Nagashima, H. Yasumitsu, K. Sugahara and K. Miyazaki. 1996. Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. Proc. Natl. Acad. Sci. USA. 93(16), 8384-9.

Andreasen, P. A., R. Egelund and H. H. Petersen. 2000. The plasminogen activation system in tumor growth, invasion, and metastasis. Cell. Mol. Life Sci. 57(1), 25-40.

Armulik, A., A. Abramsson and C. Betsholtz. 2005. Endothelial/pericyte interactions. Circ. Res. 97(6), 512-23.

Auguste, P., S. Lemiere, F. Larrieu-Lahargue and A. Bikfalvi. 2005. Molecular mechanisms of tumor vascularization. Crit. Rev. Oncol. Hematol. 54(1), 53-61.

Autiero, M., J. Waltenberger, D. Communi, A. Kranz, L. Moons, D. Lambrechts, J. Kroll, S. Plaisance, M. De Mol, F. Bono, S. Kliche, G. Fellbrich, K. Ballmer-Hofer, D. Maglione, U. Mayr-Beyrle, M. Dewerchin, S. Dombrowski, D. Stanimirovic, P. Van Hummelen, C. Dehio, D. J. Hicklin, G. Persico, J. M. Herbert, D. Communi, M. Shibuya, D. Collen, E. M. Conway and P. Carmeliet. 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat. Med. 9(7), 936-43.

Baker, A. H., D. R. Edwards and G. Murphy. 2002. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J. Cell Sci. 115(Pt 19), 3719-27.

Baluk, P., S. Morikawa, A. Haskell, M. Mancuso and D. M. McDonald. 2003. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. 163(5), 1801-15.

Banfi, A., G. von Degenfeld and H. M. Blau. 2005. Critical role of microenvironmental factors in angiogenesis. Curr. Atheroscler. Rep. 7(3), 227-34.

Bansal, K., M. L. Liang and J. T. Rutka. 2006. Molecular biology of human gliomas. Technol. Cancer Res. Treat. 5(3), 185-94.

Barker, T. H., G. Baneyx, M. Cardo-Vila, G. A. Workman, M. Weaver, P. M. Menon, S. Dedhar, S. A. Rempel, W. Arap, R. Pasqualini, V. Vogel and E. H. Sage. 2005. SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity. J. Biol. Chem. 280(43), 36483-93.

Batchelor, T. T., A. G. Sorensen, E. di Tomaso, W. T. Zhang, D. G. Duda, K. S. Cohen, K. R. Kozak, D. P. Cahill, P. J. Chen, M. Zhu, M. Ancukiewicz, M. M. Mrugala, S. Plotkin, J. Drappatz, D. N. Louis, P. Ivy, D. T. Scadden, T. Benner, J. S. Loeffler, P. Y. Wen and R. K. Jain. 2007. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell. 11(1), 83-95.

Beaty, R. M., J. B. Edwards, K. Boon, I. M. Siu, J. E. Conway and G. J. Riggins. 2007. PLXDC1 (TEM7) is identified in a genome-wide expression screen of glioblastoma endothelium. J. Neurooncol. 81(3), 241-8.

Beer, A. J. and M. Schwaiger. 2008. Imaging of integrin alphavbeta3 expression. Cancer Metastasis Rev. 27(4), 631-44.

Ben-Ze'ev, A., M. Shtutman and J. Zhurinsky. 2000. The integration of cell adhesion with gene expression: the role of beta-catenin. Exp. Cell Res. 261(1), 75-82.

Benjamin, L. E., D. Golijanin, A. Itin, D. Pode and E. Keshet. 1999. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J. Clin. Invest. 103(2), 159-65.

Bergers, G. and L. E. Benjamin. 2003. Tumorigenesis and the angiogenic switch. Nat. Rev. Cancer. 3(6), 401-10.

Bergers, G. and S. Song. 2005. The role of pericytes in blood-vessel formation and maintenance. Neuro-oncol. 7(4), 452-64.

Bergers, G., S. Song, N. Meyer-Morse, E. Bergsland and D. Hanahan. 2003. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J. Clin. Invest. 111(9), 1287-95.

Bernstein, J. J., W. J. Goldberg and E. R. Laws, Jr. 1989. Human malignant astrocytoma xenografts migrate in rat brain: a model for central nervous system cancer research. J. Neurosci. Res. 22(2), 134-43.

Bernstein, J. J. and C. A. Woodard. 1995. Glioblastoma cells do not intravasate into blood vessels. Neurosurgery. 36(1), 124-32; discussion 132.

Bhati, R., C. Patterson, C. A. Livasy, C. Fan, D. Ketelsen, Z. Hu, E. Reynolds, C. Tanner, D. T. Moore, F. Gabrielli, C. M. Perou and N. Klauber-DeMore. 2008. Molecular characterization of human breast tumor vascular cells. Am. J. Pathol. 172(5), 1381-90.

Binder, B. R., J. Mihaly and G. W. Prager. 2007. uPAR-uPA-PAI-1 interactions and signaling: a vascular biologist's view. Thromb. Haemost. 97(3), 336-42.

Blanchette, F., N. Rivard, P. Rudd, F. Grondin, L. Attisano and C. M. Dubois. 2001. Cross-talk between the p42/p44 MAP kinase and Smad pathways in transforming growth factor beta 1-induced furin gene transactivation. J. Biol. Chem. 276(36), 33986-94.

Boucher, Y., H. Salehi, B. Witwer, G. R. t. Harsh and R. K. Jain. 1997. Interstitial fluid pressure in intracranial tumours in patients and in rodents. Br. J. Cancer. 75(6), 829-36.

Brat, D. J., A. C. Bellail and E. G. Van Meir. 2005. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro. Oncol. 7(2), 122-33.

Brat, D. J., A. A. Castellano-Sanchez, S. B. Hunter, M. Pecot, C. Cohen, E. H. Hammond, S. N. Devi, B. Kaur and E. G. Van Meir. 2004. Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. Cancer. Res. 64(3), 920-7.

Brat, D. J. and T. B. Mapstone. 2003. Malignant glioma physiology: cellular response to hypoxia and its role in tumor progression. Ann. Intern. Med. 138(8), 659-68.

Brat, D. J. and E. G. Van Meir. 2001. Glomeruloid microvascular proliferation orchestrated by VPF/VEGF: a new world of angiogenesis research. Am. J. Pathol. 158(3), 789-96.

Brat, D. J. and E. G. Van Meir. 2004. Vaso-occlusive and prothrombotic mechanisms associated with tumor hypoxia, necrosis, and accelerated growth in glioblastoma. Lab. Invest. 84(4), 397-405.

Brekken, R. A., X. Huang, S. W. King and P. E. Thorpe. 1998. Vascular endothelial growth factor as a marker of tumor endothelium. Cancer Res. 58(9), 1952-9.

Brekken, R. A. and E. H. Sage. 2001. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol. 19(8), 816-27.

Buckanovich, R. J., D. Sasaroli, A. O'Brien-Jenkins, J. Botbyl, R. Hammond, D. Katsaros, R. Sandaltzopoulos, L. A. Liotta, P. A. Gimotty and G. Coukos. 2007. Tumor vascular proteins as biomarkers in ovarian cancer. J. Clin. Oncol. 25(7), 852-61.

Burger, A. M., D. D. Spyropoulos, H. J. Kahn and e. al. 2005. Development of an insulin-like growth factor binding protein-related protein 1 knockout mouse. Proc. Amer. Assoc. Cancer Res. 46, 1080.

Burger, A. M., X. Zhang, H. Li, J. L. Ostrowski, B. Beatty, M. Venanzoni, T. Papas and A. Seth. 1998. Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. Oncogene. 16(19), 2459-67.

Burger, P. C. and P. Kleihues. 1989. Cytologic composition of the untreated glioblastoma with implications for evaluation of needle biopsies. Cancer. 63(10), 2014-23.

Burrows, F. J., E. J. Derbyshire, P. L. Tazzari, P. Amlot, A. F. Gazdar, S. W. King, M. Letarte, E. S. Vitetta and P. E. Thorpe. 1995. Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy. Clin. Cancer Res. 1(12), 1623-34.

Carmeliet, P. 2000. Mechanisms of angiogenesis and arteriogenesis. Nat. Med. 6(4), 389-95.

Carmeliet, P. 2003. Angiogenesis in health and disease. Nat. Med. 9(6), 653-60.

Carmeliet, P. 2004. Manipulating angiogenesis in medicine. J. Intern. Med. 255(5), 538-61.

Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau and A. Nagy. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 380(6573), 435-9.

Carmeliet, P. and R. K. Jain. 2000. Angiogenesis in cancer and other diseases. Nature. 407(6801), 249-57.

Carmeliet, P., L. Moons, A. Luttun, V. Vincenti, V. Compernolle, M. De Mol, Y. Wu, F. Bono, L. Devy, H. Beck, D. Scholz, T. Acker, T. DiPalma, M. Dewerchin, A. Noel, I. Stalmans, A. Barra, S. Blacher, T. Vandendriessche, A. Ponten, U. Eriksson, K. H. Plate, J. M. Foidart, W. Schaper, D. S. Charnock-Jones, D. J. Hicklin, J. M. Herbert, D. Collen and M. G. Persico. 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat. Med. 7(5), 575-83.

Cecchelli, R., V. Berezowski, S. Lundquist, M. Culot, M. Renftel, M. P. Dehouck and L. Fenart. 2007. Modelling of the blood-brain barrier in drug discovery and development. Nat. Rev. Drug Discov. 6(8), 650-61.

Chang, S. S., D. S. O'Keefe, D. J. Bacich, V. E. Reuter, W. D. Heston and P. B. Gaudin. 1999. Prostate-specific membrane antigen is produced in tumor-associated neovasculature. Clin. Cancer Res. 5(10), 2674-81.

Chang, Y. S., E. di Tomaso, D. M. McDonald, R. Jones, R. K. Jain and L. L. Munn. 2000. Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. Proc. Natl. Acad. Sci. U S A. 97(26), 14608-13.

Chantrain, C. F., P. Henriet, S. Jodele, H. Emonard, O. Feron, P. J. Courtoy, Y. A. DeClerck and E. Marbaix. 2006. Mechanisms of pericyte recruitment in tumour angiogenesis: a new role for metalloproteinases. Eur. J. Cancer. 42(3), 310-8.

Charalambous, C., T. C. Chen and F. M. Hofman. 2006. Characteristics of tumor-associated endothelial cells derived from glioblastoma multiforme. Neurosurg. Focus. 20(4), E22.

Chen, Y., M. Pacyna-Gengelbach, F. Ye, T. Knosel, P. Lund, N. Deutschmann, K. Schluns, W. F. Kotb, C. Sers, H. Yasumoto, T. Usui and I. Petersen. 2007. Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) has potential tumour-suppressive activity in human lung cancer. J. Pathol. 211(4), 431-8.

Chen, Y. G. and J. Massague. 1999. Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. J. Biol. Chem. 274(6), 3672-7.

Cheng, S. Y., H. J. Huang, M. Nagane, X. D. Ji, D. Wang, C. C. Shih, W. Arap, C. M. Huang and W. K. Cavenee. 1996. Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. Proc. Natl. Acad. Sci. U S A. 93(16), 8502-7.

Chiarugi, P. and T. Fiaschi. 2007. Redox signalling in anchorage-dependent cell growth. Cell Signal. 19(4), 672-82.

Choe, G., S. Horvath, T. F. Cloughesy, K. Crosby, D. Seligson, A. Palotie, L. Inge, B. L. Smith, C. L. Sawyers and P. S. Mischel. 2003. Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo. Cancer Res. 63(11), 2742-6.

Colorado, P. C., A. Torre, G. Kamphaus, Y. Maeshima, H. Hopfer, K. Takahashi, R. Volk, E. D. Zamborsky, S. Herman, P. K. Sarkar, M. B. Ericksen, M. Dhanabal, M. Simons, M. Post, D. W. Kufe, R. R. Weichselbaum, V. P. Sukhatme and R. Kalluri. 2000. Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res. 60(9), 2520-6.

Constam, D. B., J. Philipp, U. V. Malipiero, P. ten Dijke, M. Schachner and A. Fontana. 1992. Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. J. Immunol. 148(5), 1404-10.

Crone, C. and S. P. Olesen. 1982. Electrical resistance of brain microvascular endothelium. Brain Res. 241(1), 49-55.

Dai, C. and E. C. Holland. 2001. Glioma models. Biochim. Biophys. Acta. 1551(1), M19-27.

Danen, E. H. and K. M. Yamada. 2001. Fibronectin, integrins, and growth control. J. Cell Physiol. 189(1), 1-13.

Davies, D. C. 2002. Blood-brain barrier breakdown in septic encephalopathy and brain tumours. J. Anat. 200(6), 639-46.

Davis, G. E., K. J. Bayless and A. Mavila. 2002. Molecular basis of endothelial cell morphogenesis in three-dimensional extracellular matrices. Anat. Rec. 268(3), 252-75.

Davis, G. E. and C. W. Camarillo. 1996. An alpha 2 beta 1 integrin-dependent pinocytic mechanism involving intracellular vacuole formation and coalescence regulates capillary lumen and tube formation in three-dimensional collagen matrix. Exp. Cell Res. 224(1), 39-51.

Davis, G. E. and D. R. Senger. 2005. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ. Res. 97(11), 1093-107.

de Bouard, S., P. Herlin, J. G. Christensen, E. Lemoisson, P. Gauduchon, E. Raymond and J. S. Guillamo. 2007. Antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastoma. Neuro. Oncol. 9(4), 412-23.

de Caestecker, M. 2004. The transforming growth factor-beta superfamily of receptors. Cytokine Growth Factor Rev. 15(1), 1-11.

Deane, B. R. and P. L. Lantos. 1981. The vasculature of experimental brain tumours. Part 2. A quantitative assessment of morphological abnormalities. J. Neurol. Sci. 49(1), 67-77.

Deb-Rinker, P., D. Ly, A. Jezierski, M. Sikorska and P. R. Walker. 2005. Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. J. Biol. Chem. 280(8), 6257-60.

Degeorges, A., F. Wang, H. F. Frierson, Jr., A. Seth and R. A. Sikes. 2000. Distribution of IGFBP-rP1 in normal human tissues. J. Histochem. Cytochem. 48(6), 747-54.

Demeule, M., D. Shedid, E. Beaulieu, R. F. Del Maestro, A. Moghrabi, P. B. Ghosn, R. Moumdjian, F. Berthelet and R. Beliveau. 2001. Expression of multidrug-resistance P-glycoprotein (MDR1) in human brain tumors. Int. J. Cancer. 93(1), 62-6.

Demuth, T. and M. E. Berens. 2004. Molecular mechanisms of glioma cell migration and invasion. J. Neurooncol. 70(2), 217-28.

Derynck, R., Y. Zhang and X. H. Feng. 1998. Smads: transcriptional activators of TGF-beta responses. Cell. 95(6), 737-40.

Desbaillets, I., A. C. Diserens, N. Tribolet, M. F. Hamou and E. G. Van Meir. 1997. Upregulation of interleukin 8 by oxygen-deprived cells in glioblastoma suggests a role in leukocyte activation, chemotaxis, and angiogenesis. J. Exp. Med. 186(8), 1201-12.

Dinda, A. K., C. Sarkar, S. Roy, K. Kharbanda, M. Mathur, A. K. Khosla and A. K. Banerji. 1993. A transmission and scanning electron microscopic study of tumoral and peritumoral microblood vessels in human gliomas. J. Neurooncol. 16(2), 149-58.

Djonov, V. G., H. Kurz and P. H. Burri. 2002. Optimality in the developing vascular system: branching remodeling by means of intussusception as an efficient adaptation mechanism. Dev. Dyn. 224(4), 391-402.

Doolittle, N. D., M. E. Miner, W. A. Hall, T. Siegal, E. Jerome, E. Osztie, L. D. McAllister, J. S. Bubalo, D. F. Kraemer, D. Fortin, R. Nixon, L. L. Muldoon and E. A. Neuwelt. 2000. Safety and efficacy of a multicenter study using intraarterial chemotherapy in conjunction with osmotic opening of the blood-brain barrier for the treatment of patients with malignant brain tumors. Cancer. 88(3), 637-47.

Dor, Y., V. Djonov and E. Keshet. 2003. Making vascular networks in the adult: branching morphogenesis without a roadmap. Trends Cell Biol. 13(3), 131-6.

Drivdahl, R., K. H. Haugk, C. C. Sprenger, P. S. Nelson, M. K. Tennant and S. R. Plymate. 2004. Suppression of growth and tumorigenicity in the prostate tumor cell line M12 by overexpression of the transcription factor SOX9. Oncogene. 23(26), 4584-93.

Duda, D. G., K. S. Cohen, S. V. Kozin, J. Y. Perentes, D. Fukumura, D. T. Scadden and R. K. Jain. 2006. Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. Blood. 107(7), 2774-6.

Dvorak, H. F. 2006. Discovery of vascular permeability factor (VPF). Exp. Cell Res. 312(5), 522-6.

Eberhard, A., S. Kahlert, V. Goede, B. Hemmerlein, K. H. Plate and H. G. Augustin. 2000. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res. 60(5), 1388-93.

Egginton, S. and M. Gerritsen. 2003. Lumen formation: in vivo versus in vitro observations. Microcirculation. 10(1), 45-61.

Eliceiri, B. P., R. Paul, P. L. Schwartzberg, J. D. Hood, J. Leng and D. A. Cheresh. 1999. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol. Cell. 4(6), 915-24.

Engel, M. E., M. A. McDonnell, B. K. Law and H. L. Moses. 1999. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J. Biol. Chem. 274(52), 37413-20.

Engelhardt, B. 2003. Development of the blood-brain barrier. Cell Tissue Res. 314(1), 119-29.

Ergun, S., N. Kilic, J. H. Wurmbach, A. Ebrahimnejad, M. Fernando, S. Sevinc, E. Kilic, F. Chalajour, W. Fiedler, H. Lauke, K. Lamszus, P. Hammerer, J. Weil, H. Herbst and J. Folkman. 2001. Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes. Angiogenesis. 4(3), 193-206.

Fenstermacher, J., P. Gross, N. Sposito, V. Acuff, S. Pettersen and K. Gruber. 1988. Structural and functional variations in capillary systems within the brain. Ann. N. Y. Acad. Sci. 529, 21-30.

Ferrara, N. 2004. Vascular endothelial growth factor as a target for anticancer therapy. Oncologist. 9 Suppl 1, 2-10.

Ferrara, N. 2004. Vascular endothelial growth factor: basic science and clinical progress. Endocr. Rev. 25(4), 581-611.

Folkman, J. 1971. Tumor angiogenesis: therapeutic implications. N. Engl. J. Med. 285(21), 1182-6.

Folkman, J. 2004. Endogenous angiogenesis inhibitors. APMIS. 112(7-8), 496-507.

Fortin, D., A. Desjardins, A. Benko, T. Niyonsega and M. Boudrias. 2005. Enhanced chemotherapy delivery by intraarterial infusion and blood-brain barrier disruption in malignant brain tumors: the Sherbrooke experience. Cancer. 103(12), 2606-15.

Fukumura, D. and R. K. Jain. 2007. Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. Microvasc. Res. 74(2-3), 72-84.

Fulci, G., M. Labuhn, D. Maier, Y. Lachat, O. Hausmann, M. E. Hegi, R. C. Janzer, A. Merlo and E. G. Van Meir. 2000. p53 gene mutation and ink4a-arf deletion appear to be two mutually exclusive events in human glioblastoma. Oncogene. 19(33), 3816-22.

Furnari, F. B., T. Fenton, R. M. Bachoo, A. Mukasa, J. M. Stommel, A. Stegh, W. C. Hahn, K. L. Ligon, D. N. Louis, C. Brennan, L. Chin, R. A. DePinho and W. K. Cavenee. 2007. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev. 21(21), 2683-710.

Gale, N. W. and G. D. Yancopoulos. 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev. 13(9), 1055-66.

Galli, R., E. Binda, U. Orfanelli, B. Cipelletti, A. Gritti, S. De Vitis, R. Fiocco, C. Foroni, F. Dimeco and A. Vescovi. 2004. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res. 64(19), 7011-21.

Ge, A. Z. and E. C. Butcher. 1994. Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF-beta ligand. Gene. 138(1-2), 201-6.

Gengrinovitch, S., B. Berman, G. David, L. Witte, G. Neufeld and D. Ron. 1999. Glypican-1 is a VEGF165 binding proteoglycan that acts as an extracellular chaperone for VEGF165. J. Biol. Chem. 274(16), 10816-22.

Gerber, H. P., A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit and N. Ferrara. 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. 273(46), 30336-43.

Gerhardt, H. and C. Betsholtz. 2003. Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res. 314(1), 15-23.

Ghilardi, C., G. Chiorino, R. Dossi, Z. Nagy, R. Giavazzi and M. Bani. 2008. Identification of novel vascular markers through gene expression profiling of tumor-derived endothelium. BMC Genomics. 9, 201.

Giese, A., B. Laube, S. Zapf, U. Mangold and M. Westphal. 1998. Glioma cell adhesion and migration on human brain sections. Anticancer Res. 18(4A), 2435-47.

Giese, A., M. A. Loo, S. A. Norman, S. Treasurywala and M. E. Berens. 1996. Contrasting migratory response of astrocytoma cells to tenascin mediated by different integrins. J. Cell Sci. 109 (Pt 8) 2161-8.

Gilles, C., J. A. Bassuk, H. Pulyaeva, E. H. Sage, J. M. Foidart and E. W. Thompson. 1998. SPARC/osteonectin induces matrix metalloproteinase 2 activation in human breast cancer cell lines. Cancer Res. 58(23), 5529-36.

Gladson, C. L. 1996. Expression of integrin alpha v beta 3 in small blood vessels of glioblastoma tumors. J. Neuropathol. Exp. Neurol. 55(11), 1143-9.

Glunde, K., A. P. Pathak and Z. M. Bhujwalla. 2007. Molecular-functional imaging of cancer: to image and imagine. Trends Mol. Med. 13(7), 287-97.

Goldman, E. 1907. The growth of malignant disease in man and the lower animals with special reference to the vascular system. Lancet. 2, 1236-1240.

Goudar, R. K., Q. Shi, M. D. Hjelmeland, S. T. Keir, R. E. McLendon, C. J. Wikstrand, E. D. Reese, C. A. Conrad, P. Traxler, H. A. Lane, D. A. Reardon, W. K. Cavenee, X. F. Wang, D. D. Bigner, H. S. Friedman and J. N. Rich. 2005. Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition. Mol. Cancer Ther. 4(1), 101-12.

Goumans, M. J., F. Lebrin and G. Valdimarsdottir. 2003. Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. Trends Cardiovasc. Med. 13(7), 301-7.

Goumans, M. J., G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras and P. ten Dijke. 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J. 21(7), 1743-53.

Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe and A. J. Giaccia. 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature. 379(6560), 88-91.

Greenberg, D. A. and K. Jin. 2005. From angiogenesis to neuropathology. Nature. 438(7070), 954-9.

Guha, A., K. Dashner, P. M. Black, J. A. Wagner and C. D. Stiles. 1995. Expression of PDGF and PDGF receptors in human astrocytoma operation specimens supports the existence of an autocrine loop. Int. J. Cancer. 60(2), 168-73.

Guo, W. and F. G. Giancotti. 2004. Integrin signalling during tumour progression. Nat Rev. Mol. Cell Biol. 5(10), 816-26.

Hagedorn, M., S. Javerzat, D. Gilges, A. Meyre, B. de Lafarge, A. Eichmann and A. Bikfalvi. 2005. Accessing key steps of human tumor progression in vivo by using an avian embryo model. Proc. Natl. Acad. Sci. U S A. 102(5), 1643-8.

Hamid, S. A., C. Daly and S. Campbell. 2003. Visualization of live endothelial cells ex vivo and in vitro. Microvasc. Res. 66(2), 159-63.

Hashizume, H., P. Baluk, S. Morikawa, J. W. McLean, G. Thurston, S. Roberge, R. K. Jain and D. M. McDonald. 2000. Openings between defective endothelial cells explain tumor vessel leakiness. Am. J. Pathol. 156(4), 1363-80.

Hata, Y., A. Clermont, T. Yamauchi, E. A. Pierce, I. Suzuma, H. Kagokawa, H. Yoshikawa, G. S. Robinson, T. Ishibashi, T. Hashimoto, F. Umeda, S. E. Bursell and L. P. Aiello. 2000. Retinal expression, regulation, and functional bioactivity of prostacyclin-stimulating factor. J. Clin. Invest. 106(4), 541-50.

Haubner, R., W. A. Weber, A. J. Beer, E. Vabuliene, D. Reim, M. Sarbia, K. F. Becker, M. Goebel, R. Hein, H. J. Wester, H. Kessler and M. Schwaiger. 2005. Noninvasive visualization of the activated alphavbeta3 integrin in cancer patients by positron emission tomography and [18F]Galacto-RGD. PLoS Med. 2(3), e70.

Hawkins, B. T. and T. P. Davis. 2005. The blood-brain barrier/neurovascular unit in health and disease. Pharmacol. Rev. 57(2), 173-85.

Helmlinger, G., F. Yuan, M. Dellian and R. K. Jain. 1997. Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat. Med. 3(2), 177-82.

Hermanson, M., K. Funa, M. Hartman, L. Claesson-Welsh, C. H. Heldin, B. Westermark and M. Nister. 1992. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res. 52(11), 3213-9.

Hiles, J. J. and J. M. Kolesar. 2008. Role of sunitinib and sorafenib in the treatment of metastatic renal cell carcinoma. Am. J. Health. Syst. Pharm. 65(2), 123-31.

Hirano, A. and T. Matsui. 1975. Vascular structures in brain tumors. Hum. Pathol. 6(5), 611-21.

Holash, J., P. C. Maisonpierre, D. Compton, P. Boland, C. R. Alexander, D. Zagzag, G. D. Yancopoulos and S. J. Wiegand. 1999. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science. 284(5422), 1994-8.

Hori, S., S. Ohtsuki, K. Hosoya, E. Nakashima and T. Terasaki. 2004. A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. J. Neurochem. 89(2), 503-13.

Hoschuetzky, H., H. Aberle and R. Kemler. 1994. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J. Cell Biol. 127(5), 1375-80.

How, H. K., A. Yeoh, T. C. Quah, Y. Oh, R. G. Rosenfeld and K. O. Lee. 1999. Insulin-like growth factor binding proteins (IGFBPs) and IGFBP-related protein 1-levels in cerebrospinal fluid of children with acute lymphoblastic leukemia. J. Clin. Endocrinol. Metab. 84(4), 1283-7.

Huang, H., S. Colella, M. Kurrer, Y. Yonekawa, P. Kleihues and H. Ohgaki. 2000. Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays. Cancer Res. 60(24), 6868-74.

Hwa, V., Y. Oh and R. G. Rosenfeld. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr. Rev. 20(6), 761-87.

Hwa, V., C. Tomasini-Sprenger, A. L. Bermejo, R. G. Rosenfeld and S. R. Plymate. 1998. Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells. J. Clin. Endocrinol Metab. 83(12), 4355-62.

Ignatova, T. N., V. G. Kukekov, E. D. Laywell, O. N. Suslov, F. D. Vrionis and D. A. Steindler. 2002. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia. 39(3), 193-206.

Inman, G. J., F. J. Nicolas, J. F. Callahan, J. D. Harling, L. M. Gaster, A. D. Reith, N. J. Laping and C. S. Hill. 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. 62(1), 65-74.

Isaka, T., T. Yoshimine, M. Maruno, R. Kuroda, H. Ishii and T. Hayakawa. 1994. Altered expression of antithrombotic molecules in human glioma vessels. Acta Neuropathol. 87(1), 81-5.

Iscove, N. N., M. Barbara, M. Gu, M. Gibson, C. Modi and N. Winegarden. 2002. Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. Nat. Biotechnol. 20(9), 940-3.

Jackson, C. 2002. Matrix metalloproteinases and angiogenesis. Curr. Opin. Nephrol. Hypertens. 11(3), 295-9.

Jain, R. K. 1994. Barriers to drug delivery in solid tumors. Sci. Am. 271(1), 58-65.

Jain, R. K. 2001. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat. Med. 7(9), 987-9.

Jain, R. K. 2003. Molecular regulation of vessel maturation. Nat. Med. 9(6), 685-93.

Jain, R. K. 2005. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 307(5706), 58-62.

Jain, R. K., E. di Tomaso, D. G. Duda, J. S. Loeffler, A. G. Sorensen and T. T. Batchelor. 2007. Angiogenesis in brain tumours. Nat. Rev. Neurosci. 8(8), 610-22.

Jansen, M., P. C. de Witt Hamer, A. N. Witmer, D. Troost and C. J. van Noorden. 2004. Current perspectives on antiangiogenesis strategies in the treatment of malignant gliomas. Brain Res. Brain Res. Rev. 45(3), 143-63.

Jimenez, B., O. V. Volpert, S. E. Crawford, M. Febbraio, R. L. Silverstein and N. Bouck. 2000. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat. Med. 6(1), 41-8.

Johanson, C. E. 1980. Permeability and vascularity of the developing brain: cerebellum vs cerebral cortex. Brain Res. 190(1), 3-16.

Jones, J. I. and D. R. Clemmons. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev. 16(1), 3-34.

Jung, Y. D., S. A. Ahmad, W. Liu, N. Reinmuth, A. Parikh, O. Stoeltzing, F. Fan and L. M. Ellis. 2002. The role of the microenvironment and intercellular cross-talk in tumor angiogenesis. Semin. Cancer Biol. 12(2), 105-12.

Kalluri, R. 2003. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat. Rev. Cancer. 3(6), 422-33.

Kamphaus, G. D., P. C. Colorado, D. J. Panka, H. Hopfer, R. Ramchandran, A. Torre, Y. Maeshima, J. W. Mier, V. P. Sukhatme and R. Kalluri. 2000. Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J. Biol. Chem. 275(2), 1209-15.

Karkkainen, M. J., T. Makinen and K. Alitalo. 2002. Lymphatic endothelium: a new frontier of metastasis research. Nat. Cell Biol. 4(1), E2-5.

Kato, M. V., H. Sato, T. Tsukada, Y. Ikawa, S. Aizawa and M. Nagayoshi. 1996. A follistatin-like gene, mac25, may act as a growth suppressor of osteosarcoma cells. Oncogene. 12(6), 1361-4.

Kaur, B., F. W. Khwaja, E. A. Severson, S. L. Matheny, D. J. Brat and E. G. Van Meir. 2005. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. Neuro. Oncol. 7(2), 134-53.

Keeton, M. R., S. A. Curriden, A. J. van Zonneveld and D. J. Loskutoff. 1991. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. J. Biol. Chem. 266(34), 23048-52.

Kilic, N., H. Lauke, W. Fiedler, T. Pottek, E. Kilic, M. R. Freeman and S. Ergun. 1999. Angiogenic switch and vascular stability in human Leydig cell tumours. Angiogenesis. 3(3), 231-40.

Kim, H. S., S. R. Nagalla, Y. Oh, E. Wilson, C. T. Roberts, Jr. and R. G. Rosenfeld. 1997. Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc. Natl. Acad. Sci. U S A. 94(24), 12981-6.

Kim, S., K. Bell, S. A. Mousa and J. A. Varner. 2000. Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am. J. Pathol. 156(4), 1345-62.

Kishibe, J., S. Yamada, Y. Okada, J. Sato, A. Ito, K. Miyazaki and K. Sugahara. 2000. Structural requirements of heparan sulfate for the binding to the tumor-derived adhesion factor/angiomodulin that induces cord-like structures to ECV-304 human carcinoma cells. J. Biol. Chem. 275(20), 15321-9.

Kleihues, P. and H. Ohgaki. 1999. Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro. Oncol. 1(1), 44-51.

Kleihues, P., F. Soylemezoglu, B. Schauble, B. W. Scheithauer and P. C. Burger. 1995. Histopathology, classification, and grading of gliomas. Glia. 15(3), 211-21.

Kniesel, U. and H. Wolburg. 2000. Tight junctions of the blood-brain barrier. Cell Mol. Neurobiol. 20(1), 57-76.

Knighton, D., D. Ausprunk, D. Tapper and J. Folkman. 1977. Avascular and vascular phases of tumour growth in the chick embryo. Br. J. Cancer. 35(3), 347-56.

Koivunen, E., W. Arap, H. Valtanen, A. Rainisalo, O. P. Medina, P. Heikkila, C. Kantor, C. G. Gahmberg, T. Salo, Y. T. Konttinen, T. Sorsa, E. Ruoslahti and R. Pasqualini. 1999. Tumor targeting with a selective gelatinase inhibitor. Nat. Biotechnol. 17(8), 768-74.

Komatsu, S., Y. Okazaki, M. Tateno, J. Kawai, H. Konno, M. Kusakabe, A. Yoshiki, M. Muramatsu, W. A. Held and Y. Hayashizaki. 2000. Methylation and downregulated expression of mac25/insulin-like growth factor binding protein-7 is associated with liver tumorigenesis in SV40T/t antigen transgenic mice, screened by restriction landmark genomic scanning for methylation (RLGS-M). Biochem. Biophys. Res. Commun. 267(1), 109-17.

Konerding, M. A., W. Malkusch, B. Klapthor, C. van Ackern, E. Fait, S. A. Hill, C. Parkins, D. J. Chaplin, M. Presta and J. Denekamp. 1999. Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. Br. J. Cancer. 80(5-6), 724-32.

Konig, R., D. Baldessari, N. Pollet, C. Niehrs and R. Eils. 2004. Reliability of gene expression ratios for cDNA microarrays in multiconditional experiments with a reference design. Nucleic Acids Res. 32(3), e29.

Kubes, P. 1995. Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. Microcirculation. 2(3), 235-44.

Kwak, H. J., M. J. Park, H. Cho, C. M. Park, S. I. Moon, H. C. Lee, I. C. Park, M. S. Kim, C. H. Rhee and S. I. Hong. 2006. Transforming growth factor-beta1 induces tissue inhibitor of metalloproteinase-1 expression via activation of extracellular signal-regulated kinase and Sp1 in human fibrosarcoma cells. Mol. Cancer Res. 4(3), 209-20.

Landberg, G., H. Ostlund, N. H. Nielsen, G. Roos, S. Emdin, A. M. Burger and A. Seth. 2001. Downregulation of the potential suppressor gene IGFBP-rP1 in human breast cancer is associated with inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation in estrogen receptor negative tumors. Oncogene. 20(27), 3497-505.

Lang, F. F., D. C. Miller, M. Koslow and E. W. Newcomb. 1994. Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. J. Neurosurg. 81(3), 427-36.

Lebrin, F., M. Deckers, P. Bertolino and P. Ten Dijke. 2005. TGF-beta receptor function in the endothelium. Cardiovasc. Res. 65(3), 599-608.

Lee, W. S., M. K. Jain, B. M. Arkonac, D. Zhang, S. Y. Shaw, S. Kashiki, K. Maemura, S. L. Lee, N. K. Hollenberg, M. E. Lee and E. Haber. 1998. Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. Circ. Res. 82(8), 845-51.

Leenders, W. P., B. Kusters and R. M. de Waal. 2002. Vessel co-option: how tumors obtain blood supply in the absence of sprouting angiogenesis. Endothelium. 9(2), 83-7.

Lefranc, F., J. Brotchi and R. Kiss. 2005. Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. J. Clin. Oncol. 23(10), 2411-22.

Leon, S. P., R. D. Folkerth and P. M. Black. 1996. Microvessel density is a prognostic indicator for patients with astroglial brain tumors. Cancer. 77(2), 362-72.

Lesniak, M. S., R. Langer and H. Brem. 2001. Drug delivery to tumors of the central nervous system. Curr. Neurol. Neurosci. Rep. 1(3), 210-6.

Liang, Y., M. Diehn, N. Watson, A. W. Bollen, K. D. Aldape, M. K. Nicholas, K. R. Lamborn, M. S. Berger, D. Botstein, P. O. Brown and M. A. Israel. 2005. Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. Proc. Natl. Acad. Sci. U S A. 102(16), 5814-9.

Liebner, S., A. Fischmann, G. Rascher, F. Duffner, E. H. Grote, H. Kalbacher and H. Wolburg. 2000. Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. Acta Neuropathol. (Berl). 100(3), 323-31.

Lin, J., M. Lai, Q. Huang, Y. Ma, J. Cui and W. Ruan. 2007. Methylation patterns of IGFBP7 in colon cancer cell lines are associated with levels of gene expression. J. Pathol. 212(1), 83-90.

Long, D. M. 1970. Capillary ultrastructure and the blood-brain barrier in human malignant brain tumors. J. Neurosurg. 32(2), 127-44.

Louis, D. N. 2006. Molecular pathology of malignant gliomas. Annu. Rev. Pathol. 1, 97-117.

Luwor, R. B., A. H. Kaye and H. J. Zhu. 2008. Transforming growth factor-beta (TGF-beta) and brain tumours. J. Clin. Neurosci. 15(8), 845-55.

Lyden, D., K. Hattori, S. Dias, C. Costa, P. Blaikie, L. Butros, A. Chadburn, B. Heissig, W. Marks, L. Witte, Y. Wu, D. Hicklin, Z. Zhu, N. R. Hackett, R. G. Crystal, M. A. Moore, K. A. Hajjar, K. Manova, R. Benezra and S. Rafii. 2001. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat. Med. 7(11), 1194-201.

MacDonald, T. J., T. Taga, H. Shimada, P. Tabrizi, B. V. Zlokovic, D. A. Cheresh and W. E. Laug. 2001. Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery. 48(1), 151-7.

Machein, M. R., J. Kullmer, B. L. Fiebich, K. H. Plate and P. C. Warnke. 1999. Vascular endothelial growth factor expression, vascular volume, and, capillary permeability in human brain tumors. Neurosurgery. 44(4), 732-40; discussion 740-1.

Madden, S. L., B. P. Cook, M. Nacht, W. D. Weber, M. R. Callahan, Y. Jiang, M. R. Dufault, X. Zhang, W. Zhang, J. Walter-Yohrling, C. Rouleau, V. R. Akmaev, C. J. Wang, X. Cao, T. B. St Martin, B. L. Roberts, B. A. Teicher, K. W. Klinger, R. V. Stan, B. Lucey, E. B. Carson-Walter, J. Laterra and K. A. Walter. 2004. Vascular gene expression in nonneoplastic and malignant brain. Am. J. Pathol. 165(2), 601-8.

Maniotis, A. J., R. Folberg, A. Hess, E. A. Seftor, L. M. Gardner, J. Pe'er, J. M. Trent, P. S. Meltzer and M. J. Hendrix. 1999. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. Am. J. Pathol. 155(3), 739-52.

Mariani, L., C. Beaudry, W. S. McDonough, D. B. Hoelzinger, T. Demuth, K. R. Ross, T. Berens, S. W. Coons, G. Watts, J. M. Trent, J. S. Wei, A. Giese and M. E. Berens. 2001. Glioma cell motility is associated with reduced transcription of proapoptotic and proliferation genes: a cDNA microarray analysis. J. Neurooncol. 53(2), 161-76.

Mason, W. P. and J. G. Cairncross. 2005. Drug Insight: temozolomide as a treatment for malignant glioma--impact of a recent trial. Nat. Clin. Pract. Neurol. 1(2), 88-95.

Massague, J. 1998. TGF-beta signal transduction. Annu. Rev. Biochem. 67, 753-91.

Max, R., R. R. Gerritsen, P. T. Nooijen, S. L. Goodman, A. Sutter, U. Keilholz, D. J. Ruiter and R. M. De Waal. 1997. Immunohistochemical analysis of integrin alpha vbeta3 expression on tumor-associated vessels of human carcinomas. Int. J. Cancer. 71(3), 320-4.

McCarty, J. H., R. A. Monahan-Earley, L. F. Brown, M. Keller, H. Gerhardt, K. Rubin, M. Shani, H. F. Dvorak, H. Wolburg, B. L. Bader, A. M. Dvorak and R. O. Hynes. 2002. Defective associations between blood vessels and brain parenchyma lead to cerebral hemorrhage in mice lacking alphav integrins. Mol. Cell Biol. 22(21), 7667-77.

McDonald, K. L., M. G. O'Sullivan, J. F. Parkinson, J. M. Shaw, C. A. Payne, J. M. Brewer, L. Young, D. J. Reader, H. T. Wheeler, R. J. Cook, M. T. Biggs, N. S. Little, C. Teo, G. Stone and B. G. Robinson. 2007. IQGAP1 and IGFBP2: valuable biomarkers for determining prognosis in glioma patients. J. Neuropathol. Exp Neurol. 66(5), 405-17.

Mettouchi, A., S. Klein, W. Guo, M. Lopez-Lago, E. Lemichez, J. K. Westwick and F. G. Giancotti. 2001. Integrin-specific activation of Rac controls progression through the G(1) phase of the cell cycle. Mol. Cell. 8(1), 115-27.

Milkiewicz, M., E. Ispanovic, J. L. Doyle and T. L. Haas. 2006. Regulators of angiogenesis and strategies for their therapeutic manipulation. Int. J. Biochem. Cell Biol. 38(3), 333-57.

Miller, C. R. and A. Perry. 2007. Glioblastoma. Arch. Pathol. Lab. Med. 131(3), 397-406.

Miller, S. A., D. D. Dykes and H. F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16(3), 1215.

Mischel, P. S., S. F. Nelson and T. F. Cloughesy. 2003. Molecular analysis of glioblastoma: pathway profiling and its implications for patient therapy. Cancer Biol. Ther. 2(3), 242-7.

Misra, A., S. Ganesh, A. Shahiwala and S. P. Shah. 2003. Drug delivery to the central nervous system: a review. J. Pharm. Pharm. Sci. 6(2), 252-73.

Miyagami, M., M. Tazoe and S. Nakamura. 1998. Expression of vascular endothelial growth factor and p53 protein in association with neovascularization in human malignant gliomas. Brain Tumor Pathol. 15(2), 95-100.

Mojsilovic-Petrovic, J., M. Nesic, A. Pen, W. Zhang and D. Stanimirovic. 2004. Development of rapid staining protocols for laser-capture microdissection of brain vessels from human and rat coupled to gene expression analyses. J. Neurosci. Methods. 133(1-2), 39-48.

Moreno, M. J., M. Ball, M. F. Andrade, A. McDermid and D. B. Stanimirovic. 2006. Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells. Glia. 53(8), 845-57.

Morikawa, S., P. Baluk, T. Kaidoh, A. Haskell, R. K. Jain and D. M. McDonald. 2002. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. 160(3), 985-1000.

Murphy, M., M. J. Pykett, P. Harnish, K. D. Zang and D. L. George. 1993. Identification and characterization of genes differentially expressed in meningiomas. Cell Growth Differ. 4(9), 715-22.

Mutaguchi, K., H. Yasumoto, K. Mita, A. Matsubara, H. Shiina, M. Igawa, R. Dahiya and T. Usui. 2003. Restoration of insulin-like growth factor binding protein-related protein 1 has a tumor-suppressive activity through induction of apoptosis in human prostate cancer. Cancer Res. 63(22), 7717-23.

Nagakubo, D., T. Murai, T. Tanaka, T. Usui, M. Matsumoto, K. Sekiguchi and M. Miyasaka. 2003. A high endothelial venule secretory protein, mac25/angiomodulin, interacts with multiple high endothelial venule-associated molecules including chemokines. J. Immunol. 171(2), 553-61.

Nagane, M., H. Lin, W. K. Cavenee and H. J. Huang. 2001. Aberrant receptor signaling in human malignant gliomas: mechanisms and therapeutic implications. Cancer Lett. 162, Suppl S17-S21.

Nakamura, M., K. Shimada, E. Ishida, H. Nakase and N. Konishi. 2007. Genetic analysis to complement histopathological diagnosis of brain tumors. Histol. Histopathol. 22(3), 327-35.

Newcomb, E. W., H. Cohen, S. R. Lee, S. K. Bhalla, J. Bloom, R. L. Hayes and D. C. Miller. 1998. Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFR, MDM2 or Bcl-2 genes. Brain Pathol. 8(4), 655-67.

Nico, B., V. Benagiano, D. Mangieri, N. Maruotti, A. Vacca and D. Ribatti. 2008. Evaluation of microvascular density in tumors: pro and contra. Histol Histopathol. 23(5), 601-7.

Nicosia, R. F., S. V. Nicosia and M. Smith. 1994. Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. Am. J. Pathol. 145(5), 1023-9.

Nisato, R. E., J. C. Tille, A. Jonczyk, S. L. Goodman and M. S. Pepper. 2003. alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. Angiogenesis. 6(2), 105-19.

Nishikawa, R., X. D. Ji, R. C. Harmon, C. S. Lazar, G. N. Gill, W. K. Cavenee and H. J. Huang. 1994. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc. Natl. Acad. Sci. U S A. 91(16), 7727-31.

Nishishita, T. and P. C. Lin. 2004. Angiopoietin 1, PDGF-B, and TGF-beta gene regulation in endothelial cell and smooth muscle cell interaction. J. Cell Biochem. 91(3), 584-93.

Norris, A. W. and C. R. Kahn. 2006. Analysis of gene expression in pathophysiological states: balancing false discovery and false negative rates. Proc. Natl. Acad. Sci. U S A. 103(3), 649-53.

Nutt, C. L., D. R. Mani, R. A. Betensky, P. Tamayo, J. G. Cairncross, C. Ladd, U. Pohl, C. Hartmann, M. E. McLaughlin, T. T. Batchelor, P. M. Black, A. von Deimling, S. L. Pomeroy, T. R. Golub and D. N. Louis. 2003. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. Cancer Res. 63(7), 1602-7.

O'Reilly, M. S., T. Boehm, Y. Shing, N. Fukai, G. Vasios, W. S. Lane, E. Flynn, J. R. Birkhead, B. R. Olsen and J. Folkman. 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell. 88(2), 277-85.

O'Reilly, M. S., L. Holmgren, Y. Shing, C. Chen, R. A. Rosenthal, M. Moses, W. S. Lane, Y. Cao, E. H. Sage and J. Folkman. 1994. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell. 79(2), 315-28.

Oh, Y., S. R. Nagalla, Y. Yamanaka, H. S. Kim, E. Wilson and R. G. Rosenfeld. 1996. Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II. J. Biol. Chem. 271(48), 30322-5.

Oldendorf, W. H., M. E. Cornford and W. J. Brown. 1977. The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. Ann. Neurol. 1(5), 409-17.

Olofsson, A., K. Miyazono, T. Kanzaki, P. Colosetti, U. Engstrom and C. H. Heldin. 1992. Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. J. Biol. Chem. 267(27), 19482-8.

Papadopoulos, M. C., S. Saadoun, D. K. Binder, G. T. Manley, S. Krishna and A. S. Verkman. 2004. Molecular mechanisms of brain tumor edema. Neuroscience. 129(4), 1011-20.

Papadopoulos, M. C., S. Saadoun, C. J. Woodrow, D. C. Davies, P. Costa-Martins, R. F. Moss, S. Krishna and B. A. Bell. 2001. Occludin expression in microvessels of neoplastic and non-neoplastic human brain. Neuropathol. Appl. Neurobiol. 27(5), 384-95.

Pardali, K. and A. Moustakas. 2007. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim. Biophys. Acta. 1775(1), 21-62.

Park, J. E., H. H. Chen, J. Winer, K. A. Houck and N. Ferrara. 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J. Biol. Chem. 269(41), 25646-54.

Park, M. and S. T. Lee. 1999. The fourth immunoglobulin-like loop in the extracellular domain of FLT-1, a VEGF receptor, includes a major heparin-binding site. Biochem. Biophys. Res. Commun. 264(3), 730-4.

Patan, S., L. L. Munn and R. K. Jain. 1996. Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: a novel mechanism of tumor angiogenesis. Microvasc. Res. 51(2), 260-72.

Pen, A., M. J. Moreno, Y. Durocher, P. Deb-Rinker and D. B. Stanimirovic. 2008. Glioblastoma-secreted factors induce IGFBP7 and angiogenesis by modulating Smad-2-dependent TGF-beta signaling. Oncogene. 27(54), 6834-44.

Pen, A., M. J. Moreno, J. Martin and D. B. Stanimirovic. 2007. Molecular markers of extracellular matrix remodeling in glioblastoma vessels: microarray study of laser-captured glioblastoma vessels. Glia. 55(6), 559-72.

Pepper, M. S. 1997. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev. 8(1), 21-43.

Pepper, M. S. 2001. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler. Thromb. Vasc. Biol. 21(7), 1104-17.

Pepper, M. S., N. Ferrara, L. Orci and R. Montesano. 1991. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem. Biophys. Res. Commun. 181(2), 902-6.

Pfeifer, A., T. Kessler, S. Silletti, D. A. Cheresh and I. M. Verma. 2000. Suppression of angiogenesis by lentiviral delivery of PEX, a noncatalytic fragment of matrix metalloproteinase 2. Proc. Natl. Acad. Sci. U S A. 97(22), 12227-32.

Plate, K. H. 1999. Mechanisms of angiogenesis in the brain. J. Neuropathol. Exp. Neurol. 58(4), 313-20.

Plymate, S. R., K. H. Haugk, C. C. Sprenger, P. S. Nelson, M. K. Tennant, Y. Zhang, L. W. Oberley, W. Zhong, R. Drivdahl and T. D. Oberley. 2003. Increased manganese superoxide dismutase (SOD-2) is part of the mechanism for prostate tumor suppression by Mac25/insulin-like growth factor binding-protein-related protein-1. Oncogene. 22(7), 1024-34.

Poncelet, A. C. and H. W. Schnaper. 1998. Regulation of human mesangial cell collagen expression by transforming growth factor-beta1. Am. J. Physiol. 275(3 Pt 2), F458-66.

Pope, W. B., A. Lai, P. Nghiemphu, P. Mischel and T. F. Cloughesy. 2006. MRI in patients with high-grade gliomas treated with bevacizumab and chemotherapy. Neurology. 66(8), 1258-60.

Pugh, C. W. and P. J. Ratcliffe. 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat. Med. 9(6), 677-84.

Ramsauer, M. and P. A. D'Amore. 2007. Contextual role for angiopoietins and TGFbeta1 in blood vessel stabilization. J. Cell Sci. 120(Pt 10), 1810-7.

Rao, J. S. 2003. Molecular mechanisms of glioma invasiveness: the role of proteases. Nat. Rev. Cancer. 3(7), 489-501.

Reed, M. J., R. B. Vernon, I. B. Abrass and E. H. Sage. 1994. TGF-beta 1 induces the expression of type I collagen and SPARC, and enhances contraction of collagen gels, by fibroblasts from young and aged donors. J. Cell Physiol. 158(1), 169-79.

Rempel, S. A., W. A. Golembieski, S. Ge, N. Lemke, K. Elisevich, T. Mikkelsen and J. A. Gutierrez. 1998. SPARC: a signal of astrocytic neoplastic transformation and reactive response in human primary and xenograft gliomas. J. Neuropathol. Exp. Neurol. 57(12), 1112-21.

Reynolds, B. A. and S. Weiss. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 255(5052), 1707-10.

Ribatti, D., B. Nico, A. Vacca, L. Roncali, P. H. Burri and V. Djonov. 2001. Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat. Rec. 264(4), 317-24.

Rich, J. N., C. Hans, B. Jones, E. S. Iversen, R. E. McLendon, B. K. Rasheed, A. Dobra, H. K. Dressman, D. D. Bigner, J. R. Nevins and M. West. 2005. Gene expression profiling and genetic markers in glioblastoma survival. Cancer Res. 65(10), 4051-8.

Risau, W. 1997. Mechanisms of angiogenesis. Nature. 386(6626), 671-4.

Ronnov-Jessen, L. and O. W. Petersen. 1996. A function for filamentous alpha-smooth muscle actin: retardation of motility in fibroblasts. J. Cell. Biol. 134(1), 67-80.

Rossant, J. and L. Howard. 2002. Signaling pathways in vascular development. Annu. Rev. Cell Dev. Biol. 18, 541-73.

Ruan, W., E. Xu, F. Xu, Y. Ma, H. Deng, Q. Huang, B. Lv, H. Hu, J. Lin, J. Cui, M. Di, J. Dong and M. Lai. 2007. IGFBP7 plays a potential tumor suppressor role in colorectal carcinogenesis. Cancer Biol. Ther. 6(3), 354-9.

Sage, E. H., M. Reed, S. E. Funk, T. Truong, M. Steadele, P. Puolakkainen, D. H. Maurice and J. A. Bassuk. 2003. Cleavage of the matricellular protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis. J. Biol. Chem. 278(39), 37849-57.

Saksela, O. and D. B. Rifkin. 1990. Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. J. Cell Biol. 110(3), 767-75.

Saleh, M., S. A. Stacker and A. F. Wilks. 1996. Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial growth factor sequence. Cancer Res. 56(2), 393-401.

Sallinen, S. L., P. K. Sallinen, H. K. Haapasalo, H. J. Helin, P. T. Helen, P. Schraml, O. P. Kallioniemi and J. Kononen. 2000. Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. Cancer Res. 60(23), 6617-22.

Santarelli, J. G., V. Udani, Y. C. Yung, S. Cheshier, A. Wagers, R. A. Brekken, I. Weissman and V. Tse. 2006. Incorporation of bone marrow-derived Flk-1-expressing CD34+ cells in the endothelium of tumor vessels in the mouse brain. Neurosurgery. 59(2), 374-82; discussion 374-82.

Sasaki, H., S. Moriyama, H. Yukiue, Y. Kobayashi, Y. Nakashima, M. Kaji, I. Fukai, M. Kiriyama, Y. Yamakawa and Y. Fujii. 2001. Expression of the antiapoptosis gene, AAC-11, as a prognosis marker in non-small cell lung cancer. Lung Cancer. 34(1), 53-7.

Sathornsumetee, S., D. A. Reardon, A. Desjardins, J. A. Quinn, J. J. Vredenburgh and J. N. Rich. 2007. Molecularly targeted therapy for malignant glioma. Cancer. 110(1), 13-24.

Sato, J., S. Hasegawa, K. Akaogi, H. Yasumitsu, S. Yamada, K. Sugahara and K. Miyazaki. 1999. Identification of cell-binding site of angiomodulin (AGM/TAF/Mac25) that interacts with heparan sulfates on cell surface. J. Cell Biochem. 75(2), 187-95.

Sato, Y., Z. Chen and K. Miyazaki. 2007. Strong suppression of tumor growth by insulin-like growth factor-binding protein-related protein 1/tumor-derived cell adhesion factor/mac25. Cancer Sci. 98(7), 1055-63.

Sawada, T., Y. Kato, M. Kobayashi and Y. Takekekawa. 2000. Immunohistochemical study of tight junction-related protein in neovasculature in astrocytic tumor. Brain Tumor Pathol. 17(1), 1-6.

Schiera, G., E. Bono, M. P. Raffa, A. Gallo, G. L. Pitarresi, I. Di Liegro and G. Savettieri. 2003. Synergistic effects of neurons and astrocytes on the differentiation of brain capillary endothelial cells in culture. J. Cell. Mol. Med. 7(2), 165-70.

Schinkel, A. H., J. J. Smit, O. van Tellingen, J. H. Beijnen, E. Wagenaar, L. van Deemter, C. A. Mol, M. A. van der Valk, E. C. Robanus-Maandag, H. P. te Riele and et al. 1994. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell. 77(4), 491-502.

Schlageter, K. E., P. Molnar, G. D. Lapin and D. R. Groothuis. 1999. Microvessel organization and structure in experimental brain tumors: microvessel populations with distinctive structural and functional properties. Microvasc Res. 58(3), 312-28.

Schultz, C., N. Lemke, S. Ge, W. A. Golembieski and S. A. Rempel. 2002. Secreted protein acidic and rich in cysteine promotes glioma invasion and delays tumor growth in vivo. Cancer Res. 62(21), 6270-7.

Seaman, S., J. Stevens, M. Y. Yang, D. Logsdon, C. Graff-Cherry and B. St Croix. 2007. Genes that distinguish physiological and pathological angiogenesis. Cancer Cell. 11(6), 539-54.

Sedlakova, R., R. R. Shivers and R. F. Del Maestro. 1999. Ultrastructure of the blood-brain barrier in the rabbit. J. Submicrosc. Cytol. Pathol. 31(1), 149-61.

Sharma, S., M. C. Sharma, D. K. Gupta and C. Sarkar. 2006. Angiogenic patterns and their quantitation in high grade astrocytic tumors. J. Neurooncol. 79(1), 19-30.

Shweiki, D., A. Itin, D. Soffer and E. Keshet. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature. 359(6398), 843-5.

Singh, S. K., I. D. Clarke, M. Terasaki, V. E. Bonn, C. Hawkins, J. Squire and P. B. Dirks. 2003. Identification of a cancer stem cell in human brain tumors. Cancer Res. 63(18), 5821-8.

Singh, S. K., C. Hawkins, I. D. Clarke, J. A. Squire, J. Bayani, T. Hide, R. M. Henkelman, M. D. Cusimano and P. B. Dirks. 2004. Identification of human brain tumour initiating cells. Nature. 432(7015), 396-401.

Sprenger, C. C., S. E. Damon, V. Hwa, R. G. Rosenfeld and S. R. Plymate. 1999. Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. Cancer Res. 59(10), 2370-5.

Sprenger, C. C., M. E. Vail, K. Evans, J. Simurdak and S. R. Plymate. 2002. Over-expression of insulin-like growth factor binding protein-related protein-1(IGFBP-rP1/mac25) in the M12 prostate cancer cell line alters tumor growth by a delay in G1 and cyclin A associated apoptosis. Oncogene. 21(1), 140-7.

St Croix, B., C. Rago, V. Velculescu, G. Traverso, K. E. Romans, E. Montgomery, A. Lal, G. J. Riggins, C. Lengauer, B. Vogelstein and K. W. Kinzler. 2000. Genes expressed in human tumor endothelium. Science. 289(5482), 1197-202.

Stanimirovic, D., P. Morley, R. Ball, E. Hamel, G. Mealing and J. P. Durkin. 1996. Angiotensin II-induced fluid phase endocytosis in human cerebromicrovascular endothelial cells is regulated by the inositol-phosphate signaling pathway. J. Cell Physiol. 169(3), 455-67.

Stevens, A., I. Kloter and W. Roggendorf. 1988. Inflammatory infiltrates and natural killer cell presence in human brain tumors. Cancer. 61(4), 738-43.

Stewart, P. A., C. L. Farrell and R. F. Del Maestro. 1991. The effect of cellular microenvironment on vessels in the brain. Part 1: Vessel structure in tumour, peritumour and brain from humans with malignant glioma. Int. J. Radiat. Biol. 60(1-2), 125-30.

Stewart, P. A. and M. J. Wiley. 1981. Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail--chick transplantation chimeras. Dev. Biol. 84(1), 183-92.

Stiver, S. I., X. Tan, L. F. Brown, E. T. Hedley-Whyte and H. F. Dvorak. 2004. VEGF-A angiogenesis induces a stable neovasculature in adult murine brain. J. Neuropathol. Exp. Neurol. 63(8), 841-55.

Stratmann, A., W. Risau and K. H. Plate. 1998. Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. Am. J. Pathol. 153(5), 1459-66.

Stubbs, M. T., M. Renatus and W. Bode. 1998. An active zymogen: unravelling the mystery of tissue-type plasminogen activator. Biol. Chem. 379(2), 95-103.

Su, E. J., C. L. Cioffi, S. Stefansson, N. Mittereder, M. Garay, D. Hreniuk and G. Liau. 2003. Gene therapy vector-mediated expression of insulin-like growth factors protects cardiomyocytes from apoptosis and enhances neovascularization. Am. J. Physiol. Heart Circ. Physiol. 284(4), H1429-40.

Swisshelm, K., K. Ryan, K. Tsuchiya and R. Sager. 1995. Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. Proc. Natl. Acad. Sci. U S A. 92(10), 4472-6.

Takamiya, Y., Y. Abe, Y. Tanaka, A. Tsugu, M. Kazuno, Y. Oshika, K. Maruo, Y. Ohnishi, O. Sato, H. Yamazaki, H. Kijima, Y. Ueyama, N. Tamaoki and M. Nakamura. 1997. Murine P-glycoprotein on stromal vessels mediates multidrug resistance in intracerebral human glioma xenografts. Br. J. Cancer. 76(4), 445-50.

Tatum, J. L., G. J. Kelloff, R. J. Gillies, J. M. Arbeit, J. M. Brown, K. S. Chao, J. D. Chapman, W. C. Eckelman, A. W. Fyles, A. J. Giaccia, R. P. Hill, C. J. Koch, M. C. Krishna, K. A. Krohn, J. S. Lewis, R. P. Mason, G. Melillo, A. R. Padhani, G. Powis, J. G. Rajendran, R. Reba, S. P. Robinson, G. L. Semenza, H. M. Swartz, P. Vaupel, D. Yang, B. Croft, J. Hoffman, G. Liu, H. Stone and D. Sullivan. 2006. Hypoxia: importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. Int. J. Radiat. Biol. 82(10), 699-757.

ten Dijke, P., P. Hansen, K. K. Iwata, C. Pieler and J. G. Foulkes. 1988. Identification of another member of the transforming growth factor type beta gene family. Proc. Natl. Acad. Sci. USA. 85(13), 4715-9.

Tong, R. T., Y. Boucher, S. V. Kozin, F. Winkler, D. J. Hicklin and R. K. Jain. 2004. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res. 64(11), 3731-6.

Trojan, J., J. F. Cloix, M. Y. Ardourel, M. Chatel and D. D. Anthony. 2007. Insulin-like growth factor type I biology and targeting in malignant gliomas. Neuroscience. 145(3), 795-811.

Tuor, U. I., G. Kurpita and C. Simone. 1994. Correlation of local changes in cerebral blood flow, capillary density, and cytochrome oxidase during development. J. Comp. Neurol. 342(3), 439-48.

Uhm, J. H., C. L. Gladson and J. S. Rao. 1999. The role of integrins in the malignant phenotype of gliomas. Front. Biosci. 4, D188-99.

Umeda, F., Y. Ono, N. Sekiguchi, T. Hashimoto, M. Masakado, K. Nakamura, Y. Chijiiwa and H. Nawata. 1998. Increased mRNA expression of a novel prostacyclin-stimulating factor in human colon cancer. J. Gastroenterol. 33(2), 213-7.

Vajkoczy, P., R. Goldbrunner, M. Farhadi, G. Vince, L. Schilling, J. C. Tonn, P. Schmiedek and M. D. Menger. 1999. Glioma cell migration is associated with glioma-induced angiogenesis in vivo. Int. J. Dev. Neurosci. 17(5-6), 557-63.

Vajkoczy, P. and M. D. Menger. 2000. Vascular microenvironment in gliomas. J. Neurooncol. 50(1-2), 99-108.

Vajkoczy, P., M. D. Menger, R. Goldbrunner, S. Ge, T. A. Fong, B. Vollmar, L. Schilling, A. Ullrich, K. P. Hirth, J. C. Tonn, P. Schmiedek and S. A. Rempel. 2000. Targeting angiogenesis inhibits tumor infiltration and expression of the pro-invasive protein SPARC. Int. J. Cancer. 87(2), 261-8.

van Beijnum, J. R., R. P. Dings, E. van der Linden, B. M. Zwaans, F. C. Ramaekers, K. H. Mayo and A. W. Griffioen. 2006. Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. Blood. 108(7), 2339-48.

van der Schaft, D. W., R. P. Dings, Q. G. de Lussanet, L. I. van Eijk, A. W. Nap, R. G. Beets-Tan, J. C. Bouma-Ter Steege, J. Wagstaff, K. H. Mayo and A. W. Griffioen. 2002. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. FASEB J. 16(14), 1991-3.

Vaupel, P., O. Thews, D. K. Kelleher and M. Hoeckel. 1998. Oxygenation of human tumors: the Mainz experience. Strahlenther Onkol. 174 Suppl 4, 6-12.

von Tell, D., A. Armulik and C. Betsholtz. 2006. Pericytes and vascular stability. Exp. Cell Res. 312(5), 623-9.

Vredenburgh, J. J., A. Desjardins, J. E. Herndon, 2nd, J. M. Dowell, D. A. Reardon, J. A. Quinn, J. N. Rich, S. Sathornsumetee, S. Gururangan, M. Wagner, D. D. Bigner, A. H. Friedman and H. S. Friedman. 2007. Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. Clin. Cancer Res. 13(4), 1253-9.

Wajapeyee, N., R. W. Serra, X. Zhu, M. Mahalingam and M. R. Green. 2008. Oncogenic BRAF Induces Senescence and Apoptosis through Pathways Mediated by the Secreted Protein IGFBP7. Cell. 132(3), 363-74.

Wang, D., J. C. Anderson and C. L. Gladson. 2005. The role of the extracellular matrix in angiogenesis in malignant glioma tumors. Brain Pathol. 15(4), 318-26.

Wang, J. L., Y. H. Liu, M. C. Lee, T. M. Nguyen, C. Lee, A. Kim and M. Nguyen. 2000. Identification of tumor angiogenesis-related genes by subtractive hybridization. Microvasc. Res. 59(3), 394-7.

Wang, M. Y., K. V. Lu, S. Zhu, E. Q. Dia, I. Vivanco, G. M. Shackleford, W. K. Cavenee, I. K. Mellinghoff, T. F. Cloughesy, C. L. Sawyers and P. S. Mischel. 2006. Mammalian target of rapamycin inhibition promotes response to epidermal growth factor receptor kinase inhibitors in PTEN-deficient and PTEN-intact glioblastoma cells. Cancer Res. 66(16), 7864-9.

Ward, N. L. and J. C. Lamanna. 2004. The neurovascular unit and its growth factors: coordinated response in the vascular and nervous systems. Neurol. Res. 26(8), 870-83.

Watabe, T., A. Nishihara, K. Mishima, J. Yamashita, K. Shimizu, K. Miyazawa, S. Nishikawa and K. Miyazono. 2003. TGF-beta receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. J. Cell Biol. 163(6), 1303-11.

Watanabe, K., O. Tachibana, K. Sata, Y. Yonekawa, P. Kleihues and H. Ohgaki. 1996. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol. 6(3), 217-23; discussion 23-4.

Weidner, N. 1995. Intratumor microvessel density as a prognostic factor in cancer. Am. J. Pathol. 147(1), 9-19.

Weller, M., U. Malipiero, P. Groscurth and A. Fontana. 1995. T cell apoptosis induced by interleukin-2 deprivation or transforming growth factor-beta 2: modulation by the phosphatase inhibitors okadaic acid and calyculin A. Exp. Cell Res. 221(2), 395-403.

Wesseling, P., R. O. Schlingemann, F. J. Rietveld, M. Link, P. C. Burger and D. J. Ruiter. 1995. Early and extensive contribution of pericytes/vascular smooth muscle cells to microvascular proliferation in glioblastoma multiforme: an immuno-light and immuno-electron microscopic study. J. Neuropathol. Exp. Neurol. 54(3), 304-10.

Wick, W., M. Platten and M. Weller. 2001. Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta. J. Neurooncol. 53(2), 177-85.

Willis, C. L., C. C. Nolan, S. N. Reith, T. Lister, M. J. Prior, C. J. Guerin, G. Mavroudis and D. E. Ray. 2004. Focal astrocyte loss is followed by microvascular damage, with subsequent repair of the blood-brain barrier in the apparent absence of direct astrocytic contact. Glia. 45(4), 325-37.

Wilson, H. M., R. S. Birnbaum, M. Poot, L. S. Quinn and K. Swisshelm. 2002. Insulin-like growth factor binding protein-related protein 1 inhibits proliferation of MCF-7 breast cancer cells via a senescence-like mechanism. Cell Growth Differ. 13(5), 205-13.

Winkler, F., S. V. Kozin, R. T. Tong, S. S. Chae, M. F. Booth, I. Garkavtsev, L. Xu, D. J. Hicklin, D. Fukumura, E. di Tomaso, L. L. Munn and R. K. Jain. 2004. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell. 6(6), 553-63.

Wolburg, H. and A. Lippoldt. 2002. Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul. Pharmacol. 38(6), 323-37.

Wolburg, H., K. Wolburg-Buchholz, J. Kraus, G. Rascher-Eggstein, S. Liebner, S. Hamm, F. Duffner, E. H. Grote, W. Risau and B. Engelhardt. 2003. Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. Acta Neuropathol. (Berl.). 105(6), 586-92.

Wong, A. J., S. H. Bigner, D. D. Bigner, K. W. Kinzler, S. R. Hamilton and B. Vogelstein. 1987. Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. Proc. Natl. Acad. Sci. U S A. 84(19), 6899-903.

Wu, Y., S. Yakar, L. Zhao, L. Hennighausen and D. LeRoith. 2002. Circulating insulin-like growth factor-I levels regulate colon cancer growth and metastasis. Cancer Res. 62(4), 1030-5.

Yakymovych, I., P. Ten Dijke, C. H. Heldin and S. Souchelnytskyi. 2001. Regulation of Smad signaling by protein kinase C. FASEB J. 15(3), 553-5.

Yamada, N., M. Kato, H. Yamashita, M. Nister, K. Miyazono, C. H. Heldin and K. Funa. 1995. Enhanced expression of transforming growth factor-beta and its type-I and type-II receptors in human glioblastoma. Int. J. Cancer. 62(4), 386-92.

Yamauchi, T., F. Umeda, M. Masakado, M. Isaji, S. Mizushima and H. Nawata. 1994. Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem. J. 303 (Pt 2) 591-8.

Yan, B., H. H. Chour, B. K. Peh, C. Lim and M. Salto-Tellez. 2006. RhoA protein expression correlates positively with degree of malignancy in astrocytomas. Neurosci. Lett. 407(2), 124-6.

Yuan, X., J. Curtin, Y. Xiong, G. Liu, S. Waschsmann-Hogiu, D. L. Farkas, K. L. Black and J. S. Yu. 2004. Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene. 23(58), 9392-400.

Zachary, I. 2003. VEGF signalling: integration and multi-tasking in endothelial cell biology. Biochem. Soc. Trans. 31(Pt 6), 1171-7.

Zagzag, D., H. Zhong, J. M. Scalzitti, E. Laughner, J. W. Simons and G. L. Semenza. 2000. Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. Cancer. 88(11), 2606-18.

Zhang, W., J. Mojsilovic-Petrovic, M. F. Andrade, H. Zhang, M. Ball and D. B. Stanimirovic. 2003. The expression and functional characterization of ABCG2 in brain endothelial cells and vessels. FASEB J. 17(14), 2085-7.

Zhao, H., T. Hastie, M. L. Whitfield, A. L. Borresen-Dale and S. S. Jeffrey. 2002. Optimization and evaluation of T7 based RNA linear amplification protocols for cDNA microarray analysis. BMC Genomics. 3(1), 31.

Zumkeller, W., D. Muller, S. Muller, C. Gunther and M. Westphal. 1998. Expression and synthesis of insulin-like growth factor-binding proteins in human glioma cell lines. Int. J. Oncol. 12(1), 129-35.