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GENERAL INTRODUCTION
INTRODUCTION

Brain tumors include a wide variety of subtypes of which the most common malignant type is glioblastoma multiforme (GBM). In recent years our understanding of the genetic background of these tumors has increased. However, in spite of these advances the prognosis for patients remains poor. Epigenetic and post-transcriptional aberrations have been shown to be involved in a multitude of cancer types. In this thesis we aim to investigate epigenetic and post-transcriptional signaling in the regulation of gene expression in endothelial cells and glioma cells during angiogenesis.

Glioblastoma multiforme

The first systematic description of brain tumors was made by Virchow in 1865 on the basis of microscopic and macroscopic features. In 1926, Bailey and Cushing were the first to devise a classification system that correlated prognosis to the histogenetic features of gliomas. Currently, central nervous system (CNS) tumors are classified according to the 2007 WHO scheme which includes over 100 (sub-)types of central nervous system tumors and grades tumors on the basis of morphological characteristics and biological behavior. GBMs are classified as the most malignant - grade IV - gliomas and display histological features of glial cells.

The incidence rate of GBM is 2 to 3 per 100,000 people in the United States and Europe. The incidence rate increases with age, with most GBMs arising after age 50. However, GBM may occur in all age groups, including children. There are no well-established associations between environmental factors and GBM, however, relations with cranial irradiation and cytomegalovirus have been suggested. An increased incidence of GBM is observed in a number of rare genetic disorders including neurofibromatosis, Turcot and Li-fraumeni syndrome, tuberous sclerosis and Von Hippel Lindau disease.

GBMs are histologically characterized by increased cellularity, cellular atypia, endothelial proliferation, the presence of necrotic areas surrounded by pseudopalisading tumor cells and an infiltrative growth pattern. GBM biology is characterized by a number of tumorigenic features including: growth factor overexpression, loss of cell cycle control, apoptosis dysregulation, migration and infiltrative growth and angiogenesis.

The origin of GBM is subject to controversy. Recent studies suggest that these tumors arise from neural progenitor cells. However, the differentiation stage at which these cells derange has not yet been established. It is also possible that these tumors arise from mature glial cells that have undergone a series of oncogenic mutations. In addition, it is proposed that differentiated cells in the CNS can undergo dedifferentiation to a progenitor state upon specific genetic alterations and may then initiate tumor progression.

The differentiation between tumors that arise de novo - so-called primary - and secondary GBM, which arises from pre-existing lower grade glial tumors, was first made by Scherer. Primary GBM accounts for ~95% of GBM cases and occurs more often in older patients. Secondary GBM is more common in younger patients. Although loss of heterozygosity on chromosome arm 10q is the most frequent gene alteration for both primary and secondary GBM, the two groups differ significantly with regard to the spectrum of genetic changes involved in tumor development. Primary GBM is often characterized by overexpression or
amplification of MDM2, p16 deletions or PTEN mutations. Another frequent genetic alteration in GBM is amplification of EGFR or its mutant allele EGFRvIII. Secondary GBM typically shows TP53 mutation and PDGFR amplification or overexpression. Additional common genetic alterations in secondary GBM include loss of heterozygosity at chromosome arm 19q, loss of DCC gene expression and RB protein alterations. Mutations of IDH1 are characteristic of low grade gliomas and are also frequently present in secondary GBM and are associated with a better outcome. Recently, integrated genomic profiling of GBM revealed four distinct molecular subtypes. These subtypes are referred to as proneural, neural, classical, and mesenchymal, and differed with regard to response to aggressive therapy.

**Diagnosis and treatment**

The major diagnostic modalities in the evaluation of brain tumors are neuroradiologic imaging and histological examination of biopsy specimens. MRI is the imaging technique of choice for brain tumors and is essential for preoperative planning. Moreover, clues to a provisional diagnosis may be provided by the characteristic appearance of specific tumors in different advanced MRI modalities, including perfusion- and diffusion MRI, PET-MRI and MR-spectroscopy. The golden standard in diagnostic evaluation of a brain tumor is histological examination of the biopsy specimen. This consists of analysis of the tumor type as well as an assessment of tumor grade on the basis of tissue morphology by H&E staining and additional immunohistochemical analysis. Molecular markers such as 1p-19q status, MGMT methylation status and (mutant) IDH1 protein expression in glioma are playing an increasing role in tumor characterization and it is expected that this will allow for further treatment stratification and improved treatment results in the future.

Currently, GBMs are treated on the basis of a combined modality approach including maximal surgical resection and adjuvant radiation- and chemotherapy. A 98% resection or more of the contrast-enhancing tumor on MRI is correlated with increased survival. Recent advances in surgical techniques, such as neuronavigation, motor mapping and awake craniotomy, have helped to improve the extent of resection while preserving neurologic functions. However, complete resection is impossible as GBMs are characterized by infiltrative growth of tumor cells along white matter tracts and perivascular spaces that extend outside the tumor margin visible on MRI. As a result, tumor recurrence is inevitable, even in spite of adjuvant chemo-radiation therapy.

Currently, the oral alkylating drug temozolomide is the preferred treatment for adjuvant chemoradiotherapy in GBM. The effect of adding temozolomide to radiation therapy was first demonstrated in a study by Stupp et al, which showed that overall survival was significantly increased by a combination treatment of radiation therapy and temozolomide. Treatment toxicity was shown to be limited and did not affect quality of life. Subsequent studies confirmed this beneficial effect of combined temozolomide and radiation therapy in all clinical prognostic subgroups, including patients aged 60-70 years. Interestingly, methylation of the promoter for MGMT was correlated with outcome and benefit from temozolomide chemotherapy. This suggests MGMT promoter methylation status identifies patients most likely to benefit from the addition of temozolomide. Another target for future therapy is angiogenesis inhibition. Bevacizumab is a monoclonal antibody that binds vascular endothelial
growth factor (VEGF), which is a key mediator of GBM-associated pathological angiogenesis. Bevacizumab reduces steroid requirements and is associated with radiographic response in phase II studies in patients with recurrent glioma. However, the reported response was most likely related to a decrease in brain edema (as evidenced by a reduced contrast-enhancement) but not by tumor regression. It was also reported that bevacizumab treatment led to increased invasion of normal brain areas by tumor cells as an escape mechanism.

In spite of recent treatment improvements using concomitant temozolomide chemoradiotherapy, GBM still carries a poor prognosis with most patients succumbing to their disease within two years. An increased understanding of the molecular pathways involved in GBM progression may provide potential targets for future gene or pharmacological therapies. In addition to genetic changes, epigenetic alterations such as histone modifications and methylation, as well as post-transcriptional gene regulation by microRNAs (miRNAs), also play a role in GBM biology. Here we will discuss the role of miRNAs and epigenetic regulation in aberrant GBM angiogenesis. A brief overview of tumor angiogenesis is presented below. This is followed by a short introduction on epigenetics and miRNA.

ANGIOGENESIS

Angiogenesis is the physiological process of formation of new blood vessels from pre-existent vessels. Angiogenesis plays an important role in growth and development, as cells require oxygen and nutrients for their survival. The formation of blood vessels during embryonic development starts with vasculogenesis: a process where angiogenic progenitor cells group and subsequently differentiate to form endothelial vessels. The maturation of this primitive vascular network into a functional vascular network is achieved through the process of angiogenesis which involves the sprouting, branching or splitting of new capillaries from existent vessels and stabilization of the network by smooth muscle cells and pericytes.

The angiogenesis process is regulated by a balance between pro- and anti-angiogenic factors and is largely dormant in the healthy adult. Only in specific situations, such as in the female reproductive tract and during wound healing, are endothelial cells activated by angiogenic stimuli to undergo neovascularization. In these physiological situations the angiogenic response is tightly coordinated by temporal and spatial cues. However, in various diseases this process is derailed leading to pathological angiogenesis. Diseases associated with pathological angiogenesis include: diabetes, arthritis, atherosclerosis and, most notably, cancer.

Tumor angiogenesis

The observation that angiogenesis occurs around tumors was already made over a 100 years ago. This observation was followed by the hypothesis that tumors produce a diffusible ‘angiogenic’ substance to promote neovascularization. Subsequent research showed that cells acquire angiogenic capacity during tumor progression. This ‘angiogenic switch’ is triggered as a tumor grows beyond a critical size of 2-3 mm and requires more oxygen and nutrients than can be locally supplied through diffusion. As the tumor becomes hypoxic, the tumor cells start to produce and secrete pro-angiogenic factors such as fibroblast growth factor (FGF) and VEGF to stimulate neovascularization.
VEGF is the major growth factor controlling angiogenesis and covers a family of genes including VEGF-A, -B, -C, -D, and PIGF of which VEGF-A is the most important\textsuperscript{57}. VEGF stimulates angiogenesis through the promotion of endothelial cell migration, invasion, proliferation and survival. In addition, VEGF increases microvascular permeability and was originally discovered as vascular permeability factor\textsuperscript{58}. VEGF production is, amongst others, promoted by hypoxia-inducible transcription factor (HIF)\textsuperscript{59}, resulting in a feedback mechanism where tissue hypoxia leads to new blood vessel formation. Members of the VEGF family elicit cellular responses by binding to tyrosine kinase receptors such as Flt-1 and VEGFR-2 on the cell surface\textsuperscript{60}. VEGFR-2 receptor is predominantly located on endothelial cells and is the dominant mediator of VEGF effects\textsuperscript{61}. Although VEGF is the most significant and well-studied among these factors, many others are involved in angiogenesis.

Besides chemical or growth factor stimulation, other triggers that stimulate tumor neovascularization include mechanical stress, immune response and genetic mutations. In addition to angiogenesis and vasculogenesis, Jain and Carmeliet recently described other mechanisms that may contribute to vessel formation in tumors\textsuperscript{62}. Intussusception is the splitting of pre-existing vessels to generate daughter vessels. Vessel co-option is the process where cancer cells grow around and co-opt the existing vasculature. Vascular mimicry occurs when cancer cells get incorporated into the blood vessel wall. Tumor stem cell to endothelial cell differentiation describes the differentiation of cancer stem-like cells into endothelial cells. However, the extent to which these processes are relevant in cancer is still subject to discussion. In addition, the molecular mechanisms that drive the interplay between tumor cells and the environment in the context of angiogenesis are complex and largely unresolved.

The brain vasculature is composed of endothelial cells, pericytes and astrocytes and is characterized by a blood brain barrier (BBB). The BBB restricts the exchange of molecules between the circulating blood and the brain tissue. Similar to neoplasms elsewhere in the body, brain tumors are characterized by hypoxia, rely on a blood supply for progression and demonstrate increased endothelial cell proliferation\textsuperscript{63}. Compared to normal brain vasculature, glioma blood vessels are characterized by an irregular shape, dilatation and perivascular detachment. In addition, while the normal vasculature in the brain is characterized by a BBB, this barrier is, at least partly, disrupted in brain tumors. The increased permeability of the BBB results in vasogenic edema - the increased accumulation of fluid and plasma proteins - peritumorally and in the surrounding brain\textsuperscript{64}. Moreover, disruption of the BBB leads to contrast-enhancement of brain tumors on CT and MRI following i.v. contrast administration\textsuperscript{65}. The mechanism by which the BBB becomes compromised is not completely understood, however, VEGF-induced hyperpermeability has been shown to play a major role\textsuperscript{63}. In brain tumors VEGF is released by endothelial cells, as well as by tumor cells, and is stimulated by hypoxia but also by genetic and epigenetic changes and various signaling molecules.

The extent to which the previously described cellular mechanisms of new vessel formation are active in human GBM is not entirely clear. It is generally accepted that co-option of pre-existing vessels occurs during initial brain tumor growth\textsuperscript{66}. As the tumor grows, angiogenesis becomes the principal mechanism of neovascularization\textsuperscript{66, 63}. However, vasculogenesis has also been implicated to play a major role\textsuperscript{67}. Two studies recently described a tumor stem cell
to endothelial cell differentiation mechanism in the context of glioma\textsuperscript{68,69}, however, another study demonstrated that GBM mutant vascular cells are extremely rare\textsuperscript{70}.

**Angiogenesis as therapeutic target**

The hypothesis that tumor growth and metastasis are angiogenesis-dependent, and that blocking angiogenesis could therefore be a strategy to arrest tumor growth, was put forward by Folkmann in 1971\textsuperscript{53}. Currently, anti-angiogenic drugs are used in the treatment of advanced colorectal cancer\textsuperscript{71}, non-small cell lung cancer\textsuperscript{72}, renal cell cancer\textsuperscript{73} and GBM\textsuperscript{33}. The common target for these therapies is the VEGF pathway. Various classes of drugs have been approved for clinical application. Bevacizumab is an antibody that targets and neutralizes VEGF. Sunitinib and sorafenib are VEGF receptor tyrosine kinase inhibitors. Aflibercept is a fusion protein containing VEGF binding domains and acts as a soluble decoy receptor. Sunitinib and sorafenib have shown anti-angiogenic activity as monotherapy. Aflibercept and bevacizumab on the other hand are typically used in combination with standard chemotherapy. There are several ways by which VEGF inhibition may produce its clinical effects. VEGF blockade reduces tumor microvessel density and blood flow by slowing endothelial cell proliferation\textsuperscript{74}. However, VEGF blockade also affects other cell types in the tumor microenvironment such as hematopoietic progenitor cells, dendritic cells and the tumor cells themselves\textsuperscript{75}. As an alternative hypothesis it was suggested that VEGF inhibition can temporarily ‘normalize’ tumor vasculature, resulting in enhanced delivery of chemotherapeutics\textsuperscript{76}. In GBM, anti-VEGF therapy also reduces morbidity by reduction of vascular leakage and vasogenic edema. Although anti-VEGF treatment resulted in improved progression free survival, an increase in overall survival has not yet been demonstrated and the overall results of anti-VEGF therapy are more modest than predicted by most preclinical studies\textsuperscript{36,77}. Resistance to anti-VEGF therapy may be caused by activation of alternative pro-angiogenic pathways. In addition, some of the previously discussed alternative mechanisms for new vessel formation such as co-option may not be as sensitive to VEGF inhibitor treatment\textsuperscript{36,48}. Moreover, preclinical data has suggested that anti-angiogenic treatment may stimulate cancer invasiveness and metastasis by increasing hypoxia\textsuperscript{35,36}.

**EPIGENETIC AND POST-TRANSCRIPTIONAL MODIFICATION**

Epigenetics refers to changes in gene expression or cellular phenotype caused by mechanisms that do not involve changes in the underlying nucleotide sequence. This explains how differentiated cells in an organism express different features whilst containing the same DNA sequence. For example, a neuron and a muscle cell have completely different appearance and functionality but both stem from the same primary precursor cell – the zygote – and carry the same DNA sequence. Various mechanisms of epigenetic and post-transcriptional modifications have recently been discovered, including DNA methylation, histone modifications and miRNAs.

**Epigenetic mechanisms**

Epigenetic changes modify the activity of genes without altering the DNA sequence, occur at various levels of cellular biology and make use of different molecular mechanisms. DNA
methylation and histone modifications are involved in remodeling of the chromatin structure - the complex of DNA and protein that comprises the chromosomes. Chromatin is organized into nucleosomes, these are repeating units that consist of DNA wrapped around a sphere/octamer of histone proteins. Chromatin can consist in multiple configurations. Heterochromatin is highly compacted, transcriptionally silent and the most common form in mammalian cells. Euchromatin is less condensed and may be transcriptionally active or inactive. Post-transcriptional modifications include processes in which certain subtypes of RNA molecules - such as miRNA - inhibit gene expression through degradation of specific mRNA.

**DNA methylation**

DNA methylation is the addition of a methyl group to the DNA at a cytosines-preceding guanides (CpG) site and converts cytosine to 5-methylcytosine. DNA methylation was the first epigenetic mechanism associated with suppression of gene expression and is catalyzed by DNA methyltransferases (DNMTs). Promoter hypermethylation inhibits transcription factor binding and consequently transcription initiation. In addition, methylated DNA attracts methylcytosine binding proteins that cause condensation of chromatin, resulting in transcriptional repression and thus suppression of gene expression. Most methylated CpG sites are located in non-coding regions and may serve as a defense mechanism to silence the genome of foreign origin such as viruses. However, clusters of CpG sites that are located in gene promoter regions are called CpG islands and are typically unmethylated to allow gene transcription.

**Histone modifications**

Histones are spheres consisting of an octamer of histone proteins (H2A, H2B, H3 and H4) and act as spools around which the DNA binds. Histones can undergo a number of different modifications including acetylation, methylation, ubiquitylation, phosphorylation and sumoylation which affect the ability for chromatin to condense. These modifications typically occur at the histone-tails. Acetylation and methylation are the most studied modifications. Acetylation is induced by histone acetyltransferases and weakens the charge attraction between DNA and the histone. This causes the chromatin to decondense and facilitates the binding of transcription factors. On the other hand, histone deacetylases cause chromatin condensation and transcriptional repression. Histone methylation can be either activating or repressive depending on its location. Modifications - or “histone marks” - that are related to active transcription include histone 3 lysine 9 acetylation (H3K9ac) and lysine 4 trimethylation (H3K4me3). Common repressive histone marks include trimethylation of lysines 9 and 27 of histone 3 (H3K9me3 and H3K27me3). These marks are established through the combined function of specific groups of enzymes including histone acetyltransferases (HATs) and histone deacetylases (HDACs) that add and remove acetylgroups, or histone methyltransferases (HMTs) and demethylases (HDMs) that add and remove methyl groups. The mechanism in which changes to the histone have a direct effect on the DNA is called the “cis” model of epigenetic regulation. Alternatively, in the “trans” model changes to the histone tails create binding sites for chromatin-modifying enzymes. Multiple activating and repressive histone modifications can occur simultaneously to regulate specific cellular processes such as transcription or replication. The combination of these modifications is referred to as the histone code.
Polycomb group proteins

Polycomb group (PcG) proteins are key effectors of chromatin remodeling and thereby maintaining cellular identity. PcG proteins are involved in the regulation of developmental control genes, stem cell identity and proliferation. PcG proteins are classified into two groups, the Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2). These complexes function together to establish a silenced chromatin state. PRC2 consists of amongst others SUZ12, EED and EZH2 proteins and is involved in the initiation of gene repression. EZH2 is the catalytically active component of PRC2 and is capable of trimethylating Lysine 27 and 9 of Histone 3 (H3K27 and H3K9). This results in the recruitment of the more diverse PRC1 complex and subsequent transcriptional repression. EZH2 expression has been associated with increased risk and aggressiveness of multiple types of cancer.

Epigenetics in cancer

It has become clear that besides genetic lesions, epigenetic lesions or mutations that accumulate over a person’s lifetime can also result in the loss of control of cell proliferation and the development of cancer. Aberrant DNA methylation patterns have been described in most human cancers. Hypo- and hypermethylation can both contribute to carcinogenesis. DNA hypomethylation was the first epigenetic modification associated with cancer and may result in the activation of previously silenced oncogenes or lead to loss of imprinting. Furthermore, hypomethylation has been shown to contribute to genomic instability. On the other hand, DNA hypermethylation of CpG islands in the promoter region can cause aberrant silencing of tumor suppressor genes. In addition to changes in DNA methylation, histone modifications cause changes in chromatin structure and the accessibility of the DNA to transcription factors, and are implicated in many forms of cancer. Multiple epigenetic mechanisms may be involved simultaneously, for example genome-wide hypomethylation is associated with CpG island hypermethylation and promoter hypermethylation often occurs simultaneously with histone modifications.

The increased understanding of epigenetic mechanisms underlying carcinogenesis may lead to the discovery of epigenetic biomarkers for early detection, and to the prediction of prognosis and response to therapy. Epigenetic alterations are common in GBM. Hypomethylation was shown to occur in roughly 80% of primary GBM and the extent of hypomethylation was correlated with increased proliferation. Various studies in GBM report silencing of genes involved in cell cycle regulation, apoptosis and invasion by CpG island hypermethylation. In addition, hypermethylation of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) increases therapeutic response to DNA-alkylating agents such as temozolomide and is associated with significantly longer survival in GBM and low grade glioma treated with radiation and temozolomide.

Epigenetic therapies for cancer

The deregulation of genes involved in carcinogenesis by epigenetic mechanisms may provide potential new targets for therapy or diagnostics. In principal, epigenetic changes should be easier to revert than mutations affecting the genetic code. Many trials are currently investigating the effect of various epigenetic agents in the treatment of cancer.
of a variety of cancers. At the moment, most progress has been made with studies that involve DNA methyltransferase inhibitors and histone deacetylase inhibitors. For example, azacitidine and decitabine are compounds that inhibit DNA methylation by interaction with DNA methyltransferases and have recently been approved for clinical application in the treatment of myelodysplastic syndrome\textsuperscript{113, 114}. In addition, vorinostat - a more specific inhibitor of histone deacetylases - was recently approved for the treatment of cutaneous T-cell lymphoma\textsuperscript{115}. Since multiple epigenetic mechanisms may be involved simultaneously, several trials are now being conducted that combine different epigenetic agents in an effort to obtain synergistic effects. Given the preclinical evidence for potential therapeutic benefit of inhibition of histone methylation, drugs targeting the enzymes involved in this modification are being developed\textsuperscript{116, 117}. Preclinical studies on EZH2 inhibition have used the S-adenosylhomocysteine hydrolase inhibitor DZNep, which interferes with the methionine cycle and thereby hampers EZH2-induced histone methylation. However, so far no compounds are currently approved for treatment or clinical trials\textsuperscript{118}.

**Post-transcriptional regulation**

MicroRNAs (miRNAs), together with short interfering RNAs, are part of the RNA interference pathway\textsuperscript{119}. This pathway is an important mechanism by which the expression of specific genes or genomic regions can be modified through RNA silencing. MicroRNAs are small, single-stranded, non-coding RNAs, ~22 nucleotides in length, that can block mRNA translation or negatively regulate mRNA stability, and thereby play a central role in the regulation of gene expression\textsuperscript{120}. Because miRNAs have the ability to silence a gene through partial complementary binding to its mRNA, one single miRNA may target and regulate over 100 different mRNAs\textsuperscript{121}. As a result, miRNAs play a part in virtually all cellular processes such as proliferation, differentiation, apoptosis and migration. It is also becoming clear that deregulated miRNA expression is a common feature of human diseases, especially in specific forms of cancer\textsuperscript{122, 123}.

**miRNA in glioma and angiogenesis**

Several miRNA expression profiling studies have identified miRNAs which are up- or downregulated in GBM\textsuperscript{40, 42-47}. Many studies have tried to reveal the functional role of these miRNAs in pathological processes, such as cellular proliferation, differentiation, invasion, and apoptosis. GBM is characterized by “neo-angiogenesis” and microvascular proliferation, and these processes have been a major focus for novel therapeutics. Recently, it was shown that angiogenesis is also controlled by miRNAs\textsuperscript{124, 125}. AngiomiRs, the miRNAs involved in angiogenesis, could represent potential therapeutic targets for anti-angiogenesis therapy or improved delivery of chemotherapeutic agents through vessel normalization, thereby increasing the therapeutic window by improving drug distribution\textsuperscript{76}.

As previously discussed, tumor angiogenesis is driven by a complex balance between pro-angiogenic and anti-angiogenic factors that act on tumor cells and adjacent stroma. In GBM it was shown that VEGF signaling in primary endothelial cell cultures caused overexpression of VEGFR2 in a positive feed-forward loop, which is at least partly regulated by loss of miRNA-mediated control of VEGF receptor degradation. Moreover, it was found that GBM cells, notorious for their VEGF production, elicited a similar response when co-cultured with endothelial cells\textsuperscript{126}. \textsuperscript{16}
It has been hypothesized that miRNA involved in normal angiogenesis and vascular development may also have functions in pathological glioma angiogenesis. However, the full spectrum of miRNAs that play a part in tumor angiogenesis remains to be defined.

**Prospect for clinical applications**

Decisions regarding treatment modalities (resection, chemotherapy, radiotherapy) for brain tumors are currently based on imaging and tumor histology. Therapy stratification might be improved by the use of molecular markers\textsuperscript{127}. MicroRNA expression profiles may differ significantly between normal tissue and tumor, as well as between tumor subtypes. As such, miRNA may play a role in improved diagnosis and prognosis\textsuperscript{128, 129}. This was underscored by the observation that a miRNA-based classifier produced more robust results than a classifier based on mRNA expression\textsuperscript{130}. It was also shown that miRNA expression can discriminate between intrinsic and metastatic tumors in the brain\textsuperscript{131}. In prostate cancer a miRNA expression profile was also shown to predict response to therapy\textsuperscript{132}. MicroRNA expression has also been related to glioma response to radiation therapy\textsuperscript{133} or chemotherapy\textsuperscript{134}.

MicroRNAs can be detected in several bodily fluids including plasma, serum\textsuperscript{135} and cerebrospinal fluid (CSF)\textsuperscript{136, 137} and have been shown to remain stable after being subjected to severe conditions (temperature, pH). In addition, miRNAs can be detected in microvesicles\textsuperscript{138}. As such, miRNA may act as a relatively non-invasive biomarker.

So far, the only miRNA-based therapy tested in humans is anti-miR-122 LNA for treating hepatitis C virus (HCV) infection. The safety and antiviral activity of weekly subcutaneous anti-miR-122 doses was assessed in a phase 2 trial, demonstrating reduction in HCV RNA without any serious adverse effects recorded\textsuperscript{139, 140}.

**Microvesicles**

Microvesicles - or exosomes - are nano-sized vesicles secreted by a wide range of cells and have been detected in virtually all body fluids such as plasma, CSF and urine\textsuperscript{141, 142}. The content of microvesicles differs from cell to cell and consists of various molecular constituents of the cells they originate from, including DNA, mRNA and microRNA\textsuperscript{138, 143}. Neighboring, or distant, cells can take up exosomes and absorb their contents. In this way, microvesicles can influence the behavior of the recipient cell\textsuperscript{138, 141}. Microvesicle transfer has been shown to affect various cellular processes such as drug- and imunoresponse, and migration and invasion\textsuperscript{141}. As such, the role of microvesicles as a potential biomarker, as well as their role in cell-to-cell signaling, is actively being researched.

**AIMS AND OUTLINE**

Over recent years the knowledge of the molecular background of brain tumors such as glioblastoma and medulloblastoma has increased significantly. As a result, we now better understand how these tumors develop and evolve. These insights may provide novel targets for future treatments.

The aim of this thesis was to study epigenetic and miRNA signaling in the regulation of gene expression in endothelial cells and glioma cells during angiogenesis.
Chapter 2 briefly reviews glioma angiogenesis and miRNA biology. Subsequently, we will provide an overview of miRNAs involved in glioma angiogenesis and therapeutic applications will be discussed.

In chapter 3 we will investigate if the polycomb group protein EZH2 is differentially expressed in glioblastoma and if this has implications for biologic processes such as proliferation, migration, invasion and angiogenesis. In addition, we will explore if miRNA signaling plays a part in deregulated EZH2 expression. We also test if inhibition of EZH2 inhibits glioblastoma growth in vitro and in vivo.

Chapter 4 further investigates the role of miR-101 and EZH2 in glioblastoma and addresses the question if miR-101 / EZH2 signaling has a function in glioma-associated endothelial cells and angiogenesis. Furthermore, we analyze if the pro-angiogenic factor VEGF has an effect on miR-101 and EZH2 expression in glioma associated endothelial cells.

In chapter 5 we will explore the function of miR-125b in glioblastoma-related angiogenesis. We analyze if VEGF also has an effect on miR-125b expression in glioma-associated endothelial cells and study the effect of miR-125b on MAZ, a transcription factor responsible for the activation of VEGF.

In chapter 6 we will investigate if exosomes can function as mediators of intercellular angiogenic signaling by transferring pro-angiogenic miRNA from one endothelial cell to another.

In chapter 7 we switch our attention from epigenetic signaling in angiogenesis and explore by in silico analysis which genes are commonly downregulated in medulloblastoma. For one of these we will investigate its role as a prognostic marker.

Finally, the findings presented in this thesis will be discussed in chapter 8.

REFERENCES


A SYSTEMATIC REVIEW OF ANGIOmiRS IN GLIOBLASTOMA NEOVASCULARIZATION

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ABSTRACT

MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression post-transcriptionally by binding to target mRNAs. miRNAs play an essential role in the regulation of several physiological and pathological processes, including cell proliferation, cell cycle regulation, apoptosis, invasion, and stem cell behavior. miRNAs involved in neovascularization, termed angiomiRs, are reviewed here. We emphasize on the role of angiomiRs in tumor neovascularization, and in particular we focus on glioblastoma, the most malignant and vascularized brain tumor.
**BIOGENESIS AND FUNCTION OF miRNAs**

miRNAs are together with short interfering RNAs (siRNAs) part of the RNA interference (RNAi) pathway\(^1\). This pathway is an important mechanism by which the expression of specific genes or genomic regions can be modified through RNA silencing. The miRNA biogenesis pathway is complex and it has been revealed that there is not one general pathway universal to all miRNAs\(^2\). miRNAs are non-coding, endogenous, short, single-stranded RNA molecules of approximately 20-23 nucleotides\(^3\). The miRNA sequences can reside in introns of protein-coding transcripts or in specific miRNA genes and are commonly transcribed by RNA polymerase II\(^2\). After transcription and RNA splicing in case of an intronic miRNA, a pri-miRNA is generated containing one or more hairpin loops. These hairpin structures are resected in the nucleus by the Drosha/DGCR8 complex, an RNase III endonuclease, which results in the formation of the pre-miRNA\(^4\). This miRNA precursor is approximately 70 nucleotides long and subsequently transported to the cytoplasm by Exportin-5-Ran-GTP. Here, the pre-miRNA is cleaved by Dicer, another RNase III endonuclease, to remove the hairpin loop. This generates a mature, functional, double-stranded miRNA molecule\(^1, 4\).

In order to regulate gene expression the miRNA duplex is associated with Ago proteins to form a multi-protein RNA-induced silencing complex (RISC). Ago proteins are part of the Argonaute protein family and are important players in RNA interference pathways as they elicit mRNA destabilization or translational repression, resulting in post-transcriptional gene silencing\(^5\). The association of a duplex miRNA with Ago proteins will result in the unwinding of the miRNA duplex and dismissal of the passenger strand\(^1\). The miRNA or guide strand is retained in the complex and subsequently guides the miRISC to the target transcript, which will regulate post-transcriptional gene expression. miRNAs can bind to their target mRNA in the 3’UTR region, where the regulation of gene expression is dependent on the degree of complementarity between the miRNA and its binding sites in this region. Perfect complementary binding of the target transcript with the miRISC may result in mRNA degradation, while partial complementary binding can induce repression of translation by the Ago proteins. Another possibility in the silencing of the target mRNA is mRNA deadenylation which will destabilize the target transcript\(^6\). Because miRNAs have the ability to silence a gene through partial complementary binding to its mRNA, one single miRNA can target and regulate over 100 different mRNAs\(^6\). Therefore, miRNAs are able to control the regulation of many physiological and pathological processes, including development and cancer\(^6\).

**miRNAs IN CANCER**

The role of miRNAs in the progression of cancer is undisputed, and there is a correlation between cancer development and the expression of many miRNAs\(^8-11\). These miRNAs can modulate the expression of oncogenes or tumor suppressor genes and stimulate tumor formation, or target other genes involved in the initiation and progression of cancer by acting as onco-miRs or tumor suppressor miRs\(^9, 12\). Additionally, it was revealed that the majority of miRNA genes are located in cancer associated genomic regions or in fragile sites, and that miRNA expression profiles correlate with prognosis and clinical outcome in various cancers\(^13\). Although many studies have been performed on how miRNAs regulate their targets, much less is
known about the induction of expression of the miRNAs themselves. The regulation of miRNAs can take place at the transcriptional or at the post-transcriptional level. At the transcriptional level, the function of RNA polymerase II and associated transcription factors is implicated to be important for maintaining proper miRNA production. Also, numerous miRNAs reside in introns of coding genes, these miRNAs might be regulated by the promoter of this gene. Furthermore, it is postulated that miRNAs are regulated by the epigenetic machinery. For example DNA hypermethylation is reported to inactivate the expression of several miRNAs. Another mechanism which could regulate miRNA function is the occurrence of single nucleotide polymorphisms (SNPs) in miRNA-coding genes or miRNA-binding sites in target mRNAs. For example, it was reported that a SNP in the sequence of pre-miR-146a decreased the creation of mature miR-146 and caused less inhibition of its target. At the post-transcriptional level, the processing of pri-miRNA to pre-miRNA seems to be an important step in the process of miRNA regulation. The significance of the regulation of miRNA processing in tumorigenesis was revealed when knockdown of Drosha, DGCR8 or Dicer resulted in the promotion of cellular transformation. Another mechanism at the post-transcriptional level represents miRNA editing, where adenosines (A) are modified into inosines (I) by adenosine deaminases acting on RNA (ADARs) in the primary transcripts of miRNAs. Editing of miRNA precursors might influence further processing and the ability to recognize its mRNA targets.

**GLIOBLASTOMA ANGIOGENESIS**

Glioblastoma is the most common primary malignant brain tumor. These tumors may develop at any age, with a peak incidence around 50 – 70 years. The clinical outcome is poor, with an overall 5-year survival of less than 10%. This poor clinical outcome is largely due to the diffusely infiltrative nature of glioblastoma, which makes complete surgical resection of the tumor impossible. Aberrant neovascularization is an important characteristic of glioblastoma, as the tumor blood vessels stimulate the delivery of nutrients and oxygen to the tumor and facilitate tumor cell migration to other parts of the brain.

To activate the process of tumor angiogenesis glioblastoma cells must undergo the so-called angiogenic switch. One of the primary activator mechanisms of the angiogenic switch in brain tumors is tissue hypoxia. Under hypoxic conditions the transcription factor hypoxia inducible factor 1α (HIF-1α) is upregulated, activating certain DNA promoter regions called hypoxia response elements (HREs). These response elements cause transcription of more than 100 different genes which aid a cell to overcome a hypoxic environment. An important gene regulated by HIF-1α is the vascular endothelial growth factor (VEGF). VEGF-A is found to be upregulated in glioblastoma, where it is responsible for the stimulation of blood vessel formation by regulating endothelial cell survival, proliferation and migration. The process of angiogenesis requires several distinct steps. The first step involves the breakdown of existing blood vessels. The tumor cells nearby the existing cerebral blood vessels are responsible for the disruption of the contact between endothelial cells and the basement membrane, as they remove the astrocytic foot processes from the blood vessels. This disruption results in destabilization of the vessel wall. The second step in the angiogenic process requires degradation of the basement membrane and surrounding
extracellular matrix (ECM), which will facilitate the invasion and migration of endothelial cells towards the tumor cells. Matrix metalloproteinases (MMPs) are important enzymes responsible for the degradation of the ECM. Especially MMP-2 and MMP-9 are found to be upregulated in malignant gliomas, and their expression levels are correlated with tumor grade. Additionally, the third step involves the proliferation and migration of endothelial cells and subsequently the formation of new blood vessels to supply the tumor mass with oxygen and nutrients. The pro-angiogenic compounds secreted by glioblastoma cells under hypoxic conditions after the angiogenic switch are sensed by endothelial tip cells. This leads to the migration of endothelial cells towards the tumor and the formation of new premature vessels. However, these fragile new vessels also require pericytes and smooth muscle cells to form capillary tubes. The newly formed capillary network is irregular and not well structured, albeit sufficient to provide the tumor with oxygen and nutrients.

It is evident that the interaction between endothelial cells and glioblastoma cells is an important aspect in the process of angiogenesis. To mediate this process, the expression of specific molecules is required which demands proper functioning of gene expression. Several mechanisms are known to regulate gene expression, such as the regulation of gene promoter activity. However, small non-coding miRNA molecules can also be used by cells as molecular switches, regulating the expression profile of a cell through binding to target mRNAs. It has become increasingly clear that miRNAs are important players in the regulation of gene expression in, amongst others, endothelial cells and glioblastoma cells during angiogenesis.

ANGIOMIRs IN VARIOUS FORMS OF VASCULAR DEVELOPMENT

In recent years, an increasing number of studies have provided evidence for the involvement of miRNAs in endothelial cell function and various forms of vascular development including embryonic blood vessel development, adaptive neovascularization in cardiovascular disease and pathological neovascularization in different types of cancer. The miRNAs involved in these various processes, angiomiRs, have been discussed in a number of excellent studies and reviews and are summarized in Table 1. Since the focus of this review is on glioblastoma angiogenesis, only a few of these angiomiRs will be discussed below.

miRNAs WITH A DEMONSTRATED ROLE IN ANGIOGENESIS

miR-126

miRNA-126 is among the most frequently detected miRNAs in endothelial cells. The expression of miR-126 is upregulated in endothelial cells and is implicated in angiogenesis. Increased expression of miR-126 was detected in human umbilical vein endothelial cells (HUVECs) stimulated with VEGF-A. In addition, miR-126 was highly expressed in HBMVECs, however, its expression was decreased in HBMVECs co-cultured with U87 glioma cells. Inhibition of miR-126 in vitro resulted in decreased endothelial cell proliferation, sprouting, migration and tube formation. In vivo inhibition of miR-126 resulted in compromised vessel integrity and caused leaky vessels and hemorrhaging, while the deletion of miR-126 in mice was associated with impaired angiogenesis activity.
Table 1. miRNAs with a role in angiogenesis or endothelial cell function.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Celltype</th>
<th>Expression</th>
<th>Targets</th>
<th>miRNA function</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>miR-15-16</td>
<td>EC, MM</td>
<td>down</td>
<td>VEGF, FGF2</td>
<td>anti-angiogenic</td>
<td>33, 69, 104, 105</td>
</tr>
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<td>miR-21</td>
<td>EC, PC</td>
<td>up / down</td>
<td>PTEN, RhoB</td>
<td>both</td>
<td>55-58</td>
</tr>
<tr>
<td>miR-22</td>
<td>CC</td>
<td>down</td>
<td>HIF1a, MMP2, MMP9</td>
<td>anti-angiogenic</td>
<td>72, 73</td>
</tr>
<tr>
<td>miR-23b</td>
<td>EC, CC</td>
<td>down</td>
<td>TSP1, CTGF, TIMP1, ITGAS, JAK1/STAT3, E2F, VEGF</td>
<td>anti-angiogenic</td>
<td>74, 75</td>
</tr>
<tr>
<td>miR-17-92</td>
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<td>both</td>
<td>TSP1, CTGF, TIMP1, ITGAS, JAK1/STAT3, E2F, VEGF</td>
<td>both</td>
<td>69, 106-110</td>
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<tr>
<td>miR-27a</td>
<td>EC, BC</td>
<td>up</td>
<td>SEMA6A, ZBTB10</td>
<td>pro-angiogenic</td>
<td>77, 78</td>
</tr>
<tr>
<td>miR-29a</td>
<td>EC</td>
<td>up</td>
<td>PTEN, HBP1</td>
<td>pro-angiogenic</td>
<td>79, 111</td>
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<tr>
<td>miR-30b</td>
<td>EC</td>
<td>n.r. / up</td>
<td>DLL4</td>
<td>excessive sprouting</td>
<td>45, 80</td>
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<td>miR-31</td>
<td>EC</td>
<td>up</td>
<td>FAT4, E-selectin</td>
<td>pro-angiogenic</td>
<td>81, 82</td>
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<td>miR-34</td>
<td>EPC</td>
<td>n.r.</td>
<td>SIRT1</td>
<td>induces senescence</td>
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<tr>
<td>miR-100</td>
<td>EC</td>
<td>down</td>
<td>mTOR</td>
<td>anti-angiogenic</td>
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</tr>
<tr>
<td>miR-103</td>
<td>EC</td>
<td>up</td>
<td>AGO1</td>
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<tr>
<td>miR-125b</td>
<td>OC, HCC</td>
<td>down</td>
<td>PIGF, VE-cadherin</td>
<td>anti-angiogenic</td>
<td>64-66</td>
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<tr>
<td>miR-126</td>
<td>EC</td>
<td>up</td>
<td>SPRED1, PIK3R2, VCAM-1</td>
<td>pro-angiogenic</td>
<td>34-36</td>
</tr>
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<td>miR-130a</td>
<td>EC</td>
<td>up</td>
<td>GAX, HOXA5</td>
<td>pro-angiogenic</td>
<td>41</td>
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<td>miR-132</td>
<td>EC</td>
<td>up</td>
<td>p120RasGap</td>
<td>pro-angiogenic</td>
<td>42</td>
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<tr>
<td>miR-155</td>
<td>EC, BC</td>
<td>up</td>
<td>VHL, ETS1, AT1R</td>
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<td>114, 115</td>
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<tr>
<td>miR-181a</td>
<td>EC</td>
<td>n.r.</td>
<td>PROX1</td>
<td>EC differentiation</td>
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<td>miR-200b</td>
<td>EC</td>
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<td>ETS1, VEGF, GATA2, VEGFR2</td>
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<tr>
<td>miR-210</td>
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<td>up</td>
<td>EFNA3, NPTX1</td>
<td>pro-angiogenic</td>
<td>44-46</td>
</tr>
<tr>
<td>miR-217</td>
<td>EC</td>
<td>up with age</td>
<td>SIRT1</td>
<td>induces senescence</td>
<td>119</td>
</tr>
<tr>
<td>miR-221/222</td>
<td>EC</td>
<td>up</td>
<td>c-Kit, STAa5, p27(Kip1)</td>
<td>anti-angiogenic</td>
<td>48, 49, 120</td>
</tr>
<tr>
<td>miR-320</td>
<td>EC</td>
<td>n.r.</td>
<td>IGF1</td>
<td>anti-angiogenic</td>
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</tr>
<tr>
<td>miR-378</td>
<td>Cancer CL</td>
<td>n.r.</td>
<td>SUFU, FUS1</td>
<td>pro-angiogenic</td>
<td>122</td>
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<tr>
<td>miR-424</td>
<td>EC</td>
<td>up / down</td>
<td>CUL2, MEK1, cyclin-E1</td>
<td>pro-angiogenic</td>
<td>123, 124</td>
</tr>
</tbody>
</table>


during physiological and pathophysiological processes\(^{35, 39}\). These findings indicate the role of miR-126 in maintaining vascular structure during ongoing angiogenesis. Three targets have been identified for miR-126, all involved in endothelial cell biology. Sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) are directly repressed by miR-126. SPRED1 and PIK3R2 are both negative regulators of the VEGF pathway, as SPRED1 is responsible for blocking the MAP kinase pathway by binding RAF1, and PIK3R2 negatively regulates the activity of PI3 kinase\(^{46}\). Furthermore, the vascular cell adhesion molecule 1 (VCAM1) was identified as the third target of miR-126. VCAM1 is involved in leukocyte adhesion to the vessel wall and inhibition of VCAM1 by miR-126 results in decreased adhesion of leukocytes and downregulation of vascular inflammation\(^{34}\). Recent
studies have implicated miR-126 in several types of cancer and report of a suppressive role in tumor formation. This might indicate on the one hand a tumor suppressive role for miR-126 through controlling tumor cell proliferation, migration, invasion and survival, whereas on the other hand promotion of tumor growth through modulation of blood vessel formation.

**miR-130a**

Another miRNA which is frequently upregulated in endothelial cells is miR-130a. This miRNA is located on chromosome 11 and is upregulated under high serum culture conditions. Overexpression of miR-130a in HUVECs resulted in proliferation, tube formation and migration, which supports the role of miR-130a in angiogenesis. It was revealed that miR-130a downregulates the anti-angiogenic homeobox genes GAX and HOXA5. It has been suggested that GAX inhibits nuclear factor-κB (NF-κB) signalling in endothelial cells. Recently, it was also shown that GAX induces G0 cell cycle arrest by activating p21^WAF1/CIP1^.

Chen et al. have revealed that miR-130a is an important downregulator of GAX and HOXA5 in response to mitogens, pro-angiogenic factors and pro-inflammatory factors. This downregulation might be a necessary requirement for endothelial cells to re-enter the cell cycle and become angiogenic, as GAX normally functions to maintain endothelial cells in their quiescent state.

**miR-132**

A more recently discovered miRNA in endothelial cells is miR-132, which is located on chromosome 17. Although its expression in brain endothelium remains to be elucidated, it has been shown that angiogenic growth factors such as VEGF, bFGF and conditioned media from various tumors results in the transcription of miR-132 in endothelial cells. Ectopic expression of miR-132 in vitro increased endothelial cell proliferation and tubulogenesis, whereas inhibition of miR-132 resulted in decreased proliferation and tubulogenesis. The inhibition of miR-132 also contributed to developmental and pathological angiogenesis in vivo. p120RasGAP has been identified as a primary target of miR-132. This GTPase-activating protein reduces p21 Ras activity. Overexpression of miR-132 can remove the endogenous brake on Ras activity and subsequently increase Ras signalling, which can lead to an increased angiogenic activity of endothelial cells and activate quiescent endothelial cells.

**miR-210**

Another potential angiomiR detected in endothelial cells is miR-210. The expression of this miRNA is strongly upregulated upon exposure to hypoxia in HUVECs. Overexpression of miR-210 in normoxic endothelial cells resulted in enhanced tubulogenesis and VEGF-induced cell migration, whereas blockade of miR-210 inhibited these processes. Transfection of anti-miR-210 resulted in inhibition of proliferation and induced apoptosis, under both normoxic and hypoxic conditions. It was demonstrated that the transcripts of Ephrin-A3 (EFNA3) and neuronal pentraxin-1 (NPTX1) are targets of miR-210. Ephrins and Ephrin-related receptors have previously been implicated in mediating the development of the cardiovascular system and vascular remodeling and it was shown that the pro-angiogenesis effects of miR-210 are mainly caused by downregulation of EFNA3 as miR-210 mediated stimulation of tubulogenesis and chemotaxis could be overcome by EFNA3 restoration.

In addition, miR-210 was induced by VEGF in CD34+ cells, and inhibition of miR-210 reduced
the angiogenic capacity of these cells. This suggests miR-210 might play an important role in angiogenesis and endothelial cell survival in hypoxic conditions. The role of miR-210 in glioma angiogenesis remains to be determined as - similar to miR-132 and miR-130a expression - no significant miR-210 expression was detected in brain endothelial cells.

**miR-221 and miR-222**

Both miR-221 and miR-222 are located on chromosome X11.3. These miRNAs are frequently upregulated in glioblastoma and in other cancers, and target the cyclin dependent kinase (CDK) inhibitor p27kip1 which negatively regulates cell cycle progression. Overexpression of miR-221 and miR-222 in U87 glioblastoma cells resulted in continuous cell proliferation. Moreover, these miRNAs have also been detected in endothelial cells. Overexpression of the miR-221/miR-222 family in HUVECs was shown to decrease cell migration. Interestingly, it was demonstrated that miR-221 and miR-222 affect the expression of c-kit, which is a stem cell factor (SCF) receptor. SCF is a growth factor which is implicated to be involved in angiogenesis. Several studies report that overexpression of miR-221 and miR-222 in HUVECs reduces tube formation and migration in response to SCF. The interaction between miR-221/miR-222 and c-kit might be part of a complex network that controls the formation of new vessels during tumor growth. Altogether, these findings indicate that miR-221/miR-222 might have an angiostatic effect on endothelial cells. Since both miR-221 and miR-222 are significantly expressed in brain endothelial cells it would be of interest to study their function during glioma angiogenesis.

**ANGIOMiRs IN GLIOBLASTOMA ANGIOGENESIS**

In this review we focused on the role of angiomiRs in glioblastoma angiogenesis. In glioblastoma angiomiRs could represent potential therapeutic targets for anti-angiogenesis therapy or improved delivery of chemotherapeutic agents through vessel normalization, thereby increasing the therapeutic window by improving the drug distribution. A number of studies have identified angiomiRs which are deregulated in glioblastoma or glioblastoma associated endothelial cells. Moreover a number of the angiomiRs described in the previous paragraph may also be implicated in glioblastoma neovascularization. Here, angiomiRs are discussed that may have a role in glioblastoma angiogenesis (Table 2). Some of these miRNA were shown to be involved in glioblastoma angiogenesis, whereas for others the evidence so far is limited or contradictory.

**miRNAs WITH A DEMONSTRATED ROLE IN GLIOBLASTOMA ANGIOGENESIS**

**miR-296**

The first angiomiR implicated in glioblastoma angiogenesis was miR-296. Its expression was revealed to be upregulated in endothelial cells derived from human glioblastomas when compared to endothelial cells derived from non-neoplastic brain. Additionally, when human brain primary microvascular endothelial cells (HBMVECs) where co-cultured with U87 glioblastoma cells, a significant upregulation of miR-296 was observed in these
Table 2. miRNAs detected in glioblastoma or glioblastoma associated endothelial cells with a (potential) role in angiogenesis.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Celltype</th>
<th>Expression</th>
<th>Targets</th>
<th>miRNA function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-10b</td>
<td>Glioma CL</td>
<td>up</td>
<td>CYLD, TP53, FOXO3A, PAX6, NOTCH1, PTCH1</td>
<td>pro-angiogenic</td>
<td>67</td>
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<tr>
<td>miR-15b</td>
<td>Glioma CL</td>
<td>n.r.</td>
<td>NRP2</td>
<td>anti-angiogenic</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Other (Table 1)</td>
<td>down</td>
<td></td>
<td>anti-angiogenic</td>
<td></td>
</tr>
<tr>
<td>miR-21</td>
<td>GBM</td>
<td>up</td>
<td>RECK, TIMP3, PTEN</td>
<td>pro-angiogenic</td>
<td>53-55</td>
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<td></td>
<td>EC*</td>
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<td></td>
<td>anti-angiogenic</td>
<td>38, 56-58</td>
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<tr>
<td></td>
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<td>miR-22</td>
<td>EC*</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>miR-23b</td>
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<td>up / down</td>
<td>VHL, PYK2</td>
<td>pro-angiogenic / n.r.</td>
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<td>EC*</td>
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<td></td>
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<td></td>
<td>Other (Table 1)</td>
<td>n.r. / up</td>
<td>excessive sprouting</td>
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<td></td>
<td>pro-angiogenic</td>
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<td>miR-93</td>
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<td>Integrin-β8</td>
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<td>miR-101</td>
<td>GBM, GBM EC</td>
<td>down</td>
<td>EZH2</td>
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<td></td>
<td>EC*</td>
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<td>EZH2</td>
<td>anti-angiogenic</td>
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<td>EC*, GBM EC</td>
<td>down</td>
<td>MAZ</td>
<td>anti-angiogenic</td>
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<td>miR-126</td>
<td>EC*</td>
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<td>miR-181a</td>
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<td>EC differentiation</td>
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<td>miR-296</td>
<td>EC*, GBM EC</td>
<td>up</td>
<td>HGS</td>
<td>pro-angiogenic</td>
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</table>

GBM EC: glioblastoma endothelial cells; EC*: endothelial cells after co-culture with U87 glioma cells; GBM: glioblastoma cells; Glioma CL: glioma cell-line. n.r.: not reported.
cells\textsuperscript{38}. Culturing of HBMVECs with U87 glioma-conditioned culture medium also resulted in miR-296 upregulation, which suggests that factors released by U87 cells may increase miR-296 expression in endothelial cells. Overexpression of miR-296 in HBMVECs resulted in a marked increase in tubule formation. Furthermore, it was revealed that miR-296 was significantly upregulated in HBMVECs in response to VEGF and EGF. Interestingly, when miR-296 was inhibited, a decrease in endothelial tubule branching, total tubule length, and migration of HBMVECs was observed, suggesting a role for miR-296 in the regulation of glioblastoma angiogenesis. It was demonstrated that miR-296 could directly block translation of hepatocyte growth factor regulated tyrosine kinase substrate (HGS) through a functional binding site in its 3’UTR. HGS has a function in regulating the levels of angiogenic growth factor receptors PDGFR\textbeta\textsuperscript{51} and VEGFR2\textsuperscript{52}. miR-296 induced inhibition of HGS results in increased PDGFR\textbeta and VEGFR2 protein levels and a proangiogenic response. These results suggest that miR-296 may play a significant role in glioblastoma angiogenesis.

\textbf{miR-21}

Overexpression of miR-21 has frequently been reported in glioblastoma and other solid tumors\textsuperscript{53}. It is suggested that miR-21 plays a role in glioma cell proliferation, apoptosis, migration and angiogenesis, which makes it an attractive therapeutic target. Multiple targets have been described for miR-21 in these aspects of carcinogenesis. Here, the target genes which are suggested to be involved in glioma angiogenesis will be described. First, the MMP inhibitors RECK and TIMP3 were revealed as direct targets of miR-21 in glioblastoma\textsuperscript{53}, resulting in an elevated expression of MMPs. This leads to an increased degradation of the ECM, contributing to glioma cell invasion, metastasis and angiogenesis\textsuperscript{54}. Another target of miR-21 involved in angiogenesis is the tumor suppressor PTEN. The overexpression of miR-21 results in downregulation of PTEN, which leads to activated AKT and ERK1/2 signalling. These signalling pathways are amongst others responsible for enhanced HIF-1\textalpha and VEGF expression, which in turn promotes angiogenesis\textsuperscript{55}. Of note, the previously described pro-angiogenic mechanisms have been demonstrated in glioma cells. In contrast, in endothelial cells anti-angiogenic functions have been reported for miR-21\textsuperscript{56,58}. In response to miR-21 upregulation, the expression of RhoB is decreased in endothelial cells resulting in decreased cell migration and tubulogenesis\textsuperscript{56,57}. RhoB is part of the RhoGTPase family and has been implicated as a key regulator of several angiogenic processes, such as vascular permeability, ECM remodeling, migration and proliferation\textsuperscript{59}. In addition, overexpression of miR-21 in endothelial progenitor cells (EPC), caused EPC senescence and resulted in impaired EPC angiogenesis \textit{in vitro} and \textit{in vivo}\textsuperscript{58}. Whether these mechanisms also apply in glioma associated endothelial cells remains to be determined.

\textbf{miR-101}

miR-101 was recently discovered to be downregulated in glioblastoma and glioma-associated endothelial cells\textsuperscript{38,60,61}. Moreover, it was revealed that this downregulation occurs in a glioma grade dependent manner\textsuperscript{60,61}. miR-101 is - at least partly - responsible for the translational repression of the Polycomb group (PcG) protein EZH2, which is a histone methyltransferase that functions as an important epigenetic regulator. EZH2 is part of the Polycomb repressive
complex 2, which can silence specific genes through chromatin modifications. Increased expression of EZH2 has been shown to correlate with glioma grade and recurrence. The downregulation of miR-101 in glioblastoma and glioma-associated endothelial cells results in the overexpression of EZH2 and increased histone methyltransferase activity. This overexpression results in glioma proliferation, migration and angiogenesis, stimulating glioma progression. In vitro experiments revealed that upregulation of miR-101 in endothelial cells caused reduced sprouting and migratory properties. Interestingly, miR-101 was also downregulated in cultured endothelial cells upon exposure to VEGF. Inhibition of miR-101 target EZH2 in vivo resulted in reduced glioma growth and blood vessel formation. The anti-angiogenic effect of EZH2 inhibition is potentially mediated through reduced silencing of anti-angiogenic vasoinhibin-1 (VASH1) in tumor associated endothelial cells. A similar mechanism of EZH2 regulated tumor angiogenesis was demonstrated in ovarian cancer. Collectively, this suggests a potential role for EZH2 inhibitors or miR-101 mimics in glioblastoma treatment.

miR-125b

We previously demonstrated that miR-125b is the most significantly downregulated miRNA in endothelial cells after exposure to U87-CFP glioma cells. Moreover we showed that miR-125b directly inhibits translation of Myc-associated zinc finger (MAZ), a transcription factor that regulates VEGF, and that reintroducing miR-125b attenuated migration and tubule formation of primary human brain endothelial cells in vitro. He et al. showed that miR-125b expression was also downregulated and inversely correlated with VEGF expression in ovarian cancer and that overexpression of miR-125b inhibited tumor induced blood vessel formation. In a study of miR-125b function in the context of hepatocellular cancer, introducing miR-125b significantly decreased angiogenic capacity in vitro through direct inhibition of PlGF. In addition it was also shown that reduced expression miR-125b could be restored by 5-aza-2'-deoxycytidine treatment, suggesting miR-125b expression can be epigenetically modulated. Recently it was shown that miR-125b inhibits translation of vascular endothelial cadherin in endothelial cells. This resulted in reduced in vitro tube formation by endothelial cells. Moreover, miR-125b injection induced non-functional blood vessel formation and reduced tumor growth in vivo. Interestingly in this study miR-125b expression was transiently induced by stimulation with VEGF or by ischemia.

miRNAs WITH ANGIOGENIC FUNCTION IN GLIOMA CELL LINES

miR-10b

Inhibition of miR-10b results in a distinct increase in apoptosis with a concurrent suppression of glioma cell invasion and angiogenesis in vitro and in vivo. Moreover, it was demonstrated that miRNA-10b’s pleiotropic nature may be due to its suppression of multiple tumor suppressors, including FOXO3, CYLD, HOXD10, TP53, PAX6, PTCH1 and NOTCH1.

miR-15b

In a single study performed in glioma cell line 9L the introduction of miR-15b caused downregulation of neuropilin-2 (NRP2), a transmembrane receptor that functions as
a mediator of angiogenesis by binding VEGF. In addition, in vitro tube formation and invasiveness were reduced. In the context of nasopharyngeal cancer it was demonstrated that miR-15b was downregulated under hypoxia and that miR-15b directly targets the 3'-UTR of VEGF. This suggests miR-15b has anti-angiogenic effects. However, the expression and function of miR-15b in primary glioblastoma samples or glioblastoma endothelial cells has not been investigated so far.

**miR-93**

When miR-93 overexpressing U87 glioma cells were co-cultured with endothelial cells they significantly stimulated endothelial cell spreading, growth, migration and tubule formation. miR-93 also stimulated blood vessel formation in vivo. It was further demonstrated that these effects were (in part) exerted through the inhibition of integrin-β8, a negative regulator of cell proliferation.

**miR-128**

Evidence for miR-128’s involvement in glioma angiogenesis is limited to one study in which it is demonstrated that miR-128 expression levels were decreased in glioma cell lines and tissue samples in a grade dependent matter. Furthermore, it was shown that miR-128 inhibits the expression of p70S6K1, a regulator of HIF-1α, and that overexpression of miR-128 in glioma cells subsequently reduced VEGF expression in vitro and tumor angiogenesis in vivo.

**miRNAs DIFFERENTIALLY EXPRESSED IN BRAIN ENDOTHELIAL CELLS**

**miR-22**

To date, a function for miR-22 in angiogenesis has only been reported in colon cancer models. miR-22 was shown to inhibit HIF-1α expression, repressing VEGF production during hypoxia in a colon cancer cell line. Conditioned medium from cells overexpressing miR-22 contained less VEGF protein and induced less endothelial cell invasion and growth compared to conditioned medium derived from control cells. In a different study it was confirmed that miR-22 inhibits VEGF production and demonstrated that the expression of pro-invasive gene matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) were also reduced by miR-22.

**miR-23b**

miR-23b inhibited tubule formation by HUVECs in vitro as well as angiogenesis in vivo in a subcutaneous colon cancer model. Although miR-23b didn’t target VEGF directly, VEGF mRNA and secreted VEGF levels were decreased in a colon cancer cell line treated with miR-23b mimics. This inhibitory effect on VEGF levels was possibly exerted through miR-23b’s inhibition of FZD7 and MAP3K1. In addition to its inhibitory effect on VEGF levels, it was shown that miR-23b is a mediator of growth arrest in endothelial cells after pulsatile shear flow. Pulsatile shear flow resulted in increased miR-23b levels in endothelial cells which in turn decreased expression of E2F1 and caused hypophosphorylation of retinoblastoma protein and suppressed endothelial cell growth. Intriguingly, in the context of glioma elevated miR-23b levels were reported, while down regulation of miR-23b resulted in decreased expression of HIF-1α and VEGF opposite to the effects found in colon cancer.
miR-27a
Overexpression of miR-27a significantly increased endothelial cell sprouting while its inhibition impaired angiogenic activity of endothelial cells in vitro and in vivo. The proangiogenic effect of miR-27a was produced at least in part as a result of its inhibition of SEMA6A, a known inhibitor of angiogenesis. Another proposed mechanism by which miR-27a contributes to a proangiogenic phenotype was put forward by Mertens-Talcott et al., they showed that miR-27a regulates ZBTB10, a putative repressor of specificity proteins (Sp). Transfection of a breast cancer cell line with antisense miR-27a caused increased expression of ZBTB10 mRNA and in turn decreased expression of Sp and Sp-dependent angiogenic genes including VEGF and VEGFR.

miR-29a
It was recently demonstrated that miR-29a can be upregulated in endothelial cells by TGF-β/SMAD4 signalling. Increased expression of miR-29a stimulated blood vessel formation in vitro and in a chick chorioallantoic membrane assay through the inhibition of PTEN and subsequent activation of the AKT pathway.

miR-30b
Overexpression of miR-30b in endothelial cells led to increased vessel number and length in an in vitro model of sprouting angiogenesis and was shown to inhibit DLL4, a membrane bound ligand belonging to the Notch signalling family. DLL4 plays a fundamental role in vascular development and angiogenesis by regulating vessel sprouting and branching. Although inhibition of DLL4 induces sprouting angiogenesis, it is also associated with suppressed tumor growth as the resulting vasculature is nonfunctional. Indeed, injection of miR-30 mimics resulted in suppression of DLL4 and excessive sprouting of intersegmental vessels but reduction of dorsal aorta diameter in vivo using a zebrafish model. In a different study, miR-30b was found upregulated in HUVECs in response to hypoxia, however this effect was not confirmed in a similar study.

miR-31
A number of mechanisms for miR-31 induced endothelial cell migration and angiogenic function have been reported. Wu et al. showed that miR-31 can induce endothelial cell motility by targeting FAT4, a novel tumor suppressor that decreases endothelial cell motility by increasing adhesiveness. Another study by Suarez et al. showed that miR-31 is able to inhibit the adhesion molecule E-selectin in HUVECs. This has a proangiogenic effect as E-selectin is involved in the inhibition of angiostatin - induced angiogenesis. In a study that compared lymphatic endothelial cells with blood vessel endothelial cells, miR-31 expression was significantly higher in the blood vessel endothelial cells. Indeed, miR-31 actually prevented the acquisition of a lymphatic endothelial cell phenotype by inhibition of PROX1.

miR-103
Chen et al. recently showed that hypoxia strongly induces miR-103 in vascular endothelial cells. In addition they showed that miR-103 targets argonaute1, a key element of the miRNA silencing complex (miRISC). This resulted in translational desuppression of VEGF and a proangiogenic
response in vitro and in vivo. Previous studies also observed that miR-130 was present in endothelial cells but failed to demonstrate significant upregulation upon hypoxia.

miR-181a

miR-181a expression is frequently detected in endothelial cells, however its role in angiogenesis is so far not well established. Increased miR-181a activity in primary embryonic lymphatic endothelial cells resulted in substantially reduced PROX1 mRNA and protein levels and reprogramming of lymphatic endothelial cells towards a blood vascular phenotype.

THERAPEUTIC APPLICATIONS AND FUTURE DIRECTIONS

An increasing number of miRNAs that play a part in angiogenesis have been identified. This highlights the potential of miRNAs to act as potential regulatory switches controlling glioblastoma and endothelial cell function during tumor angiogenesis. Additionally, it has been suggested that since a single miRNA is able to control multiple protein-coding genes, the use of miRNAs in angiogenesis modulation may be more efficient due to a "one-hit multiple target" mechanism where multiple messages will be silenced at once. miRNA based anti-angiogenesis cancer therapy, or miRNA therapeutics, may be performed in several ways. First, the activity of pro-angiogenic miRNAs can be inhibited by using so-called anti-miRs which bind to the miRNA itself, or by blocking the binding site on the mRNA target transcript. These anti-miRs represent an attractive way of inactivating pathological miRNAs, as they represent an oligonucleotide which is complementary to the miRNA and competes with the mRNA target transcript. It has been reported that mice injected with a single high dose of anti-miR-126 and exposed to ischemia showed a reduced angiogenic response. This study confirms the efficacy of silencing endothelial specific miRNAs by the use of miRNA inhibitors in vivo. Alternatively, anti-angiogenic miRNAs may be delivered to the location where tumor angiogenesis occurs. This may result in reduced expression of pro-angiogenic proteins. Furthermore, RNA-binding proteins or mimics might be another option to target miRNAs. For example, it has been shown that the RNA-binding protein Dnd1 inhibits the access of miRNAs to target mRNAs, which prevents miRNA-mediated silencing.

A major challenge for the use of miRNA inhibitors or mimics in vivo is to engineer such molecules that limit off-target effects. To achieve this, several chemical modifications have been developed. One of these modifications is the conjugation of miRNA inhibitors (or mimics) to cholesterol, which facilitates the cellular uptake. These miRNA inhibitors are called antagomiRs and are studied widely for their use as miRNA therapeutics. For example, it has been shown that the delivery of a cholesterol-conjugated miR-296 antagomiR resulted in decreased tumor neovascularization in nude mice-bearing subcutaneous U87 glioma xenografts. Another modification is the use of miRNA inhibitors with locked nucleotides acid (LNA), also called LNA-antimiRs. LNA nucleotides contain a modified ribose moiety which results in a locked ribose conformation, and this subsequently increases the hybridization properties of oligonucleotides. Furthermore, the conjugation of 2′-O-methoxyethyl phosphorothioate (2′-MOE) to a miRNA inhibitor also increased the inhibition efficacy,
either by facilitating the cellular uptake, or by increasing the inhibitor stability, or both. This modification is responsible for enhancing the stability of the oligonucleotide.95

Another important aspect in miRNA therapeutics is the fact that the effects of miRNAs can be different depending on cell type. For example, miR-221 and miR-222 target the pro-angiogenesis regulator c-kit in endothelial cells and the tumor suppressor p27 in cancer cells.48 Additional research is needed for strategies to deliver miRNA therapeutics to the site of tumor angiogenesis. A possible approach could be the use of similar strategies developed for siRNA therapeutics. For example, siRNA carrying antibodies that bind cell-specific surface receptors and subsequently undergo internalization is a promising approach which could also apply for miRNAs.92 Additionally, delivery mechanisms used for viral therapeutics could also be a promising strategy.93 Furthermore, the use of nanoparticles and liposomal formulas as miRNA carriers is under investigation.94-96 Moreover, it was recently revealed that glioblastoma cells release microvesicles that contain proteins, mRNAs and miRNAs. These microvesicles are taken up by neighboring cells and have a profound influence on the translational profile of these cells.97, 98 Targeting these microvesicles to the tumor endothelium might be a way for the delivery of miRNA therapeutics. Interestingly, these tumor-derived microvesicles from glioblastoma patients may also hold important diagnostic information which might be useful for therapeutic outcome, which underlines their great potential.97

With the discovery of miRNAs and the regulatory role they play in tumor angiogenesis, new potential therapeutic and diagnostic opportunities could be generated for cancer patients. Interestingly, and adding further complexity, new evidence is emerging on the role of glioblastoma-stem-like-cells (GSCs) in the formation of new blood vessels in brain tumors. Several recent studies describe that glioblastoma cells are able to transform into endothelial-like cells, a process called vascular mimicry.99, 101 These studies demonstrate that a variable number (20-90%) of endothelial cells in glioblastoma have similar genetic alterations as glioblastoma cells. These genetic aberrations in endothelial cells include EGFR amplification and alterations of chromosome 10 and 19, which are typical genomic alterations observed in glioblastoma.102 The endothelial-like cells are derived out of a subpopulation of glioblastoma cells, the CD133+ GSCs which have stem cell characteristics and are likely to be responsible for tumorigenesis.103 It is described that these CD133+ GSCs can differentiate into mutated CD133+/CD144+ endothelial progenitor cells and after stimulation with certain growth factors, including VEGF, can differentiate into mutant mature endothelial cells. The ability of GSCs to alter the tumor vasculature by endothelial cell differentiation represents a new mechanism of angiogenesis.100 These mutated endothelial cells might react differently to current anti-angiogenic treatments that are based on targeting the VEGF pathway.101 The role of miRNAs in the process of GSCs differentiation and in mutant endothelial-like cell functioning has not been elucidated. Hence, it would be of interest to study the differentiation of GSCs into mutated endothelial cells through interference with miRNA function, which might offer new therapeutic options for anti-angiogenesis treatment.
REFERENCES


104. Sun CY, She XM, Qin Y, et al. miR-15a and miR-16 affect the angiogenesis of multiple myeloma by targeting VEGF. Carcinogenesis 2013;34:426-435.


miR-101 IS DOWN-REGULATED IN GLIOBLASTOMA RESULTING IN EZH2-INDUCED PROLIFERATION, MIGRATION AND ANGIOGENESIS


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ABSTRACT

Background
Glioblastoma (GBM) is a malignant brain tumor with dismal prognosis. GBM patients have a median survival of less than 2 years. GBM is characterized by fast cell proliferation, infiltrative migration, and by the induction of angiogenesis. MicroRNAs and polycomb group (PcG) proteins have emerged as important regulators of gene expression.

Methods
Here we determined that miR-101 is down-regulated in GBM, resulting in overexpression of the miR-101 target PcG protein EZH2, a histone methyltransferase affecting gene expression profiles in an epigenetic manner.

Results
Inhibition of EZH2 in vitro by pre-miR-101, EZH2 siRNA, or small molecule DZNep, attenuated GBM cell growth, migration/invasion, and GBM-induced endothelial tubule formation. In addition, for each biological process we identified ontology-associated transcripts that significantly correlate with EZH2 expression. Inhibition of EZH2 in vivo by systemic DZNep administration in a U87-Fluc-mCherry GBM xenograft mouse imaging model resulted in reduced tumor growth.

Conclusion
Our results indicate that EZH2 has a versatile function in GBM progression and that its overexpression is at least partly due to decreased miR-101 expression. Inhibition of EZH2 may be a potential therapeutic strategy to target GBM proliferation, migration, and angiogenesis.
INTRODUCTION

GBM remains among the most devastating cancers with a median survival of less than 15 months and virtually no survival beyond five years\(^1\). GBM is the grade IV glioma and can arise \textit{de novo} or through progression of lower grade gliomas. Evidence supporting the critical role of proliferation, migration and angiogenesis in the biological behavior of these tumors has led to a variety of studies on the basic mechanisms involved. GBM cells are highly proliferative but are also notorious because of their capacity to migrate through the brain parenchyma and their ability to induce angiogenic blood vessel sprouting. Several factors are involved in the angiogenesis process, which results in recruitment, proliferation and alignment of endothelial blood vessel cells through a complex interaction between endothelial cells and tumor cells\(^2\).

miRNAs comprise a large group of endogenous non-coding RNAs that can block mRNA translation or negatively regulate mRNA stability and thereby play a central role in the regulation of gene expression\(^3\). It is also becoming clear that deregulated miRNA expression is a common feature of human diseases, especially in specific forms of cancer\(^4,5\). Recent studies have identified several miRNAs that are altered in GBM tumor cells themselves\(^6,7\) as well as in GBM-associated endothelial cells\(^8\).

PcG proteins are important epigenetic regulators which can function as transcriptional repressors that silence specific sets of genes through chromatin modification\(^9\). PcG proteins are grouped in polycomb repressive complexes (PRC). PRC2 includes enhancer of zeste 2 (EZH2), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2 is the catalytically active component of PRC2 and is capable of trimethylating lysine 27 of histone H3 (H3K27) when in complex with SUZ12 and EED\(^10-15\). Recently, an increasing number of studies linked various oncogenic properties to EZH2, including impaired cellular differentiation and enhanced proliferation and \textit{in vivo} tumor growth\(^16-22\). EZH2 is overexpressed in various cancers, which correlates to decreased patient survival\(^16,18,19,23-25\). Although EZH2 knock down was shown to be embryonic lethal in mice\(^26\), knock down of EZH2 in cancer cells resulted in growth arrest, as well as in diminished tumor growth and reduced metastasis \textit{in vivo}\(^16,20,22\). The role of PcG proteins in GBM is not well understood, but has been described to involve bone morphogenetic protein signalling, controlling the differentiation capacity of GBM cells\(^27\).

Here we report that EZH2 expression in GBM is regulated by miR-101. We show that miR-101 is down-regulated in GBM cells, resulting in increased EZH2 expression and enhanced GBM cell proliferation, migration, and angiogenesis.

RESULTS

To evaluate the expression levels of EZH2 in GBM cells and non-neoplastic brain (NNB) we performed immunohistochemistry for EZH2 protein expression on tissue microarrays containing GBM and NNB samples. Most of the GBM samples showed fields of strong nuclear staining for EZH2 while none of the NNB samples did (Fig. 1A). Increased EZH2 expression correlated with glioma grade and glioma recurrence (Fig. 1B), suggesting that EZH2 could be a marker for glioma aggressiveness. In addition, the Rembrandt database was used to show that EZH2 expression correlated with decreased GBM patient survival.
EZH2 protein was strongly expressed in human GBM cell lines, including U251 and U87, but not in NNN (Fig. 1D).

In order to determine whether potential GBM-expressed miRNAs could affect EZH2 expression we first determined which miRNAs expressed in NNN are differentially expressed in GBM (Supplemental Table S1A). Next, we used miRbase to identify 63 miRNAs predicted to target EZH2. Upon integration of the list of miRNAs predicted to target EZH2 and the differential GBM/NNN miRNA expression ratios, we found that miR-101, miR-98, miR-137, and miR-139 were down-regulated in GBM tissue as compared to NNN and have the potential to regulate EZH2 (Supplemental Table S1B). Another miRNA which was previously found to target EZH2, miR-26a, was not included in our subset of miRNAs expressed in the brain, and therefore not part of our study.

Figure 1. miR-101 regulates EZH2 in glioma cells. (A) Representative tissue sections stained with an antibody directed against EZH2. Immunohistochemical staining shows absent nuclear staining of non-neoplastic brain (NNN), and strong nuclear staining in glioblastoma (GBM). Scale bar = 100 µm. (B) Quantification of EZH2 protein expression in glioma tissue microarrays. Negative = 0%; Weak = <5%; Intermediate 5-25%; Strong = >25% positive EZH2 staining. EZH2 expression correlates to glioma grade (left) and to glioma recurrence (right). (C) Correlation between GBM patient survival and EZH2 mRNA expression (http://caintegrator-info.nci.nih.gov/rembrandt). (D) EZH2 protein analysis by Western blot on various cell lines and non-neoplastic brain tissue (NNN).
We were particularly interested in miR-101 since it was confirmed to bind the EZH2 3'-UTR at two sites (Fig. 2A), and was recently shown to interact with EZH2 in other types of cancer\textsuperscript{30, 31}. Previous analysis showed that genomic loss of miR-101-1 and miR-101-2 alleles was observed in 18.7% of GBM cases\textsuperscript{30, 32}. Based on these findings, we decided to further analyze miR-101/EZH2 functionality in GBM. First, down-regulation of miR-101 was confirmed in primary glioma samples of different WHO grades by quantitative PCR (qRT-PCR) analysis (Fig. 2B and 2C). To establish that miR-101 affects EZH2 protein expression and histone methyltransferase activity in GBM, we transfected human U87 GBM cells with pre-miR-101 molecules and determined the levels of EZH2 protein and H3K27me3. In addition to pre-miR-101, we included EZH2 siRNA, non-related control oligonucleotides, and the S-adenosylhomocysteine hydrolase inhibitor DZNep, a potent EZH2 inhibitor\textsuperscript{33, 34}. DZNep,

![Diagram](image)

**Figure 2.** miR-101 is down-regulated in GBM and targets EZH2. (A) Predicted RNA structure of the 3'UTR of EZH2 by RNAfold software, in red the 2 miR-101 binding sites are indicated. (B and C) Down-regulation of miR-101 was confirmed by qRT-PCR analysis. RNA extracted from surgically removed gliomas from patients was analyzed by qRT-PCR. The data were normalized to the level of GAPDH mRNA in each sample. NB = Non-neoplastic brain; II, III, IV indicate WHO glioma grades. (D) EZH2 and H3K27me3 expression analysis by Western blot in U87 GBM cells following transfection with pre-miR-101, pre-miR-control, EZH2 siRNA or control, or treatment with DZNep. Data presented as relative EZH2 expression compared to non-transfected (n.t.) cells. Error bars indicate s.d. *p<0.05, **p<0.001, t test.
EZH2 siRNA, and pre-miR-101 all notably repressed EZH2 protein expression and reduced the levels of trimethylation of H3K27 (Fig. 2D), indicating inhibition of EZH2 function.

To determine the effects of EZH2 on GBM cell proliferation we first analyzed which genes associated with cell proliferation correlated with EZH2 expression in GBM and NNB. First, EZH2 was found overexpressed in most GBM samples as compared to NNB. However, in few samples the EZH2 mRNA expression was found to be in the same range as in the NNB (Fig. 3A). Out of the 1419 genes that were linked to the proliferation gene ontology as determined by AmiGO, 214 genes showed a clear correlation (>67%) with EZH2 expression in GBM (Fig. 3A). Interestingly, the GBM samples with normal EZH2 expression levels also showed less expression of the genes associated with cell proliferation. Next, cellular proliferation was studied in GBM cell cultures to determine if EZH2 influences the proliferation of GBM cells. miR-101 induction, EZH2 knock down by siRNA and treatment with DZNep significantly reduced cellular proliferation in U87-GFP GBM cells (Fig. 3B and 3C). The effect of DZNep treatment on proliferation inhibition was confirmed in other GBM cell lines in vitro (Fig. 3D).

To determine the effects of EZH2 on GBM cell migration we analyzed which genes belonging to the migration gene ontology correlated with EZH2 expression in GBM and
NNB. A significant correlation between the expression of 28 out of 279 genes associated with cell migration and EZH2 expression was observed (Fig. 4A). In order to determine whether miR-101 up-regulation or EZH2 inhibition also affected GBM cell migration, scratch assays were performed. Up-regulation of miR-101 by pre-miR-101 resulted in a significant decrease in U87 migration. The EZH2 inhibitors DZNep and EZH2 siRNA showed a similar decrease in migration (Fig. 4B and 4C). To further evaluate the effects of miR-101/EZH2 modulation on in vitro migration and invasion, a Boyden chamber assay was used. U87 cells that were

Figure 4. In silico analysis of EZH2 mRNA expression and the correlation to migration-related mRNAs. Heatmap of percentile fold change of gene expression of migration-related genes sorted by correlation with EZH2 expression (rows) in patients sorted by level of EZH2 expression (columns). Color coding is similar to Fig 3A. (B) U87 monolayer cultures were scratched. Images were acquired directly after scratching (t = 0) and 24 h later (t = 24). The migration front is indicated by the dashed lines. Scale bar = 450 µm. (C) Quantitation of cell migration into the scratch using ImageJ software. (E and F) U87 cells were transfected with pre-miR-101, EZH2 siRNA, non-related control molecules, or treated with DZNep, and analyzed for invasion capability. EZH2 inhibition decreased invasion as shown by Hoechst staining. Error bars indicate s.d. *p<0.05, ***p<0.001, t test. Scale bar = 225 µm
transfected with pre-miR-101 showed a significant decrease in ability to invade, as visualized by Hoechst staining (Fig. 4D and quantitated in 4E). Again, similar results were observed after treatment with the EZH2 inhibitor DZNep or EZH2 knock down by siRNA (Fig. 4D and 4E).

To determine the effects of EZH2 on GBM-induced angiogenesis we also analyzed which genes belonging to the angiogenesis gene ontology correlated with EZH2 expression in GBM. Again, a significant correlation between the expression of 33 out of 308 genes associated with angiogenesis and EZH2 expression was observed (Fig. 5A). Next, HBMVECs were cultured in EBM, EGM, or EBM supplemented with U87 human GBM cells expressing GFP (U87-GFP), all on a Matrigel substratum to promote tubule network formation. Tubules were visualized by a combination of light and fluorescence microscopy. After pre-treatment of the GBM cells with DZNep, or transfection with pre-miR-101, EZH2 siRNA, or non-related oligonucleotides of similar chemistry, and subsequent co-culturing with HBMVECs on Matrigel, we analyzed tubule length and tubule branching. Up-regulation of miR-101 in U87-GFP cells resulted in a substantial decrease in total tubule length and tubule branching.

Figure 5. miR-101/EZH2 affects GBM angiogenesis in vitro.
(A) In silico analysis of EZH2 mRNA expression and the correlation to angiogenesis-related mRNAs. Heatmap of percentile fold change of gene expression of angiogenesis-related genes sorted by correlation with EZH2 expression (rows) in patients sorted by level of EZH2 expression (columns). Color coding is similar to Fig 3A. (B) U87-Fluc-mCherry cells were treated and co-cultured with HBMVECs on Matrigel coated plates. Tubule formation was assessed 96 h after transfection as tubule length and branching. Scale bar = 450 µm. (C) Quantitation of tubule length and branching in (B) using ImageJ software. Error bars indicate s.d. *p<0.05, ***p<0.001, t test.
(Fig. 5B and 5C). In addition to pre-miR-101, treatment of the U87-GFP cells with EZH2 siRNA and DZNep also inhibited U87-induced tubule network formation (Fig. 5B and 5C).

Finally, to study the effects of modulation of EZH2 on GBM growth in vivo, we implanted U87 human GBM cells stably expressing Fluc and the fluorescent protein mCherry (U87-Fluc-mCherry) into the flanks of nude mice. Tumor growth was monitored over time by intravenous injection of the Fluc substrate o-luciferin and in vivo bioluminescence imaging using a CCD camera. After tumor cell implantation, we injected one set of mice (n = 5) intravenously with the EZH2 inhibitor DZNep (0.07 mg/kg) and a parallel control set (n = 5) with PBS only, at day 3, 5, and 7, followed by weekly injection. CCD camera imaging of Fluc bioluminescence activity in the tumor allowed us to monitor tumor growth over time. The tumor volume of the mice treated with PBS increased logarithmically over time, while the tumor volume in the mice treated with DZNep showed reduced growth (Fig. 6A and 6B).

**DISCUSSION**

Here we show that miR-101 is down-regulated in glioma in a grade dependent manner. The impaired translational repression of EZH2 by miR-101 causes EZH2 overexpression in GBM, which correlates with patient survival. EZH2 is a methyltransferase that affects the expression of many genes. Based on in silico expression analysis and EZH2 expression correlation
we found that EZH2 overexpression induces glioma proliferation, migration/invasion, and angiogenesis, processes driving glioma progression.

miR-101/EZH2 was found to be deregulated in several other types of cancer, including prostate cancer\textsuperscript{20}, bladder transitional cell carcinoma\textsuperscript{31}, gastric cancer\textsuperscript{37}, and described to strongly correlate with migration, invasion, and metastasis\textsuperscript{30, 37}. Here we demonstrate a role for EZH2 overexpression in GBM, which could be inhibited by miR-101, siEZH2, or small molecule inhibitor DZNep. Besides induction of migration and invasion, we identified a role for EZH2 in cellular proliferation and the induction of angiogenesis, indicating a versatile pro-tumoral function for EZH2 in GBM. Inhibition of EZH2 by DZNep resulted in reduced GBM growth \textit{in vitro} and in a limited experiment \textit{in vivo}. Altogether, these results indicate that EZH2 may be a useful drug target for the treatment of GBM.

miRNAs are known to affect cellular processes such as angiogenesis\textsuperscript{8, 38, 39}. Here we show a role for miR-101 in GBM-induced angiogenesis. Unpublished data indicate that miR-101 also regulates EZH2 in endothelial cells. We provide evidence that miR-101 down-regulation regulates angiogenesis by induction of EZH2 and a pro-angiogenic mRNA profile. However, the exact mechanisms of EZH2 function in endothelial and GBM cells remains to be investigated. Besides miR-101, we also found the predicted EZH2 targeting miRNAs miR-98, miR-137, and miR-139 to be down-regulated in GBM cells as compared to NNB tissue. It remains to be investigated whether these miRNAs truly repress EZH2, and whether other previously identified miR-101 target genes are also repressed by miR-101 in GBM cells, these may include Cox-2, Mcl-1 and Fos\textsuperscript{37}, MAGI-2\textsuperscript{26}, DNA-PKcs and ATM\textsuperscript{41}, COX-2\textsuperscript{42}. The complex interaction of reduced miR-101-mediated translational repression and increased EZH2-mediated transcriptional repression at least seem to cause pro-tumoral switches in the GBM transcriptome profile. Interestingly, our results also identified that a subset of GBMs express normal levels of EZH2 mRNA. We found that the genes associated with proliferation, migration, and angiogenesis were also expressed in the normal range, following the EZH2 expression levels. Further research in the nature and behavior of this subset of EZH2-low GBMs and its correlation to miR-101 expression is warranted.

In conclusion, our results indicate that EZH2 has a versatile pro-tumoral function in GBM and that its overexpression is at least partly due to decreased miR-101 expression. Inhibition of EZH2 may be a potential therapeutic strategy to target GBM proliferation, migration, and angiogenesis.

**MATERIALS AND METHODS**

**Cells**

Human brain microvascular endothelial cells (HBMVECs; Cell Systems ACBRI-376) were cultured in EGM medium (Lonza). C6, 293T, MCF-7, U118, U251, U373, and U87 cells (U-87 MG; ATCC) were cultured in DMEM (Lonza) containing 10% FBS and antibiotics. U87-Fluc-mCherry cells were produced by stably transducing U87 cells with CMV-controlled expression cassette using a lentiviral vector\textsuperscript{43}. 
Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) analysis was used to determine the relative expression levels of miR-101, miR-186, EZH2, and GAPDH mRNA. Total RNA was isolated using the miRVANA miRNA isolation kit. Equal amounts of RNA were converted into cDNA using miR-101, miR-186, EZH2, and GAPDH RT primers (Applied Biosystems and Qiagen, according to the manufacturer’s protocol). Subsequently, quantitative PCR was performed using primers and materials from Applied Biosystems. All experiments were performed using biological triplicates and experimental duplicates. The data was normalized to miR-186 or GAPDH expression levels.

Disclosed primers used were:

- **human EZH2**
  - 5’-CCTGAAGTATGTCGGCATCGAAAGAG-3’ (forward)
  - 5’-TGCAAAAATTCACTGGTACAAAACACT-3’ (reverse)

- **human GAPDH**
  - 5’-GTCGGAGTCAACGGATT-3’ (forward)
  - 5’-AAGCTTCCCGTTCTCAG-3’ (reverse)

Western blots and immunohistochemistry

EZH2 protein and H3K27me3 expression was detected by SDS-PAGE followed by Western blot analysis, using mouse anti-EZH2 monoclonal antibody (BD biosciences) and rabbit anti-H3K27me3 (Upstate Biotechnology). Mouse anti-Actin (Millipore) was used as a loading control. Protein levels were detected using ECL detection solution (GE healthcare) and visualized on X-ray film (GE healthcare). Paraffin sections of human GBM tissue and NNB were incubated with monoclonal mouse anti-EZH2 (BD biosciences) antibodies. Positive reactions were visualised using a secondary antibody (DAKO EnvisionHRP) and 3,3-diaminobenzidine or liquid red chromogen.

miRNA modulation

50 nM of pre-miR-101 (Ambion) or pre-miR-control (Ambion) oligonucleotides were transfected into U87 human GBM cells using Lipofectamin2000 (Invitrogen), according the manufacturer’s protocol. For the inhibition of EZH2 50 nM of EZH2 siRNA (Qiagen) was transfected into U87 cells. siRNA-AF or control oligonucleotides (Qiagen) were used as controls. After 5 h of transfection, the transfection medium was replaced by DMEM until further analysis.

In silico analysis

A RNA microarray dataset containing 81 GBM and 23 NNB samples as published by was used to obtain mRNA expression estimates. To quantify the differential level of expression for each glioblastoma sample, the significance analysis of microarrays algorithm was performed using the samr package (version 1.24 by B. Narasimhan and R. Tibshirani) in R, A Language and Environment for Statistical Computing (release 2.4.1; Vienna, Austria; http://www.R-project.org) based on randomization. Unpaired two class comparison for cancer versus normal tissue samples was performed, unless paired samples were involved, in which case a paired analysis was performed. The selection of the delta parameter was based on a median false discovery rate less than 0.05. The default number of 100 permutations was used. This resulted in fold change values for all 19769 genes on the Affymetrix microarray platform. Using the AmiGO tool of the Gene Ontology project lists of transcripts associated with the
biological processes proliferation (GO:0008283; 9/20/10), migration (GO:0030334; 9/20/10) and angiogenesis (GO:0001525; 9/20/10) were obtained. Lists of unique transcripts were prepared by removing duplicate entries. Subsequently the microarray dataset was queried for the genes in each of these ontologies. Samples were sorted on EZH2 expression level for the NNB and GBM samples separately and the average expression levels scaled on a gene by gene basis for genes significantly correlating with EZH2 expression (absolute value Pearson correlation > 0.667) were plotted as a heatmap using the gplots package in R.

**In vitro angiogenesis assay**
HBMVECs were cultured on Matrigel (BD biosciences) in EBM (Lonza) in the presence or absence of U87-Fluc-mCherry cells, or EGM (Lonza). The experiments were performed in triplicate, repeated twice and judged in a double blind fashion by at least two observers. At least 3 pictures were taken randomly of each culture well using a digital camera system (Leica). Total tubule length and number of branches were analyzed using the software program ImageJ.

**In vitro migration assay**
GBM cells were grown to confluence in 24-wells plates and were transfected with 50 nM of oligonucleotides or treated with DZNep (5 µM). At 72 h after transfection an artificial wound was created using a pipette tip after which the cells were further incubated. To analyse cell migration into the scratched area, pictures were taken at 0 and 24 h using a digital camera system coupled to a microscope. ImageJ was used to determine the migration distance (in µm) as the reduction of the width of the open area.

**In vitro invasion assay**
GBM invasion function was analyzed using a Boyden chamber assay. A 24-well Transwell system (Corning) was used, with each well containing a permeable transwell insert containing a 6.5 mm polycarbonate membrane with 8 µm pores. The inserts were coated with 4x diluted basement membrane extract (Trevigen) and incubated overnight in 5% CO₂ at 37°C. GBM cells were transfected with 50 nM of oligonucleotides for 24 h or treated with DZNep (5 µM for 24 h), starved for 24 h and harvested in serum free medium. Per insert 25,000 GBM cells were subsequently placed on the membrane. The inserts were immersed in a 24-well plate that was filled with EGM growth medium culture media. After incubation for 24 h, the membrane was washed briefly with PBS. The upper side of the membrane was then wiped gently with a cotton ball. The membrane was then fixed in 4% formaldehyde and stained with Hoechst. The magnitude of GBM cell migration was evaluated by counting the migrated cells in 3 random high-power (5x) microscope fields.

**Tumor model and bioluminescence imaging**
All animal studies were approved by the Massachusetts General Hospital Review Board. Nude mice were anesthetised with i.p. injection of xylazine (5 mg/kg) and ketamine (100 mg/kg). 50 µl containing 1 x 10⁶ U87-Fluc-mCherry cells were pre-mixed with an equal volume of matrigel (BD biosciences) and implanted in the flanks of nude mice. DZNep was administered intravenously to tumor-bearing mice at a dose of 0.07 mg/kg diluted in 100 µl PBS at day 3, 5 and 7 after tumor implantation, followed by weekly injection. Mice were
anesthetized as above and Fluc imaging was performed 10 min after intravenous injection of 150 µl beetle D-luciferin (4 mg/kg body weight) (Xenogen), and recording photon counts over 5 min using a cooled CCD camera with no illumination. Dim polychromatic illumination was used to take a light image of the animal. Visualization was performed using CMIR-Image, a program developed by the Center for Molecular Imaging Research using image display and analysis suite developed in IDL (Research Systems Inc., Boulder, CO). An intensity contour procedure to identify bioluminescence signals with intensities significantly greater than the background was used to define regions of interest. The mean, standard deviation and sum of photon counts in these regions were calculated as a measurement of Fluc activity.

**Statistics**

Difference in biological properties between parental and EZH2 downregulated cells was analysed using Student's t-test. P-values <0.05 were considered statistically significant.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


DOWN-REGULATION OF miR-101 IN ENDOTHELIAL CELLS PROMOTES BLOOD VESSEL FORMATION THROUGH REDUCED REPRESSION OF EZH2


*These authors contributed equally to this work

ABSTRACT

Angiogenesis is a balanced process controlled by pro- and anti-angiogenic molecules of which the regulation is not fully understood. Besides classical gene regulation, miRNAs have emerged as post-transcriptional regulators of angiogenesis. Furthermore, epigenetic changes caused by histone-modifying enzymes were shown to modulate angiogenesis as well. However, a possible interplay between miRNAs and histone-modulating enzymes during angiogenesis has not been described. Here we show that VEGF-mediated down-regulation of miR-101 caused pro-angiogenic effects. We found that the pro-angiogenic effects are partly mediated through reduced repression by miR-101 of the histone-methyltransferase EZH2, a member of the Polycomb group family, thereby increasing methylation of histone H3 at lysine 27 and transcriptome alterations. In vitro, the sprouting and migratory properties of primary endothelial cell cultures were reduced by inhibiting EZH2 through up-regulation of miR-101, siRNA-mediated knockdown of EZH2, or treatment with 3-Deazaneplanocin-A (DZNep), a small molecule inhibitor of EZH2 methyltransferase activity. In addition, we found that systemic DZNep administration reduced the number of blood vessels in a subcutaneous glioblastoma mouse model, without showing adverse toxicities. Altogether, by identifying a pro-angiogenic VEGF/miR-101/EZH2 axis in endothelial cells we provide evidence for a functional link between growth factor-mediated signalling, post-transcriptional silencing, and histone-methylation in the angiogenesis process. Inhibition of EZH2 may prove therapeutic in diseases in which aberrant vascularization plays a role.
INTRODUCTION

Angiogenesis - the formation of new blood vessels - occurs during tissue growth and development, but also during wound healing and cancer. Angiogenesis is a balanced process controlled by pro- and anti-angiogenic molecules. VEGF has been identified as one of the most potent factors involved in angiogenesis. VEGF, produced in large amounts by cancer cells during tumor growth, interacts with its receptors VEGFR1 and VEGFR2 thereby causing endothelial cell survival, proliferation, and sprouting.

Recently, it was shown that angiogenesis is also controlled by miRNAs. miRNAs comprise a large group of endogenous non-coding RNAs that can block mRNA translation or negatively regulate mRNA stability and thereby play a central role in regulating gene expression. We previously showed that VEGF signalling in primary endothelial cell cultures caused overexpression of VEGFR2 in a positive feed-forward loop, which is at least partly regulated by loss of miRNA-mediated control of VEGF receptor degradation. Moreover, we found that glioblastoma cells, notorious for their VEGF production, elicited a similar response when co-cultured with endothelial cells.

Another class of potential regulators of gene expression is the group of chromatin modulators involved in histone modification, such as histone deacetylases and histone methyltransferases. Polycomb group proteins (PcG) function as transcriptional repressors that silence specific sets of genes through chromatin modification. PcG proteins act together in polycomb repressive complexes (PRC). PRC2 includes enhancer of zeste 2 (EZH2), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2 is the catalytically active component of PRC2 and is capable of trimethylating histone H3 at lysine 27 (H3K27me3). Expression profiling indicated that EZH2 transcripts are up-regulated in ovarian carcinoma-associated endothelial cells, and modulated EZH2 expression was described to affect genes associated with endothelial differentiation. It was also described that EZH2 expression is regulated by miRNAs including miR-26, miR-214, and miR-101.

Here we report that EZH2 is up-regulated in angiogenic endothelial cells and that miR-101 is down-regulated in primary endothelial cells exposed to VEGF or glioblastoma cells, as well as in blood vessel endothelial cells that were isolated from vascularized glioblastomas from patient samples. We confirm that EZH2 is a direct post-transcriptional target of miR-101 and that VEGF-mediated down-regulation of miR-101 results in increased expression of the histone methyltransferase EZH2 in angiogenic endothelial cells. Finally, we show that inhibition of EZH2 histone methyltransferase activity inhibits vascularization in glioblastomas in vivo, suggesting a possible therapeutic potential for EZH2 inhibitors in the many diseases in which aberrant vascularization plays a role.

RESULTS

miR-101 directly targets EZH2 and is down-regulated in tumor-associated endothelial cells

We recently showed that various miRNAs are deregulated in human brain microvascular endothelial cells (HBMVECs) co-cultured with U87 glioblastoma cells. Here, a ~3-fold down-
regulation of miR-101 was confirmed by qRT-PCR analysis using miR-186 and GAPDH as normalization controls, both of which were uniformly expressed in endothelial cells in the presence or absence of tumor cells (Fig. 1A). In addition, we examined the expression of miR-101 in angiogenic endothelial cells derived from gliomas resected from patients. Here, endothelial cells were isolated from blood vessels derived from three glioblastomas and three grade II gliomas, as well as from non-neoplastic brain. All tumor samples were highly angiogenic as confirmed by CD31 immunohistochemical analysis. We observed reduced miR-101 expression levels in glioma blood vessels as compared to normal brain vessels, as determined by qRT-PCR analysis (Fig. 1B). We were particularly interested in miR-101 since this miRNA was recently shown to interact with EZH2 in different types of cancer, and predicted to bind the EZH2 3'-UTR at two sites. To confirm that miR-101 regulates the 3'-UTR of EZH2, we transfected a firefly luciferase (Fluc) reporter vector encoding the wild type 3'-UTR of EZH2 or lacking the EZH2 3'-UTR (control) into 293T cells. Overexpression of miR-101 by pre-miR-101, but not by pre-miR-control, resulted in a decrease in Fluc expression in the cells expressing the reporter with the 3'-UTR of EZH2. No effects were observed using the control vectors lacking the EZH2 3'-UTR (Fig. 1C).

**EZH2 is up-regulated in tumor-associated endothelial cells**

To determine whether EZH2 is overexpressed in angiogenic endothelial cells we analyzed EZH2 mRNA and protein expression. We found the expression of EZH2 mRNA to be increased in HBMVECs after co-culture with human U87 glioblastoma cells (Fig. 2A). In addition, we analyzed EZH2 mRNA expression in glioblastoma blood vessel endothelial cells resected from patient samples as described above. qRT-PCR revealed that EZH2 expression levels were up-regulated in glioblastoma blood vessels as compared to normal brain vessels (Fig. 2B). The overexpression of EZH2 mRNA in the glioma-associated endothelial cells was in line with previously described mRNA expression data of ovarian carcinoma-associated endothelial cells. Upon examination of the EZH2 immunohistochemistry of vascularized glioblastoma and non-neoplastic brain tissue slices, we noticed positive nuclear staining for EZH2 in the endothelial cells of newly formed blood vessels in the glioblastoma samples, but not in the non-neoplastic brain tissues (Fig. 2C). These results indicate an inverse correlation of miR-101 and EZH2 expression levels in angiogenic endothelial cells and led us to further investigate the role of deregulated EZH2 expression during angiogenesis.

**VEGF down-regulates miR-101 expression in endothelial cells resulting in EZH2 up-regulation**

To determine whether EZH2 expression in HBMVECs could be suppressed by up-regulation of miR-101 we performed Western blot analysis for EZH2 after transfection of cells with pre-miR-101, siRNA directed against EZH2, or non-related control oligonucleotides. We also included lysates from cells treated with the S-adenosylhomocysteine hydrolase inhibitor DZNep, a potent inhibitor of EZH2 histone methyltransferase activity. Treatment of the endothelial cells with DZNep, EZH2 siRNA, or pre-miR-101, all markedly reduced EZH2 protein levels. EZH2 gene silencing also decreased histone 3 methylation at lysine 27, as determined by Western blot (Fig. 3A). To establish whether tumor-derived soluble factors...
are sufficient to induce differential expression of miR-101 and EZH2, HBMVEC cells were cultured in endothelial basal medium (EBM), EBM supplemented with an angiogenic cocktail (EGM), or EBM conditioned culture medium derived from U87 glioblastoma cells (U87cm). We used qRT-PCR to show down-regulation of miR-101 levels in HBMVECs cultured in U87cm or EGM (Fig. 3B). Interestingly, we were not able to measure significant and reproducible miR-101 down-regulation in HUVECs upon exposure to U87 conditioned medium, in contrast to HBMVECs (Fig. S1A). In parallel to the miR-101 down-regulation, EZH2 protein levels were up-regulated in HBMVECs grown in either U87cm or EGM (Fig. 3C) as well as in HUVECs.
Figure 2. EZH2 is up-regulated in angiogenic endothelial cells and is controlled by miR-101. (A) Overexpression of EZH2 in HBMVECs exposed to U87-GFP glioblastoma cells confirmed by qRT-PCR analysis. RNA extracted from CD31+ HBMVECs cultured in the presence or absence of U87 glioblastoma cells was analysed by qRT-PCR with primers specific for EZH2. The data were normalized to the levels of GAPDH mRNA in each sample. (B) RNA extracted from individual glioma endothelial samples was analyzed by qRT-PCR for expression levels of EZH2. All values were normalized to GAPDH mRNA levels in the same samples. (n=3) Error bars indicate s.d. *p<0.05, ***p<0.001 by t test. (C) Immunohistochemical staining for EZH2, or EZH2 and CD31, in glioblastoma blood vessels. While glioblastoma sections show positive nuclear staining for EZH2 in tumor blood vessels, staining is absent in NNB. In the top panels EZH2 stains brown and arrows indicate blood vessels. In the bottom panels EZH2 stains purple and CD31 stain brown. Scale bar = 50 µm (top panels) or 15 µm (bottom panels).
grown in EGM but not reproducibly in HUVECs grown in U87cm (Fig. S1B). EGM and U87cm both contain many growth factors. VEGF in particular, appears to play a major role in angiogenesis\textsuperscript{11, 33}. To address the role of VEGF in regulating miR-101 and EZH2 expression, HBMVECs were cultured for 24 h in EBM supplemented with different amounts of VEGF, or in EGM, after which total RNA was isolated and miR-101 and EZH2 expression levels were

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Figure 3. VEGF down-regulates miR-101 expression in endothelial cells resulting in EZH2 up-regulation. (A) HBMVECs protein expression analysis of EZH2 and H3K27me3 following transfection of HBMVECs with pre-miR-101, EZH2 siRNA, non-related control molecules or treatment with DZNep. Numbers indicate relative EZH2 protein expression normalized against β-Actin expression. (B) Inhibition of miR-101 in HBMVECs exposed to U87 secreted factors was confirmed by qRT-PCR analysis. RNA extracted from HBMVECs cultured in EBM, EGM or EBM derived from U87 glioblastoma cells was analyzed by qRT-PCR with primers specific for miR-101. The data were normalized to the levels of GAPDH mRNA in each sample. (C) HBMVECs protein expression analysis of EZH2 following culturing in either EBM, EGM or EBM derived from U87 glioblastoma cells. Numbers indicate the relative expression of EZH2 compared to cells cultured in EBM. (D) HBMVECs were grown in EBM supplemented with different amounts of VEGF for 24 h, after which RNA was isolated to determine the expression levels of miR-101 and EZH2 by qRT-PCR. The data were normalized to the level of GAPDH mRNA in each sample. (n=3) Error bars indicate s.d. *p<0.05, ***p<0.001, t test.
determined by qRT-PCR (Fig. 3D). An amount of 1 ng/ml of VEGF was sufficient to significantly down-regulate miR-101 levels in HBMVECs and at higher doses its inhibitory effect almost equalled that of the angiogenic cocktail used in EGM medium, indicating that VEGF plays an important role in miR-101 regulation in endothelial cells. In parallel to miR-101 down regulation, EZH2 levels were significantly increased after VEGF stimulation of HBMVECs.

**mRNA profiling of VEGF and EZH2 modulated endothelial cells**

To gain insights into target genes affected by EZH2 mediated gene silencing in the presence of VEGF stimulation, we suppressed EZH2 in HBMVECs cultured in the presence of VEGF and subsequently isolated the RNA. We then used Agilent 44K gene expression arrays to determine mRNA profiles and identify genes whose expression levels were changed by EZH2 silencing in the presence of VEGF. Simultaneously, we used expression arrays to identify a pro-angiogenic gene profile in HBMVECs stimulated by VEGF. VEGF stimulation significantly induced elongation of the endothelial cells (Fig. 4A) indicating angiogenic activation. EZH2 knockdown efficiency and H3K27me3 reduction in the array samples were confirmed by Western blot analysis (Fig. 4B). To identify which VEGF target genes are potentially depending on EZH2 regulation a comparison was made between the top 5 percentile genes whose expression was increased after EZH2 silencing (n=1865) and the top 5 percentile genes down-regulated by VEGF stimulation (n=1853) (Fig. 4C, D). We identified a significant overlap of 138 genes (Fig. 4E) in both sets (p < 10^-9). Of note, the recently reported EZH2 target gene VASH1 was also identified in the set of genes both regulated by VEGF and EZH2.

**Inhibition of EZH2 reduces endothelial tubule formation in vitro**

To further assess the function of EZH2 up-regulation in angiogenesis, we examined EZH2 inhibition in angiogenic HBMVECs in culture. HBMVECs were cultured in EBM, EGM, or were co-cultured with U87 glioblastoma cells expressing GFP (U87-GFP) in EBM, all on a Matrigel substratum to promote tubule formation. After treatment of HBMVECs with DZNep, or transfection with pre-miR-101, EZH2 siRNA, or controls, and subsequent culturing on Matrigel, we analyzed endothelial tubule length and tubule branching. Tubules were visualized by a combination of light and fluorescence microscopy, to visualize the endothelial cells and U87-GFP cells, respectively (Fig. 5A).

Up-regulation of miR-101 resulted in a significant decrease in total tubule length and tubule branching (Fig. 5A and B). In addition, treatment with EZH2 siRNA and DZNep also inhibited growth factor- or glioblastoma-induced HBMVEC tubule network formation (Fig. 5A and B). In HUVECs treatment with EZH2 siRNA and pre-miR-101 also led to a significant decrease in tubule length. DZNep treatment did not significantly reduce tubule length in HUVECs, although the quality of the resulting networks seemed less than in the control condition (Fig. S1C and D). These results indicate that EZH2 is involved in endothelial tubule formation.

**Inhibition of EZH2 reduces endothelial migration and invasion in vitro**

Endothelial migration and invasion are two other essential steps in the tumor vascularization process. In order to determine the effect of EZH2 inhibition on growth factor-induced endothelial cell migration, scratch assays were performed. HBMVEC cells were treated with either pre-miR-
Figure 4. mRNA profiling of VEGF and EZH2 modulated endothelial cells. (A) Primary human brain microvascular endothelial cells (HBMVECs) were cultured in the absence (top) or presence (bottom) of VEGF. (B) Western blot analysis of EZH2 and H3K27me3 expression in HBMVECs upon silencing of EZH2 by EZH2 siRNA. (C) Heatmap representation of top 5% percentile genes up-regulated by EZH2 knock down or down-regulated by VEGF stimulus, the overlapping section of genes regulated by both EZH2 knock down and VEGF stimulus is shown on the left. The top bar represents Log2 fold change ratios of EZH2kd (all >0), the bottom bar represents Log2 fold change ratios of VEGF stimulus (all<0). (D) Venn diagram showing a significant overlap of EZH2 regulated and VEGF regulated genes. Top 5% percentile genes up-regulated by EZH2 knock down or top 5% percentile genes down-regulated by VEGF stimulus were considered potential targets. (E) Detail of heatmap, showing the cluster of genes that are regulated by both EZH2 knock down and VEGF stimulus.
Figure 5. Inhibition of EZH2 reduces endothelial tubule formation in vitro. (A) HBMVECs were cultured on Matrigel coated plates in EBM, EGM, or EBM supplemented with U87-GFP cells. Scale bar = 450 µm. Inhibition of EZH2 in HBMVECs, either by transfection with pre-miR-101, EZH2 siRNA, or treatment with DZNep significantly reduced tubule formation as compared to control cells. Tubule formation was assessed as tubule length and branching. (B) Quantitation of tubule length and branching using ImageJ software. (n=3) Error bars indicate s.d. *p<0.05, **p<0.001, t test.
101, EZH2 siRNA, or DZNep, and cultured in EGM. To promote migration a scratch was made in the confluent HBMVEC monolayer and the migration distance of the HBMVECs into the scratched area was measured and quantitated 24 h later (Fig. 6A and B). Inhibition of EZH2 by pre-miR-101, EZH2 siRNA, or DZNep, resulted in a significant decrease in HBMVEC migration (Fig. 6A and B). We did not detect significant differences in HBMVEC proliferation over the same period, only after a 72 h period HBMVECs proliferation was negatively affected (Fig S2A and B).

To further evaluate the effects of miR-101/EZH2 modulation on endothelial invasion, a Boyden chamber Transwell assay was used. HBMVECs transfected with pre-miR-101 were seeded on the porous membrane of the Transwell insert and the ability of these cells to invade into the lower compartment was measured after 24 h. HBMVECs transfected with pre-miR-101 showed a 60% decrease in their ability to invade compared to control transfected cells, as visualized by Hoechst staining and quantification of the number of invaded cells (Fig. 6C and D). Similar results were observed after treatment with the EZH2 inhibitor DZNep or EZH2 knock down by siRNA (Fig. 6C and D). These results support a functional role for the miR-101/EZH2-axis in the angiogenesis process, where deregulation of EZH2, through EZH2 knockdown or by using the EZH2 small molecule inhibitor DZNep, mimics the anti-angiogenic effects observed after up-regulating miR-101 levels.

**EZH2 inhibitor DZNep reduces glioblastoma-induced angiogenesis in vivo**

To study the anti-angiogenic effect elicited by EZH2 inhibition in vivo, we used a subcutaneous glioblastoma mouse model and the EZH2 inhibitor DZNep. U87 glioblastoma cells, stably expressing the bioluminescence reporter Fluc and the fluorescence reporter mCherry, were implanted in the flank of nude mice (N = 10). Intravenous (i.v.) injections of the EZH2 histone methyltransferase inhibitor DZNep (0.07 mg/kg) were performed on day 3, 5 and 7 after implantation of the cells followed by weekly injection. Tumor growth was monitored using in vivo Fluc bioluminescence imaging after injection of its substrate D-Luciferin and acquiring photon counts using a CCD camera. Tumor volume in control mice increased exponentially over time, while tumors in mice treated with DZNep showed reduced growth (Fig. 7A). Ki67 staining of tumor sections revealed areas with proliferating cells in both DZNep treated and control mice (Fig. S3A). Prior to sacrifice, the mice were injected with Lectin-FITC to mark blood vessels. Upon immunofluorescence analysis of tumor tissue slices we noticed that DZNep treatment significantly reduced both the number and the size of the tumor blood vessels, as illustrated by Lectin-FITC blood vessels of mCherry-expressing glioblastoma sections (Fig. 7B and Fig. 7C). This indicates that the EZH2 inhibitor DZNep can inhibit angiogenesis in vivo, and suggests a possible therapeutic potential for EZH2 inhibitors in the many diseases in which aberrant angiogenesis plays a critical role.

**DISCUSSION**

We have analyzed miR-101 expression in primary HBMVECs. We showed that VEGF down-regulates the expression of miR-101 resulting in increased EZH2 protein expression and causing a pro-angiogenic response in the endothelial cells. Further, we show that this response can be overcome by pharmacological targeting of EZH2 in vitro and in vivo using
Figure 6. Inhibition of EZH2 reduces endothelial migration and invasion in vitro. (A) HBMVEC monolayer cultures were scratched. Images were acquired directly after scratching (t = 0) and 24 h later (t = 24). The migration front is indicated by the dashed lines. Scale bar = 450 µm. Inhibition of EZH2 in HBMVECs, either by transfection with pre-miR-101, EZH2 siRNA or treatment with DZNep significantly reduced migration as compared to control. (B) Quantitation of endothelial cell migration into the scratched area using ImageJ software. (C and D) HBMVECs were transfected with pre-miR-101, EZH2 siRNA, non-related control molecules, or treated with DZNep, incubated on a Transwell system and subsequently analyzed for invasion capability. EZH2 inhibition, either through pre-miR-101, EZH2 siRNA or DZNep, significantly decreased invasion as shown by Hoechst staining. Scale bar = 225 µm. (n=3) Error bars indicate s.d. *p<0.05, **p<0.01, t test.
the histone methyltransferase inhibitor DZNep. Altogether these results support a role for diminished miR-101-mediated suppression of EZH2 in promoting neovascularization.

Formation of new blood vessels requires endothelial cells to undergo a balanced angiogenic switch. This involves increased expression and secretion of growth factors like VEGF. VEGF modulates destabilization, proliferation, invasion, and sprouting of vessels, thereby orchestrating the formation of neovascularization via signalling through its receptors VEGFR1 and VEGFR2. The proper execution of these processes relies on a concerted action of multiple proteins. It is now clear that miRNAs can orchestrate specific biological processes through post-transcriptional regulation of gene expression. It has also been shown that specific miRNAs are responsible for regulation of endothelial gene expression during angiogenesis\textsuperscript{36-38}. To determine the miRNA signature of endothelial cells, Poliseno and colleagues generated miRNA expression profiles of HUVECs. They identified 27 highly expressed miRNAs, 15 of which were predicted to regulate the expression of receptors for angiogenic factors\textsuperscript{8}. We recently showed that the miRNA expression profile of HBMVECs was modulated upon exposure of these brain endothelial cells to glioblastoma cells\textsuperscript{11}. Further, it was shown that endothelial miRNAs can be deregulated by exposure to VEGF\textsuperscript{11, 36, 39}. Here we show that U87 conditioned medium and VEGF cause down-regulation of miR-101 in HBMVECs, subsequently resulting in up-regulation of the PcG protein EZH2. Interestingly, we were not able to measure significant and reproducible miR-101 down-regulation in HUVECs upon exposure to U87 conditioned medium, in contrast to HBMVECs. These results indicate a discrepancy between miR-101 regulation in HUVECs and HBMVECs in the context of glioma. However, also in HUVECs EZH2 protein levels could be modulated by EZH2 siRNA, pre-miR-101 or DZNep, and this affected tubule formation. In addition to the mechanism of U87cm and VEGF-suppressed miR-101-mediated translational repression of EZH2 that we describe, Lu et al. have recently shown that VEGF can also increase EZH2 promoter activity\textsuperscript{35}. Further research...
is warranted in order to investigate the different modes of EZH2 regulation in different types of endothelial cells and whether these mechanisms are mutual exclusive.

We confirmed that EZH2 translation is suppressed by miR-101. It was recently shown that miR-101 can regulate EZH2 expression in cancer cells and affects cancer cell migration and invasion\textsuperscript{26,28}. Moreover, it was described that EZH2 can be regulated by miR-26\textsuperscript{24} and miR-214\textsuperscript{25}. Of note, we found both miR-26 and miR-214 to be expressed in HBMVECs (data not shown). However, in primary HBMVECs exposed to glioblastoma cells we found miR-26 and miR-214 not to be deregulated\textsuperscript{11}. Here, we show that EZH2 up-regulation in angiogenic brain endothelial cells can be caused by reduced suppression by miR-101, although at this point we cannot exclude that miR-26, miR-214, or other miRNAs, also affect translation of EZH2 in angiogenic endothelial cells.

EZH2 promotes cancer cell proliferation \textit{in vitro} and \textit{in vivo}\textsuperscript{40-43}. This indicates a potential dual role for EZH2 in endothelial cells and in glioma cells. Regarding the effects of EZH2 inhibition \textit{in vivo}, we performed Ki67 staining on tumor slices of mice treated with or without the EZH2 inhibitor DZNep. Although we found a significant reduction in the number of blood vessels in the DZNep treated tumors we also noticed that proliferation of the glioma cells was still evident (Fig. S3), suggesting that the observed reduction in blood vessels under these conditions was - at least partly - caused by the inhibitory effect of DZNep on the blood vessel endothelial cells, paralleling the results of our \textit{in vitro} angiogenesis assays. However, we do not exclude possible partial effects of DZNep on glioma cell proliferation \textit{in vivo} and a consequent reduction in blood vessel number. It should be noted that our \textit{in vivo} results were obtained in an immune-compromised setting, it would be also of interest to study the effects of EZH2 inhibition in a syngeneic immune-competent model.

Inhibition of up-regulated EZH2 in angiogenic endothelial cells caused reduced migration, invasion, and tubule formation \textit{in vitro} and diminished blood vessel formation in glioblastoma tumors \textit{in vivo}. Here, we describe a new role for the histone methyltransferase EZH2 to stimulate a pro-angiogenic phenotype of endothelial cells. Inhibition of EZH2 by pre-miR-101, EZH2 siRNA, or the small molecule inhibitor DZNep shifted the pro-angiogenic status to a more stationary phenotype. Previously, expression profiling of EZH2-regulated genes indicated that EZH2 can act as a bona fide oncogene that stimulates an ‘active’ cell status by trimethylating lysine 27 of histone H3\textsuperscript{28,40-46}. In addition, it was described that EZH2 suppression, besides loss of H3K27me3, resulted in increased H3 acetylation, and that EZH2 modulation can affect the regulation of genes involved in endothelial differentiation\textsuperscript{22,23}. Histone deacetylase proteins were also described to affect angiogenesis\textsuperscript{12,13} and to revert EZH2-mediated gene silencing\textsuperscript{43,47}. In endothelial cells it remains to be investigated how histone modifications can affect the balance towards a more pro-angiogenic cellular state. Here we provide evidence of a functional link between growth factors, miRNAs, and the histone methyltransferase EZH2 in the angiogenesis process.

**MATERIALS AND METHODS**

**Cells**

Human brain microvascular endothelial cells (HBMVECs; Cell Systems ACBRI-376) were cultured in EGM medium (Lonza). 293T cells and U87 cells (U-87 MG; ATCC) were cultured in DMEM
containing 10% FBS and antibiotics. U87-Fluc-mCherry and U87-GFP cells were produced by stably transducing U87 cells with CMV-controlled expression cassettes using lentiviral vectors.

**RNA isolation**
Total RNA was isolated from HBMVECs, primary endothelial cells isolated from normal brain and gliomas. RNA isolation was carried out by adding 600 μl lysis buffer from the miRVANA miRNA isolation kit (Ambion) to the cell pellets. The isolation of endothelial cells from normal human brain and human gliomas is described elsewhere.

**miRNA modulation**
50 nM of pre-miR-101 (Ambion) or pre-miR-control (Ambion) oligonucleotides were transfected into HBMVECs using Lipofectamin2000 (Invitrogen), according the manufacturer’s protocol. For the inhibition of EZH2, 50 nM of EZH2 siRNA (Qiagen) was transfected into HBMVECs, siRNA-AF or non-targeting oligonucleotides (Qiagen) were used as controls. After 5 h the transfection medium was replaced by EGM until further analysis.

**Quantitive RT-PCR**
Quantitive RT-PCR (qRT-PCR) analysis was used to determine the relative expression levels of miR-101, miR-186, EZH2, and GAPDH mRNA in HBMVECs. Total RNA was isolated using the miRVANA miRNA isolation kit. Equal amounts of RNA were converted into cDNA using miR-101, miR-186, EZH2, and GAPDH RT primers (Applied Biosystems and Qiagen, according to the manufacturer’s protocol). Subsequently, quantitative PCR was performed using primers and materials from Applied Biosystems. The Ct values were used to calculate the relative fold difference in miRNA or mRNA levels. All experiments were performed using biological triplicates and experimental duplicates. The data were normalized to miR-186 and/or GAPDH expression levels. Primers used were:

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<th>Gene</th>
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<th>Reverse</th>
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<tr>
<td>human EZH2</td>
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<td>5'-TGCAAAAATTCACTGGTACAAAACACT-3'</td>
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<tr>
<td>human GAPDH</td>
<td>5'-GTCGGAGTCAACGGATT-3'</td>
<td>5'-AAGCTTCCCGTTCTCAG-3'</td>
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**Luciferase miRNA reporter assay**
293T cells were transfected with firefly luciferase reporter vectors containing EZH2 wt or control 3’-UTR. At 4 h after transfection, the cells were transfected again with 50 nM miR-101 precursor (Ambion) or controls (Ambion). The cells were lysed after 24 h, and luciferase activity was measured using a luminometer. A total of 100 ng of pCSCW-Gluc was co-transfected and used to normalize the firefly luciferase values expressed from the EZH2 3’UTR report constructs.

**Western blot analysis**
72 h after transfection with 50 nM of oligonucleotides or treatment with 5 μM DZNep, or after 24 h of culturing in either EBM, EGM or EBM conditioned culture medium derived from U87 glioblastoma cells, HBMVECs were washed, trypsin treated and harvested, centrifuged (4 min at 1200 rpm) and washed with ice-cold PBS for two times and subsequently resuspended
in RIPA buffer including protease inhibitor. After 1 h cells were centrifuged for 10 min at 10,000 rpm and 4°C. Supernatant was mixed with Laemmli buffer (Bio-RAD) including β-mercaptoethanol and heated at 97°C. 30 μg of protein was loaded on a 10% SDS-polyacrylamide gel and separated for 1 h at 100V on an electrophoresis system (Bio-Rad). Next, proteins were transferred to PVDF membranes overnight at 30V and 4°C by means of blot buffer (TRIS/glycine/methanol). After incubating the PVDF membranes with PBS for 15 min they were blocked with 5% milk in PBS-T for 1 h. Membranes were incubated for 1 h with purified mouse anti-EZH2 mAb (BD biosciences) at 1:1,000 dilution in blocking solution (1% milk in PBS-T) and mouse anti-Actin (Millipore, MAB1501R) at 1:50,000 dilution or for 24 h with rabbit anti-H3K27Me3 (1:1,000; Upstate Biotechnology). Membranes were washed with PBS-T four times for 15 min. Subsequently, membranes were incubated for 1 h with horseradish peroxidise (HRP) anti-mouse IgG (DAKO) at 1:3,000 dilution in 1% block buffer to detect primary anti-EZH2 antibody and anti-Actin antibody or HRP anti-rabbit IgG (DAKO) at 1:3,000 dilution to detect anti-H3K27Me3 antibody. Membranes were washed again with PBS-T four times for 15 min. ECL detection solution (GE healthcare) was used to detect protein levels. Levels were visualized on X-ray film (GE healthcare) and quantified using ImageJ software (NIH Image). Actin intensities were used to normalise EZH2 levels. Normalised EZH2 intensities were used to calculate the relative expression level of EZH2.

**Immunohistochemical staining**

Paraffin sections of human glioblastoma tissue and neocortex were stained with monoclonal mouse anti-EZH2 (BD biosciences) at 1:300 diluted in antibody solution (Immunologic) for 1 h at room temperature. Sections were washed in PBS three times and incubated with secondary antibody (EnvisionHRP) for 30 min at room temperature. After washing in PBS for three times, positive reactions were visualized by incubating the sections with stable 3,3-diaminobenzidine for 10 min. In case of double staining, the sections were subsequently incubated with anti-CD31 (DAKO) at 1:50 diluted in antibody diluent for 1 h at room temperature, washed three times in PBS, incubated with a biotinylated rabbit anti-mouse secondary antibody (DAKO) at 1:100 diluted in antibody diluent for 30 min at room temperature, washed in PBS three times and finally incubated with a streptavidin alkaline phosphatase complex (Roche) at 1:100 diluted in antibody diluent for 1 h at room temperature. Liquid Permanent Red chromogen was used to visualize positive reactions. After washing in distilled water, the sections were counterstained with hematoxylin for 1 min and analyzed using microscopy.

**Microarray expression analysis**

mRNA expression arrays were performed at the VUMC array core facility. HBMVECs cultured in EBM + VEGF (10 ng/ml) were compared to HBMVECs cultured in EBM only and HBMVECs transfected with EZH2 siRNA and cultured in EBM + VEGF (10 ng/ml) were compared to HBMVECs transfected with control siRNA and cultured in EBM + VEGF (10 ng/ml). Total RNA was isolated after 24 h of culturing using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA was quantified using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent) and RNA 6000 Nano LabChip kit (5065-4476). RNA integrity
numbers (RIN) of >9.0 were considered as good quality RNA. RNA samples were labeled using the Agilent Low RNA Input Linear Amplification Kit Plus (5188-5340) according to the manufacturer’s protocol. Briefly, 500 ng of total RNA was amplified and reverse transcribed to cDNA using T7-polymerase and subsequently labeled with Cy3 or Cy5. Dye incorporation was measured using a Nanodrop ND-1000 spectrophotometer. Subsequently, cRNA was hybridized using the Agilent Gene Expression Hybridization Kit (5188-5242), according to the manufacturer’s protocol. Briefly, 825 ng of Cy3 labeled cRNA was mixed with 825 ng of Cy5 labeled cRNA, fragmented for 30 min at 60°C in the dark and hybridized on an Agilent Hybridization Chamber Gasket Slide (G2S34-60011) in a rotation oven at 65°C for 17 h. Slides scanned using an Agilent Microarray Scanner (G2S65BA). Image analysis was performed using feature extraction software version 9.5 (Agilent Technologies). The Agilent GE2-v5_95 protocol was applied using default settings.

Data pre-processing and analysis was performed using the R-Bioconductor package Limma[50]. A robust Edwards background correction was applied. Within-array and between-array normalization was performed using loess and scale standardization. For EZH2 siRNA transfected versus control siRNA transfected cells the highest 5 percentile of differentially expressed log2 expression ratios [range log2 expression ratio: 0.32 – 1.76] were selected as potential target genes for EZH2. For EBM + VEGF (10ng/ml) versus EBM cultured cells the lowest 5 percentile differentially expressed log2 expression ratios [range log2 expression ratio: -4.20 – -0.48] were selected as potential target genes for VEGF induced EZH2 gene silencing. Subsequently, an overlap set of genes both induced by EZH2 knock down and reduced by VEGF was established as a VEGF-induced-EZH2-target gene set.

**In vitro Matrigel assay**

A Matrigel (BD biosciences) assay was performed to assess endothelial tube formation in vitro. 48-well Plates were coated with 75 μl of Matrigel per well and incubated at 37°C for 15 min. 72 h after transfection with 50 nM of oligonucleotides or treatment with 5 μM DZNep, HBMVECs were harvested and suspended in either EBM, EBM supplemented with U87-GFP cells or EGM. Per well, 60,000 HBMVECs were plated and maintained at 37°C. At least 3 random pictures were taken per well using a digital camera system (Leica), 16 h after plating. The images were analyzed for total tube length and number of branching points using the software program ImageJ. Experiments were performed in triplicate, repeated at least once and judged in a double blind fashion by two observers. Statistical analysis of the results of 3 experiments was performed by Student t-test.

**Wound healing assay**

HBMVECs were cultured in EGM in 24-wells plates and were transfected with 50 nM of oligonucleotides or treated with DZNep (5 μM). At 72 h after transfection an artificial wound was created using a pipette tip after which the cells were further incubated. To analyse cell migration into the wound, pictures were taken at 0 h and 24 h using a digital camera system coupled to a microscope. ImageJ was used to determine the migration distance (in μm) as the reduction of the width of the open area. Statistical analysis of the results of 3 experiments was performed by Student’s t-test.
Cell invasion assay

HBMVEC migratory function was analysed using a Boyden chamber assay. A 24-well Transwell system (Corning) was used, with each well containing a permeable Transwell insert containing a 6.5 mm polycarbonate membrane with 8 μm pores. The inserts were coated with 4x diluted basement membrane extract (Trevigen) and incubated overnight in 5% CO2 at 37°C. At 72 h after transfection with 50 nM of oligonucleotides or treatment with 5 μM DZNep cells were starved for 24 h and harvested in serum free medium. Per insert 25,000 HBMVECs were subsequently placed on the membrane. The inserts were immersed in a 24-well plate that was filled with EGM growth medium culture media. After incubation for 24 h, the membrane was washed briefly with PBS. The upper side of the membrane was then wiped gently with a cotton ball. The membrane was then fixed in 4% formaldehyde and stained with Hoechst. The magnitude of HBMVEC migration was evaluated by counting the migrated cells in 3 random high-power (5x) microscope fields. Statistical analysis of the results of 3 experiments was performed by Student’s t-test.

In vivo glioblastoma angiogenesis model

All animal studies were approved by the Massachusetts General Hospital Review Board. Protocol approved by the MGH Subcommittee on Research Animal Care (SRAC) - OLAW assurance number A3596-01. Nude mice were anesthetised with i.p. injection of xylazine (5 mg/kg) and ketamine (100 mg/kg). 50 µl containing 1 x 10^6 U87-Fluc-mCherry cells was pre-mixed with an equal volume of matrigel (BD biosciences) and implanted in the flanks of nude mice. DZNep was administered intravenously to tumor-bearing mice at a dose of 0.07 mg/kg diluted in 100 µl PBS at day 3, 5 and 7 after tumor implantation, followed by weekly injection. Mice were anesthetized as above and Fluc imaging was performed 10 min after intravenous injection of 150 µl beetle D-luciferin (4 mg/kg body weight) (Xenogen), and recording photon counts over 5 min using a cooled CCD camera with no illumination. Dim polychromatic illumination was used to take a light image of the animal. Visualization was performed using CMIR-Image, a program developed by the Center for Molecular Imaging Research using image display and analysis suite developed in IDL (Research Systems Inc., Boulder, CO). An intensity contour procedure to identify bioluminescence signals with intensities significantly greater than the background was used to define regions of interest. The mean, standard deviation and sum of photon counts in these regions were calculated as a measurement of Fluc activity. Prior to sacrifice the mice were injected intravenously with 150 µl (1 mg/ml) Lectin-FITC (Vector Laboratories) to mark the blood vessels. Animals were sacrificed by transcardial perfusion with 4% paraformaldehyde in PBS under deep anaesthesia. Tumors were removed, post-fixed in paraformaldehyde and PBS containing 30% sucrose, and sectioned into 10 µm sections that were mounted on slides and evaluated for number of blood vessels.

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REFERENCES


44. Croonquist PA, Van Ness B. The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. Oncogene 2005;24:6269-6280.


MYC-ASSOCIATED ZINC FINGER PROTEIN (MAZ) IS REGULATED BY miR-125b AND MEDIATES VEGF-INDUCED ANGIOGENESIS IN GLIOBLASTOMA
ABSTRACT

In glioblastoma patients vascular endothelial growth factor (VEGF) is a key mediator of tumor-associated angiogenesis. Glioblastomas are notorious for their capacity to induce neovascularisation, driving continued tumor growth. Here we report that miR-125b is down-regulated in glioblastoma-associated endothelial cells resulting in increased expression of its target myc-associated zinc finger protein (MAZ), a transcription factor that regulates VEGF. The down-regulation of miR-125b was also observed upon exposure of endothelial cells to glioblastoma-conditioned medium or VEGF, resulting in increased MAZ expression. Further analysis revealed that inhibition of MAZ accumulation by miR-125b, or by MAZ-specific shRNAs, attenuated primary human brain endothelial cell migration and tubule formation in vitro, phenomena considered to mimic angiogenic processes in vitro. Moreover, MAZ expression was elevated in brain blood vessels of glioblastoma patients. Altogether these results demonstrate a functional feed-forward loop in glioblastoma-related angiogenesis, in which VEGF inhibits the expression of miR-125b, resulting in increased expression of MAZ, which in its turn causes transcriptional activation of VEGF. This loop is functionally impeded by the VEGF receptor inhibitor Vandetanib and our results may contribute to the further development of inhibitors of tumor-angiogenesis.
INTRODUCTION

Glioblastoma is the most frequent malignant primary brain tumor. Despite improvements in surgery, radio- and chemotherapy, prognosis has not improved substantially over the last decade and median survival remains approximately 14 months\(^1\). Glioblastoma cells typically invade the brain parenchyma and are notorious for their capacity to induce angiogenesis, the process of sprouting of new blood vessels from pre-existing vessels\(^2\). Glioblastomas produce large amounts of the angiogenic vascular endothelial growth factor (VEGF). The endothelial cells comprising the glioblastoma vasculature express vascular endothelial growth factor receptor 2 (VEGFR2), instrumental for the signalling cascade initiated by VEGF secreted by the tumor cells and stimulating endothelial cell proliferation and capillary formation\(^2, 3\). Clinical trials with anti-VEGF/VEGFR therapy in glioblastoma patients have provided - albeit limited - supporting evidence for the therapeutic benefit of VEGF pathway inhibition\(^4\).

VEGF expression can be regulated by multiple signals, including phosphatase and tensin homolog\(^5\), nitric oxide\(^6\) and angiopoietin-2\(^7\). Many of these signals converge on the VEGF transcription factor HIF1a. Also other transcription factors including specificity protein 1 (SP1)\(^8\), and signal transducer and activator of transcription (STAT)\(^9\) can induce VEGF transcription. Myc-associated zinc finger protein (MAZ) is a ubiquitously expressed transcription factor\(^10\), that can regulate the expression of a variety of genes such as c-myc\(^11\), insulin\(^12\) and serotonin receptors\(^13\). Interestingly, MAZ-binding sites are also located in the promoter regions of various angiogenic genes\(^14-18\), including VEGF\(^19\).

MicroRNAs (miRNAs) have emerged as important regulators of angiogenesis\(^20-23\). miRNAs are short non-coding RNAs that repress gene expression by blocking the translation of their mRNA\(^24\). Previously, we identified miRNAs that modulate VEGF-mediated angiogenic signalling, including miR-296\(^23\) and miR-101\(^25\), by miRNA microarray analysis of differentially expressed miRNAs in primary glioblastoma-associated endothelial cells and non-neoplastic brain endothelial cells. Here we demonstrate that miR-125b is down-regulated in primary human brain endothelial cells exposed to glioblastoma cells, as well as in glioblastoma-associated endothelial cells isolated from surgically removed tumor tissue. Moreover, we show that MAZ is a direct post-transcriptional target of miR-125b. Finally, we identified a miR-125b/MAZ/VEGF feed-forward loop, which is functionally impeded by the VEGF receptor inhibitor Vandetanib\(^26\). Therapeutic interference in the newly identified angiogenic loop may hold promise for the treatment of cancer.

RESULTS

*miR-125b is down-regulated in glioblastoma-associated endothelial cells*

Here, we analyzed the expression levels of miR-125b in glioblastoma-associated endothelial cells. First we determined miR-125b expression in primary human brain microvascular endothelial cells (HBMVECs) cultured in the presence or absence of U87-CFP glioblastoma cells. After treatment, brain endothelial cells were separated from the tumor cells using CD31-beads and their purity was confirmed by the absence of CFP expressing U87 cells using fluorescence microscopy\(^23\). Exposure of brain endothelial cells to U87 glioblastoma
cells caused a significant reduction of miR-125b (Fig. 1A). These findings were corroborated in brain endothelial cells isolated from patients with glioma of various grades. We used qRT-PCR to determine the miR-125b expression levels in brain endothelial cells, which were isolated from three glioblastomas and three grade II gliomas, and observed reduced levels of miR-125b when compared to endothelial cells isolated from non-neoplastic brain vessels (Fig. 1B).

**miR-125b inhibits angiogenic processes in cultured endothelial cells**

miR-125b precursor or control oligonucleotides were transfected into brain endothelial cells followed by analysis of different angiogenic functions in vitro. First, light microscopy analysis of the cell monolayers demonstrated reduced elongation of the brain endothelial cells after transfection with pre-miR-125b molecules as compared to pre-miR-CTRL oligonucleotides, indicative for reduced endothelial activation (Fig. 1C). To determine the effect of miR-125b on endothelial cell migration, scratch assays were performed. HBMVEC cells were transfected with pre-miR-125b or control molecules, and cultured in normal culture medium to confluence. In order to promote migration, 48 h after transfection a scratch was placed in the HBMVEC monolayer. Twelve hours later, the migration distance of the HBMVECs into the scratched area was measured and quantified. miR-125b significantly reduced cellular growth into the scratched area (Fig. 1D). HBMVEC proliferation was not affected over the same period (data not shown). To assess the anti-angiogenic function of miR-125b, an endothelial cell tubule formation assay was performed, using pre-miR-CTRL or pre-miR-125b over-expressing brain endothelial cells cultured on a matrigel substratum. Over-expression of miR-125b resulted in a trend towards reduced total tubule length (Fig. 1E), further supporting an anti-angiogenic role for miR-125b in endothelial cells.

**miR-125b targets MAZ**

To identify a potential target of miR-125b we performed database analysis using miRBase and identified MAZ. First, to demonstrate that miR-125b targets the 3’ UTR of MAZ, we transfected a reporter vector encoding firefly luciferase (Fluc) fused to the MAZ wild type 3’ UTR or lacking this 3’ UTR (CTRL) into 293T cells. Co-transfection of pre-miR-125b, but not of pre-miR-CTRL, resulted in reduced Fluc expression levels in the cells expressing the reporter containing the 3’-UTR of MAZ. No changes in Fluc expression were observed using the control vectors lacking the MAZ 3’-UTR sequence (Fig. 2A). These results indicate that the 3’-UTR of MAZ is a target for miR-125b. Furthermore, to determine whether MAZ expression in brain endothelial cells is suppressed by miR-125b we performed qRT-PCR and Western blot analysis after transfection of brain endothelial cells with pre-miR-125b or pre-miR-CTRL molecules. Indeed, pre-miR-125b over-expression (Fig. 2B) markedly reduced MAZ mRNA and protein levels (Fig. 2C, D).

**MAZ expression is increased in angiogenic glioblastoma-associated endothelial cells**

We hypothesized that reduced expression of miR-125b in glioblastoma-associated endothelial cells leads to increased MAZ levels. To evaluate the expression of MAZ in glioma of various grades and non-neoplastic brain endothelial cells we performed immune histochemistry for MAZ on sections of grade II glioma and glioblastoma and non-neoplastic brain tissue. MAZ was
Figure 1. miR-125b is down-regulated in glioblastoma-associated endothelial cells and inhibits angiogenic phenotype in cultured endothelial cells. (A) Down-regulation of miR-125b in HBMVECs exposed to U87-GFP glioblastoma cells confirmed by qRT-PCR analysis. RNA extracted from CD31+ HBMVECs cultured in the presence or absence of U87 glioblastoma cells was analyzed by qRT-PCR with primers specific for miR-125b. The data were normalized to the level of RNU-44 in each sample. (B) RNA extracted from individual endothelial samples from non-neoplastic brain (n=3), grade II glioma (n=3) and glioblastoma (n=3) was analyzed by qRT-PCR for expression levels of miR-125b. The data were normalized as in (A). (C) The morphology of endothelial cells transfected with pre-miR-125b or pre-miR-CTRL molecules was analyzed by light microscopy. Scale bar = 125 µm. (D) HBMVEC monolayer cultures were scratched. Images were acquired directly after scratching (T=0h) and 12 h later (T=12h). The migration front is indicated by the line. Scale bar = 500 µm. Brain endothelial cell migration into the scratched area was quantified using ImageJ software. (E) HBMVECs transfected with pre-miR-125b or pre-miR-CTRL molecules were cultured on Matrigel-coated plates. Tubule formation was assessed as tubule length measured using ImageJ software. Error bars indicate s.d. *p<0.05, ***p<0.01, t-test. Scale bar = 250 µm.
expressed by glioma cells but the most distinct expression was found in the glioma-associated endothelial cells. MAZ was clearly expressed in the endothelial layer of blood vessels in the grade II glioma tissues and even more so in the glioblastoma tissues, but nearly absent in the non-neoplastic brain sections (Fig. 3A). To further assess MAZ expression in angiogenic endothelial cells we investigated MAZ mRNA expression in brain endothelial cells co-cultured with glioblastoma cells. MAZ mRNA levels were significantly increased in HBMVECs upon exposure to human U87 glioblastoma cells (Fig. 3B). These results were supported by qRT-PCR analysis of MAZ mRNA in primary endothelial cells isolated from patients with glioma of various grades described previously. MAZ expression was significantly enhanced in glioblastoma blood vessels as compared to normal brain vessels (Fig. 3C). To determine whether glioblastoma-derived factors are capable of inducing the expression of MAZ protein in brain endothelial cells, hCMEC/D3s were cultured in basal EBM-2, growth factor supplemented EGM-2, or U87-conditioned medium. Western blot analysis demonstrated that MAZ protein was increased in hCMEC/D3s cultured in U87-conditioned medium and in EGM-2, as compared to basal conditions (Fig. 3D), coinciding with the increased capacity of brain endothelial cells to form tubules on a Matrigel substrate upon MAZ up-regulation (Fig. 3E).
Glioblastoma-induced MAZ expression is mediated by VEGF

Glioblastoma cells produce large amounts of VEGF. Hence, we investigated whether VEGF is capable of controlling the miR-125b-mediated repression of MAZ. Primary HBMVECs were cultured for 24 h in EBM-2 supplemented with various amounts of VEGF. Total RNA was isolated in order to determine the expression levels of miR-125b and MAZ by qRT-PCR. Exposure of
endothelial cells to VEGF decreased miR-125b expression and increased MAZ mRNA expression in a dose-dependent manner (Fig. 4A). In addition, Western blot analysis revealed that VEGF also induced MAZ protein expression in endothelial cells (Fig. 4B). Of note, the VEGF receptor inhibitor Vandetanib diminished the induction of MAZ protein expression by U87-conditioned medium or VEGF (Fig. 4B) and significantly impaired endothelial tubule formation (Fig. 4C, D).

**MAZ mediates glioblastoma-induced tubule formation.**

We next investigated the functional role of MAZ in brain endothelial cells. MAZ expression in hCMEC/D3s was silenced by MAZ specific shRNAs. Efficient MAZ knock down was demonstrated by Western blot analysis (Fig. 5A). Subsequently, the cells expressing MAZ-specific or non-targeting control shRNAs were subjected to a tubule formation assay. MAZ knock down reduced endothelial tubule length as compared to the negative control (Fig. 5B). Further insight into the angiogenic function of MAZ was obtained by lentiviral-mediated

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**Figure 4. Glioblastoma-induced MAZ expression is mediated by VEGF.** (A) HBMVECs were grown in EBM-2 supplemented with different amounts of VEGF for 24 h. Expression levels of miR-125b, MAZ and VEGF were determined by qRT-PCR. The data were normalized to GAPDH mRNA or RNU-44 levels in each sample. (B) MAZ protein expression analysis in hCMEC/D3 cultured in EBM-2 or EBM supplemented with 50 ng/mL VEGF or U87-cm and treatment in the presence or absence of 5 µM Vandetanib. (C) hCMEC/D3 treated as in (B) cultured on Matrigel-coated plates. Scale bar = 250 µm. (D) Quantitative representation of tubule formation. Tubule formation is assessed as tubule length measured using ImageJ software. Error bars indicate s.d. *p<0.05, ***p<0.01 by t-test.
over-expression of MAZ. hCMEC/D3 cells overexpressing MAZ were subjected to the tubule formation assay, showing increased endothelial tubules and sprouts (Fig. 5C, D).

The miR-125b/MAZ/VEGF axis, a novel feed-forward loop
Ray et al. recently showed that MAZ can induce the transcriptional activation of VEGF in breast cancer cells. In line with these results we observed that MAZ knock down significantly reduced VEGF mRNA expression in hCMEC/D3s (Fig. 6A). This was corroborated in a model of endothelial hypoxia, which is associated with high levels of VEGF. hCMEC/D3 cells showed an increase in VEGF mRNA levels after exposure to hypoxia-inducing cobalt chloride, while this induction was markedly reduced in MAZ-silenced hCMEC/D3 cells (Fig. 6B). To further investigate the relation between MAZ, VEGF and miR-125b we assessed VEGF mRNA expression levels in brain endothelial cells co-cultured with glioblastoma cells and angiogenic endothelial cells derived from surgically obtained human glioma tissues. VEGF mRNA levels were significantly increased in HBMVECs upon exposure to human U87 glioblastoma cells (Fig. 6C). These results were confirmed in brain endothelial cells isolated from patient tissues. VEGF expression in glioblastoma-associated blood vessels was significantly increased as compared to normal brain vessels (Fig. 6D). Transfection of brain endothelial cells with pre-miR-125b significantly reduced VEGF mRNA expression (Fig. 6E).

DISCUSSION
Our findings point to a molecular loop which involves MAZ, VEGF and miR-125b (Fig. 6F). We demonstrated that glioma endothelial cells possess low miR-125b activity (Fig. 1A, B), and
Figure 6. The miR-125b/MAZ/VEGF axis, a novel feed-forward loop. (A) MAZ qRT-PCR analysis of hCMEC/D3 cells transduced with lentivectors encoding shRNA constructs directed against MAZ. (B) hCMEC/D3 cells as in (A) were cultured in the presence or absence of cobalt chloride to induce hypoxia and subjected to VEGF qRT-PCR analysis. The data were normalized to the level of GAPDH mRNA in each sample. (C) Over-expression of VEGF in HBMVEC exposed to U87-GFP glioblastoma cells confirmed by qRT-PCR analysis. The data was normalized to the levels of GAPDH mRNA in each sample. (D) RNA extracted from individual glioma endothelial samples was analyzed by qRT-PCR for expression levels of VEGF. All values were normalized to GAPDH mRNA levels in the same samples. (E) VEGF mRNA expression analysis in hCMEC/D3 cells following transfection with pre-miR-125b or control miRNA. (F) Schematic representation of a VEGF feed-forward loop. VEGF down-regulates the expression of miR-125b, which subsequently results in increased MAZ and VEGF expression which can be intervened by VEGF inhibitors. Error bars indicate s.d. *p<0.05, ***p<0.01 by t-test.
have relatively high levels of MAZ (Fig. 3B, C). Next, we showed that VEGF down-regulates the expression of miR-125b, subsequently resulting in increased MAZ expression (Fig. 4A), and causing a pro-angiogenic endothelial response. Of note, this response was blocked by pharmacological inhibition of the VEGF receptor using Vandetanib (Fig. 4B, C). Figure 6F shows a schematic representation of the MAZ/VEGF/miR-125b feed-forward loop.

We demonstrate that miR-125b is decreased in endothelial cells co-cultured with U87 glioblastoma cells and in endothelial cells derived from glioma of various grades. This enhances the expression of MAZ and leads to increased VEGF levels and angiogenic activity in vitro. In the last decade it has become clear that miRNAs are important regulators of angiogenesis20-23, 32. Previously, it was shown that miR-125b inhibits production of endothelin-133, a stimulator of tumor angiogenesis34, in endothelial cells derived from cardiac vascular beds. It was also reported that the pro-angiogenic transcript PLGF is inhibited by introduction of miR-125b in hepatocellular cancer cells35, leading to reduced angiogenic signalling. miR-125b also has a role in other physiological processes. Several targets of miR-125b have been identified including ERBB2 and ERBB337, E2F338, and ETS139, p5340 and Bak141. Our findings suggest that miR-125b in brain endothelial cells has an additional and indirect effect on tumor growth by affecting angiogenic processes.

We show that glioblastoma cells reduce miR-125b and as a consequence induce MAZ expression in brain endothelial cells. Importantly, this induction was dependent on VEGF as became apparent after treatment with a VEGF inhibitor. Insight into downstream pathways responsible for VEGF-mediated miR-125b downregulation requires further study. In addition, it is unlikely that MAZ is regulated solely by miR-125 and others have previously shown that MAZ expression can be regulated by interleukin-1 and -642 and tumor-necrosis factor-α16 in inflammatory disease. In addition, phosphorylation of MAZ by protein kinase A43, protein kinase C44 and mitogen activated protein kinase (MAPK)45 increases the DNA binding and transactivating potential of MAZ. Interestingly, VEGF is also known to stimulate MAPK46 and this may indicate an alternative way in which VEGF activates MAZ besides its impact on miR-125b.

Our results demonstrate that the transcription factor MAZ contributes to capillary formation by brain endothelial cells. MAZ was previously identified as a transcription factor that binds to a GA box (GGGAGGG) within the first exon of the c-myc proto-oncogene11. MAZ is a zinc-finger protein expressed ubiquitously in different human tissues10, and can regulate the expression of a variety of other genes. MAZ was identified as a tumor-associated antigen in leukemia47, and is over-expressed in breast cancer48 and hepatocellular carcinoma49. Down-regulation of MAZ suppressed cell growth and induced apoptosis through inhibition of peroxisome proliferator-activated receptor-γ1 in breast cancer50. It is of interest that MAZ-binding sites are present in the promoters of several other genes involved in angiogenesis besides VEGF19 including endothelial nitric oxide synthase14, ephrin-B218, and the matrix metalloproteinases (MMPs) MMP-119, MMP-916, and MMP-1417.

In conclusion, our findings point to a feed-forward loop in glioblastoma-associated endothelial cells, which involves MAZ, VEGF and miR-125b. Our findings may contribute to the further development of angiogenesis inhibitors.
METHODS

Cell culture
Human brain microvascular endothelial cells HBMVEC\(^2\) (Cell Systems, ACBRI-376) and hCMEC/D3\(^2\) (kindly provided by dr. P-O. Couraud (Institut Cochin, Université Paris Descartes, Paris, France), were grown in endothelial basal medium-2 (EBM-2, Lonza, Basel, Switzerland), supplemented with human epidermal growth factor (hEGF), hydrocortisone, GA-1000, VEGF, human basic fibroblast growth factor (hFGF-B), insulin-like growth factor (R\(^1\)-IGF-1), ascorbic acid and 2.5% fetal calf serum (FCS; EGM-2, Lonza, Basel, Switzerland). Human embryonic kidney cells (HEK 293T, ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin, at \(37^\circ\)C in a humidified atmosphere containing 5% CO\(_2\). U87-conditioned medium was prepared by culturing confluent U87MG cells (ATCC) in EBM for 48 h. U87-CFP cells were cultured in DMEM supplemented with serum and antibiotics as described elsewhere\(^2\).

Lentiviral shRNA expression vectors
To establish gene knock down we used plasmids encoding specific shRNAs from the TRC library (supplied by Sigma, St. Louis, MO). shRNA sequences for MAZ included clone A11 (GAGTCAAGAAACGGCTACAAT, TRCN0000015345) and clone B3 (CTCCAAAGTGTAGCGGAGGA, TRCN0000015343). To over-express MAZ, its coding sequence was amplified from hCMEC/D3 cDNA and cloned into the lentiviral vector pRRL-cPPT-CMV-X2-PRE-SIN (kindly provided by Dr. J. Seppen, Academic Medical Center, Amsterdam, the Netherlands). Lentiviral vectors were produced by co-transfecting subconfluent 293T cells with the shRNA/MAZ expression plasmid and lentiviral packaging plasmids (pMDLg/pRRE, pRSV-Rev, and pMD2.G), using calcium phosphate as a transfection reagent. Lentiviral vectors were collected 24 h after transfection. The supernatant was centrifuged to remove cell debris and stored at \(-80^\circ\)C.

Immunohistochemical staining
Histological sections of formalin-fixed paraffin-embedded human glioblastoma (n=3), human grade II glioma (n=3) and non neoplastic cerebral cortex and white matter (n=2) tissues were incubated with polyclonal rabbit anti-MAZ (Abcam, Cambridge, UK) diluted 1:100 in antibody solution (Immunologic, Duiven, the Netherlands) for 1 h at room temperature. Sections were washed three times in PBS and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (EnvisionHRP, DAKO, Glostrup, Denmark) for 30 min at room temperature. After washing in PBS for three times, positive reactions were visualized by incubating the sections with stable 3,3-diaminobenzidine for 10 min. After washing in distilled water, the sections were counterstained with hematoxylin for 1 min and analyzed by light microscopy.

Western blot analysis
HBMVEC and hCMEC/D3 cells were harvested after transduction with lentiviral vectors encoding specific shRNAs or after treatment with 5 \(\mu\)M Vandetanib (ZD6474, Zactima) or 50 nM miR-125b precursor or control (pre-miR-CTRL) molecules. Cell pellets were subsequently resuspended in RIPA buffer including protease inhibitor (Roche, Woerden, the Netherlands).
After 1 h cells were centrifuged for 10 min at 10,000 rpm and 4°C. Supernatant was mixed with Laemmli buffer (Bio-Rad, Veenendaal, the Netherlands) including 10% β-mercaptoethanol and denatured for 3 min at 97°C. Thirty μg total protein was loaded on a 10% SDS-polyacrylamide gel and separated for 1 h at 100V on an electrophoresis system (Bio-Rad). Next, proteins were transferred to PVDF membranes overnight at 30V and 4°C in blot buffer (TRIS/glycine/methanol). PVDF membranes were incubated with PBS for 15 min followed by blocking with 5% milk in PBS-T for 1 h. Membranes were incubated overnight with purified rabbit anti-MAZ (Abcam) at 1:1,000 dilution in blocking solution (1% milk in PBS-T) or for 1 h with mouse anti-actin (Millipore, Amsterdam, the Netherlands) at 1:30,000 dilution. Membranes were washed 4 times with PBS-T for 15 min. Subsequently, membranes were incubated for 1 h with horseradish peroxidase (HRP) anti-rabbit IgG (DAKO) at 1:3,000 dilution in 1% block buffer to detect primary anti-MAZ antibody, or with HRP anti-mouse IgG (DAKO) at 1:3,000 dilution to detect anti-actin antibody. Membranes were washed again as above. ECL detection solution (GE Healthcare, Diegem, Belgium) was used to detect protein levels. Levels were visualized on X-ray film (GE Healthcare) and quantified using ImageJ software (NIH Image, Bethesda, MD). Actin intensities were used to normalise MAZ levels, allowing calculation of MAZ relative expression levels.

**In vitro brain endothelial cell tube formation assay**

Matrigel assay (BD Biosciences, Breda, the Netherlands) was performed to assess brain endothelial tubule formation *in vitro*. 48-well plates were coated with 75 μl of Matrigel per well and incubated at 37°C for 15 min. HBMVEC, hCMEC/D3 cells or hCMEC/D3 cells expressing MAZ as well as non-targeting (control) or specific MAZ-targeting shRNA were harvested and suspended in either EGM-2, EBM-2 or U87-conditioned medium with or without 5 μM Vandetanib (ZD6474, Zactima). Transfected cells were harvested 72 h after transfection. Per well, 40,000 HBMVECs or 70,000 HCMEC/D3s were plated and maintained at 37°C. At 16 h after plating 3 random pictures were taken per well using a digital camera system (Leica, Wetzlar, Germany). The images were analyzed for total tube length and number of branching points using ImageJ software.

**RNA isolation**

Total RNA was isolated from HBMVECs, hCMEC/D3 and primary endothelial cells isolated from normal brain (n=3), grade II gliomas (n=3) and glioblastomas (n=3). RNA isolation was carried out using TRIZOL (Invitrogen) according to the manufacturer’s protocol. The isolation of endothelial cells from normal human brain and the human gliomas is described elsewhere.  

**Quantitive RT-PCR**

Quantitive RT-PCR (qRT-PCR) analysis was used to determine the relative expression levels of miR-125b or of RNU-44, MAZ, VEGF and GAPDH mRNA in HBMVECs or hCMEC/D3cs. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer’s protocol. Equal amounts of RNA were converted into cDNA and analyzed for miR-125b, RNU-44, MAZ, VEGF and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the manufacturer’s protocol (Applied Biosystems, Bleiswijk, the Netherlands). The Ct values were used to calculate the relative fold difference in miRNA or mRNA levels. All experiments
were performed using biological triplicates and experimental duplicates. Expression levels were normalized using the RNU-44 and/or GAPDH household genes.

Luciferase miRNA target reporter assay

The MAZ 3′ UTR was cloned from hCMEC/D3 cDNA by PCR using the forward primer 5′ -CTCGAGTGAGCTCCAAGTTGGTGCG and the reverse primer 5′ -ACTAGTCAATCCAGGCGGACAAACAG. After digestion of the PCR product by SspI and HindIII, the MAZ 3′ UTR was cloned into the SspI and HindIII sites of the multiple cloning site of pMir-Report (Ambion). This construct was co-transfected in 293T cells with 50 nM miR-125b precursor (Ambion, Applied Biosystems) or control miRNA (Ambion). After 24 h, cells were lysed and luciferase activity was measured using a luminometer (EG&G Berthold, Bad Wildbad, Germany). One hundred ng pCSCW-Gluc was co-transfected for normalization of the firefly luciferase values of the MAZ 3′ UTR report constructs.

Statistical analysis

Statistical analysis was performed with the student’s t-test (Prism 4.0; GraphPad Software, San Diego, CA), and results were considered significant if p< 0.05.

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REFERENCES


20. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXAS. *Blood* 2008;111:1217-1226.


ENDOTHELIAL CELLS REQUIRE miR-214 TO SECRETE EXOSOMES THAT SUPPRESS SENESCENCE AND INDUCE ANGIOGENESIS IN HUMAN AND MOUSE ENDOTHELIAL CELLS


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ABSTRACT
Signalling between endothelial cells, endothelial progenitor cells and stromal cells is crucial for the establishment and maintenance of vascular integrity and involves exosomes, among other signalling pathways. Exosomes are important mediators of intercellular communication in immune signalling, tumour survival, stress responses and angiogenesis. The ability of exosomes to incorporate and transfer mRNAs encoding for ‘acquired’ proteins or miRNAs repressing ‘resident’ mRNA translation suggests that they can influence the physiological behaviour of recipient cells. We here demonstrate that miR-214, a miRNA that controls endothelial cell function and angiogenesis, plays a dominant role in exosome-mediated signalling between endothelial cells. Endothelial cell-derived exosomes stimulated migration and angiogenesis in recipient cells, whereas exosomes from miR-214 depleted endothelial cells failed to stimulate these processes. Exosomes containing miR-214 repressed the expression of Ataxia Telangiectasia Mutated in recipient cells, thereby preventing senescence and allowing blood vessel formation. Concordantly, specific reduction of miR-214 content in exosome-producing endothelial cells abolishes the angiogenesis stimulatory function of the resulting exosomes. Collectively our data indicate that endothelial cells release miR-214 containing exosomes to stimulate angiogenesis through silencing of Ataxia Telangiectasia Mutated in neighbouring target cells.
INTRODUCTION

Signalling between endothelial cells, as well as signalling to bone marrow-residing endothelial progenitor cells and stromal cells\(^1\text{--}^3\), depends both on intercellular contact and exchange of secretory proteins\(^4\text{,}^5\). Additionally, endothelial cells have been demonstrated to secrete exosomes, and capture exosomes from various cell types\(^6\text{--}^9\). Transfer of signalling molecules by exosomes may thus provide a third way to regulate endothelial function\(^9\text{,}^10\). Exosomes, small vesicles secreted by a multitude of cell types, have gained much attention for their role in intercellular communication\(^11\text{,}^12\), mediating immune signalling\(^13\text{,}^14\), tumor survival\(^15\text{,}^16\), stress responses\(^17\text{,}^18\) and angiogenesis\(^9\text{,}^10\text{,}^19\text{,}^20\). The ability of exosomes to incorporate and transfer functional RNA suggests that exosomes influence the physiological phenotype of recipient cells by introducing mRNAs encoding ‘acquired’ proteins\(^10\text{,}^20\) or through delivery of miRNAs that repress ‘resident’ mRNA translation\(^21\text{--}^23\).

Several miRNAs have been demonstrated to affect endothelial function and angiogenesis\(^24\text{,}^25\), and exosomal transfer of such miRNAs may thus be instrumental for exosomes to elicit their effects on endothelial cells. One miRNA that has been identified in angiogenesis-stimulating tumor exosomes, potentially representing a miRNA important in the angiogenesis-stimulating properties of these exosomes, is miR-214\(^26\text{--}^28\). Here we demonstrate that endothelial cells secrete miR-214-enriched exosomes which promote endothelial cell migration and angiogenesis in vitro and in vivo. These exosomes repress expression of Ataxia Telangiectasia Mutated (ATM) in recipient cells in a miR-214-dependent manner, thereby avoiding senescence and allowing the stimulation of blood vessel formation.

RESULTS

**Endothelial cells produce migration- and angiogenesis-stimulating exosomes**

As a physiologically relevant in vitro model system we used human microvascular endothelial cells (HMEC-1\(^29\)). Established cultures received fresh medium, from which, after 24 hrs of culture, exosomes were isolated using a well-established differential centrifugation scheme\(^30\). When pelleted vesicles were floated into sucrose density gradients, the endothelial marker protein CD31/PECAM-1 co-migrated with the exosomal marker flotillin-1 to a density of approximately 1.11 g/ml (Figure 1A), characteristic for exosomes (1.09-1.18 g/ml)\(^12\). Transmission electron microscopy analysis showed vesicle diameters around 120 nm, consistent with our previous NTA analysis\(^37\), and a cup-shape like appearance, typical for exosomes in such analyses (Figure 1B). The capacity of endothelial exosomes to be transferred to recipient endothelial cells was studied examining the uptake of isolated exosomes labelled with the green fluorescent dye PKH67. After 2 hrs, allowing sufficient exosome uptake, a prerequisite for subsequent RNA transfer\(^10\text{,}^21\), recipient cells were washed to remove unbound exosomes, fixed and DNA was stained using DAPI. Fluorescence microscopy analysis demonstrated that PKH67-label had been taken up and was transferred to perinuclear compartments, presumably representative of late endocytic compartments (Figure 1C).

The functional relevance of exosome transfer between endothelial cells was investigated in a scratch wound migration assay (Figure 2A). Migration in basal medium (MCDB-131
without FCS, hydrocortisone and hEGF) was set at 1 (Figure 2B, basal). Migration increased 2.5 fold when conditioned medium -medium exposed to an established HMEC-1 culture for 24 hrs- was used (cond, comp). Migration was slightly but significantly impaired when the conditioned medium was depleted from exosomes by ultracentrifugation (cond, depl). Re-addition of isolated exosomes to exosome-depleted conditioned medium fully restored cell migration (cond, re-add). This stimulating effect was enhanced further by adding exosomes at a 10-fold higher concentration (cond,10x). Cell migration could also be stimulated 1.5 fold by isolated exosomes resuspended in basal medium (basal, +).

To investigate the effect of exosomes on angiogenic tubule formation, HMECs were seeded on a Matrigel substratum and incubated with either basal medium, conditioned medium or basal medium supplemented with isolated exosomes. After 18 hrs, endothelial networks had formed (Figure 2C), and average tubule lengths were determined. Compared to basal medium (Figure 2D, basal), conditioned medium (cond) and basal medium supplemented with isolated exosomes (basal, +) slightly but significantly increased the mean tube length. Also the formation of angiogenic sprouts, investigated using a bead-based sprouting assay (Figure 2E), was stimulated by both conditioned medium (Figure 2F, cond) and, to a lesser extent, by isolated exosomes (basal, +) as compared to basal medium (basal, -).
Depletion of miR-214 reduces functional effects in recipient cells

Recent studies indicate that besides proteins and lipids, also functional RNAs are packaged into and transported by various types of exosomes. To explore the RNA content of endothelial exosomes we compared RNA content of endothelial cells and endothelial cell derived exosomes. Presence of high-molecular RNA (Supplementary Information, Figure S1A,B) and more abundant small RNAs (Figure 3A,B) in endothelial exosomes was demonstrated using Agilent BioAnalyzer total-RNA and small-RNA chips.
Consistent with studies on exosomes from other cell types\textsuperscript{10, 21}, the relative abundance of ribosomal RNA in endothelial exosomes was smaller than in corresponding cells (16.50+/−5.26\% vs. 50.65+/−7.39\%; Supplementary Information, Figure S1C), whereas the relative amount of miRNAs was markedly higher in exosomes (38.75+/−4.57\% vs. 7.25+/−4.65\%; Supplementary Information, Figure S1D). qRT-PCR analysis showed a 3-fold higher abundance of miR-214 in endothelial exosomes compared to cells (normalized to RNu19; Figure 3C), and ~6-to-8-fold higher when normalized to the common mRNA housekeeping genes p0 (5.95-fold, p=0.012), β-Actin (6.14-fold, p=0.017), and GAPDH (7.68-fold, p=0.0495). Analysis of sucrose gradient fractions confirmed that at least 92\% (fractions e-j) of the miR-214 in the 100,000x\textsuperscript{g} pellet is associated with exosomes, and not with membrane-free high molecular weight complexes (Figure 3D,E). To investigate whether miR-214 is involved in the observed in vitro effects of endothelial exosomes, we reduced miR-214 levels by transfecting cells with modified single-strand RNA molecules that specifically bind and reduce miR-214 (anti-miR-214; Figure 3F). As determined by qRT-PCR (Figure 3G), this yielded ‘anti-miR-214-cells’, producing exosomes with reduced miR-214 levels (‘anti-miR-214-exosomes’). As a control, control-miR-214-cells producing, control-miR-214-exosomes, were obtained using non-targeting (NT) control RNA for transfection. Transfection of endothelial cells with miR-214 inhibitors did not affect endothelial cells with respect to cell number, endothelial identity, or angiogenic capacity, but reduced migration (Supplementary Information, Figure S2A-D). The amount of exosomes released from control- and anti-miR-214-transfected cells did not differ, although differences in exosome content other than miR-214 cannot be excluded (Supplemental Information, Figure S2E,F).

Similar to exosomes from non-transfected endothelial cells, isolated exosomes from control-miR-214-cells stimulated migration of endothelial cells in vitro. This stimulatory effect was significantly less when anti-miR-214-exosomes were used (Figure 3H). Similarly, in the angiogenesis and sprouting assays, mean tubule length and sprout length were stimulated less with anti-miR-214-exosomes compared to control-miR-214-exosomes (Figure 3I, J). Exosomes from endothelial cells in which miR-214 levels were increased by pre-miR-214 transfection did not show significant effects in either functional assay (Supplemental Information, Figure S3), suggesting that an optimal miR-214 concentration was already present in control-miR-214 cells and their exosomes.

Together, these results indicate that exosome-mediated stimulation of in vitro endothelial migration and angiogenesis is dependent on miR-214 expression in exosome-producing cells.

**Control-miR-214-exosomes prevent cell cycle arrest**

To elucidate the mechanism by which exosomal miR-214 stimulates the angiogenic program in recipient endothelial cells, we searched for early changes in gene expression by gene expression analysis on exosome recipient cells. In particular, we were interested in immediate repression of potential miR-214 target genes. Hereto, cellular RNA, isolated from endothelial cells exposed to control-miR-214- or anti-miR-214-exosomes for 6 hrs, was used for microarray analysis. Although miR-214 levels in recipients cells were not significantly affected by the amount of miR-214 delivered by exosomes (Supplemental Information, Figure S2G), 146 genes that were differentially expressed in endothelial cells that were
Figure 3. Exosomal miR-214 induces migration and angiogenesis in vitro. BioAnalyzer profiles of small RNA from (A) cells and (B) exosomes, with miRNA indicated. (C) Quantitative PCR analysis of miR-214 in endothelial cells and exosomes relative to RNu19 (relative to cell content, n=6 ± S.D., t-test). (D) MiR-214 analysis on RNA from sucrose density gradient fractions. Ct-values were subtracted from the fraction showing the highest ct-value (fraction a: ct = 39.49; B: bottom fraction). (E) Western blot detecting Flotillin-1 in the corresponding sucrose density gradient fractions, B=bottom fraction. (F) Schematic overview for the procedure to generate anti-miR-214 and control-miR-214-cells and exosomes and (G) quantitative PCR analysis of miR-214 content in anti-miR-214- and control-miR-214-cells and exosomes (values are plotted relative to samples from cells that were transfected with non-targeting (NT) negative control RNA (n=4 ± S.D., t-test). Quantification of (H) migration (n=5, ± S.D., t-test), (I) angiogenesis (n=5, ± S.D., t-test), and (J) sprouting assays (n=3, ± S.D., t-test) in which endothelial cells were treated with anti-miR-214-or control-miR-214-exosomes.
exposed to control-miR-214-exosomes, in comparison to control cells (not exposed to exosomes) were identified (Figure 4A). Importantly, 28 differentially expressed genes were observed in cells incubated with control-miR-214- versus anti-miR-214-exosomes (Figure 4B; Supplementary Information, Tables SI and SII).

Analysis of enriched GO-terms using Ontologizer confirmed that endothelial control-miR-214-exosomes stimulated general cellular functions (Supplemental Information, Table SIIa), and inhibited the response to cell cycle/death regulation. In contrast, anti-miR-214-exosomes specifically inhibited processes like angiogenesis, blood vessel development, blood vessel morphology and response to interleukin-1 in recipient cells (Figure 4C; Supplementary Information, Table SIIib). Interestingly, among the genes that were more effectively down-regulated in cells exposed to control-miR-214-exosomes compared to anti-miR-214-exosomes, the Ataxia Telangiectasia Mutated (ATM) gene contained a predicted miR-214 target sequence in its 3’-UTR (Figure 4D). We confirmed that this miR-214 target sequence is functional using luciferase reporter assays, and found that exposure of endothelial cells to control-miR-214-, but not to anti-miR-214-exosomes, down-regulates ATM protein expression (Figure 4E,F). A key role for ATM was demonstrated using a migration assay, showing that siRNA-mediated repression of ATM stimulated migration (Figure 4G). Induced ATM expression may explain the observed negative effects on endothelial function via two different mechanisms; Firstly through down-regulation of the angiogenesis-stimulating HIF-1α pathway, and secondly through induction of cell cycle arrest and senescence. qRT-PCR analysis of HIF-1α and two HIF-1α target genes (VEGFA, MMP9) revealed that expression of these genes did not differ between cells exposed to control-miR-214- or anti-miR-214-exosomes (Supplemental Information, Figure S3), indicating that the HIF-1α pathway is not regulated through ATM in recipient cells. The alternative pathway, resulting in cellular senescence, was investigated using β-galactosidase staining for senescent cells (Figure 4H). The percentage of senescent cells did not differ between cells incubated with basal medium or control-miR-214-exosomes, but was significantly higher in cells exposed to anti-miR-214-exosomes.

**Suppression of cell cycle arrest by control-miR-214 exosomes allows stimulation of blood vessel formation in vivo**

As the mature mouse mmu-miR-214 and human hsa-miR-214 are identical, we could study the effects of control-miR-214- and anti-miR-214-exosomes in vivo using a Matrigel plug assay in mice. Matrigel was mixed with vehicle (PBS), control-miR-214- or anti-miR-214-exosomes and subsequently grafted subcutaneously in mice. Plugs were excised after 2 weeks to determine infiltration of endothelial cells and formation of functional blood vessels as described by others. Interestingly, plugs containing anti-miR-214-exosomes showed significantly reduced numbers of endothelial cells that infiltrated into the Matrigel plugs compared to vehicle- and control-miR-214-containing plugs (Figure 5). In plugs containing vehicle or control-miR-214 exosomes, infiltrated cells organized into functional, red blood cell-containing blood vessels. Comparing blood vessel morphology revealed that average blood vessel areas were approximately 3-fold larger in Matrigel plugs containing control-miR-214-exosomes compared to plugs without exosomes. Furthermore, the few functional
Figure 4. Gene expression analysis of exosomal miR-214 effects on recipient endothelial cells. (A) Gene expression analysis of endothelial cells exposed to control-miR-214-or anti-miR-214-exosomes relative to endothelial cells that were incubated in the absence of purified exosomes, presented in a heat map displaying genes that were up- (blue) or down- (yellow) regulated. (B) List of the top-10 down- and up-regulated probes in recipient cells treated with anti-miR-214-exosomes compared to cells subjected to control-miR-214-exosomes. (C) List of the top-5 biological processes (with accompanying GO-terms) in recipient cells regulated by exosomal miR-214. (D) Predicted miR-214 seed sequence in the ATM 3’UTR region. (E) miR-214 ATM luciferase reporter assay (n=3, t-test). (F) ATM protein levels in lysates from cells incubated either in the absence (basal) of or with control-miR-214 (ctrl) or anti-miR-214-exosomes (anti) were determined by immunoblotting (upper panel, T=top of gel, 170 indicates MW of the highest MW marker band) and quantitation using β-actin was used as a loading control (n=3, ± S.D. ANOVA). (G) Quantitation of a migration assay with cells transfected with control- or ATM-siRNA (n=3, ± S.D., t-test). (H) β-galactosidase staining (upper panel) was used to quantify the percentage of senescent cells (lower panel; n=4, ± S.D. ANOVA).
Figure 5. Functional analysis of endothelial anti-miR-214- or control-miR-214-exosomes in vivo. Sections of dissected Matrigel plugs that contained either no exosomes (A, D), control-miR-214-exosomes (B, E), or anti-miR-214-exosomes (C, F), and had subcutaneously been grafted in mice. Sections were stained with hematoxylin and eosin (D-F, red blood cell containing blood vessel indicated with arrowheads), or CD31 (red) and DAPI (blue) (A-C). (G) Quantitation of murine endothelial cells that had infiltrated into the plugs, and (H) blood vessel surfaces. Images were recorded at RT on an Olympus CX60 microscope using an Olympus UPlan Fl 20x/0.05 objective lens which was coupled to an Olympus DP71 camera operated using CellP software. Brightness of fluorescent images was enhanced using Adobe Photoshop software.
blood vessels that were detected in plugs containing anti-miR-214-exosomes, showed an even smaller blood vessel area (Figure 5H).

**DISCUSSION**

In the study presented here, we use *in vitro* and *in vivo* approaches to elucidate the role of miR-214 in exosome-mediated cross-talk between endothelial cells. In recent years, it has become clear that besides growth factors and cytokines, also exosomes provide signalling cues for endothelial cells, stimulating activation, differentiation and angiogenesis.

We used an endothelial cell line which produces protein- and RNA-containing exosomes as an *in vitro* model to investigate autocrine effects of endothelial exosomes. As described for tumor exosomes, also exosomes from these cells contribute to conditioned medium-stimulated endothelial function, demonstrated by the reduced stimulation of migration by exosome-depleted conditioned medium. Importantly, re-addition of exosomes to exosome-depleted medium completely restored the stimulatory effect of conditioned medium, demonstrating that the exosome isolation procedure did not affect functional properties of exosomes. Not all stimulatory effects could be attributed to exosomes, and growth factors, cytokines and soluble (mi-)RNAs that are also secreted by endothelial cells are likely to contribute alongside exosomes.

Together, our *in vitro* functional assays demonstrate that endothelial exosomes stimulate the migratory and angiogenic potential of recipient endothelial cells.

Several miRNAs have already been assigned a role in endothelial function and angiogenesis (reviewed in). We focussed on miR-214, which is highly expressed in endothelial cells, has a role in angiogenesis and cell migration, and is present in angiogenesis-stimulating ovarian- and lung cancer-derived exosomes. Like miR-214 in tumour exosomes, miR-214 was enriched in endothelial cell-derived exosomes, which may indicate its importance in endothelial exosome function. By transfecting exosome-producing endothelial cells with miR-214 inhibitors, we could reduce exosome miR-214 levels without affecting the cell number or exosome secretion. Anti-miR-214-exosomes showed a reduced capacity to stimulate migration, angiogenesis and sprouting. Accordingly, genes involved in angiogenesis and blood vessel formation were significantly down-regulated in anti-miR-214 recipient cells compared to control-miR-214-exosome recipient cells. MiR-214 levels in exosome-recipient cells were not significantly affected after exposure to control-miR-214 or anti-miR-214 exosomes, indicating that the absolute miR-214 amount added through exosomes is minute compared to the total cellular RNA amount. These data suggest that senescent cells with reduced miR-214 levels may be rescued from entering senescence by incorporating miR-214 from neighbouring, miR-214 producing, cells via the exosomal shuttle.

Furthermore, this shows that in our approach exosomes should not be considered to act as transfection agents, but rather as delicate multi-component signalling devices. Nevertheless, exosome-delivered miR-214 appeared functional, as the expression of the miR-214 target gene ATM appeared up-regulated upon exposure to anti-miR-214 exosomes. Regulation of ATM appears pivotal in the effects of exosomal miR-214. ATM prevents cell cycle progression, and up-regulation of ATM in anti-miR-214-exosome recipient
cells correlated to increased cellular senescence. The HIF-1α-pathway, another described ATM target\(^{39}\) remained unaffected. As solely down-regulation of ATM in endothelial cells enhanced cell migration, it appears that exosomal miR-214 primarily represses senescence through its effect on ATM suppression. The induction of an angiogenic program may depend on other exosome-induced pathways, i.e. Notch-signalling\(^{9}\) resulting in the observed exosome-stimulatory effects.

Our data indicate that negative effects on cell cycle progression are most profound in cells exposed to anti-miR-214-exosomes, suggesting that exosomal miR-214 may stimulate the formation of blood vessels by preventing cell cycle arrest in recipient endothelial cells.

Our \textit{in vivo} studies, showing less cells and blood vessels in plugs containing anti-miR-214-exosomes, underline the physiological relevance of our \textit{in vitro} observations. Since mouse and human miR-214 are identical and that the 3′-UTR of the mouse ATM gene contains a predicted miR-214 target site\(^{38}\), effects on cell numbers can be explained by effects on cell cycle arrest, in line with our \textit{in vitro} analyses. Here basal medium and control-miR-214 exosomes did not affect the amount of senescent cells, explaining the presence of similar amounts of CD31+ cells in the Matrigel plugs. Increased senescence in anti-miR-214-exosome-recipient cells, observed \textit{in vitro}, corresponds to reduced cell amounts detected in corresponding matrigel plugs. The \textit{in vivo} effects on blood vessel number and size demonstrate that exosomal miR-214 is also functional in inducing an angiogenic program.

Previous studies showed that non-endothelial exosomes can stimulate angiogenesis\(^{10,19}\). Also, miR-214 has been previously shown to affect proliferation and differentiation of myoblasts and T-cells\(^{46,47}\) and ginsenoside-Rg1-induced angiogenesis\(^{45}\). Furthermore, our findings agree with the observed role for miR-214 in the stimulation of migration of melanoma cells\(^{46}\) and neurons\(^{48}\), and the prevention of cell cycle arrest and apoptosis in cardiomyocytes\(^{49}\). MiR-214 appeared down-regulated in endothelial cells exposed to hypoxia or TNF-α (Supplementary Information, figure S2H), however it remains to be investigated whether this is reflected in exosomes. Furthermore, the effects on exosome mRNA and protein content likely contribute to exosome function, providing a low signal-to-noise ratio studying specific miR-214 effects\(^{37}\). Anti-angiogenic effects of miR-214 have been reported in HUVEC, but these cells may be considered as ‘young’ endothelial cells, while HMECs are more ‘mature’ endothelial cells\(^{33,45}\), with subsequent different miR-214 levels\(^{50}\). For exosome function, miR-214 levels thus may provide paradoxal information. Exosomal components have been described to provide clues that are in contrast with their function in the context of cells, potentially as they are provided to target cells through a pathway different from the normal, (intra-)cellular pathway, with the inhibition of Notch signalling by Dll4-containing exosomes in endothelial cells\(^{9}\) as a prime example.

In conclusion, our results indicate an intricate exosome-mediated cross-talk interface at the vascular endothelium that prevents cell cycle arrest and regulates the angiogenic program -at least partly- via miR-214. More general, our data indicate that individual species of miRNA play a crucial role in exosome function.
MATERIALS AND METHODS

Cell culture
HMEC-1\textsuperscript{29} cells (CDC; Atlanta, GA, USA) were maintained up to passage 27 at 37°C, 5% CO\textsubscript{2} in MCDB131 medium (Life Technologies, Grand Island, NY, USA) containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 nM Hydrocortisone, and 10 mM L-Glutamine. Exosome-free medium was prepared using FCS centrifuged for at least 1 hr at 200,000xg followed by filter-sterilization (0.20 µm).

Exosome isolation
Exosomes were collected by differential centrifugation\textsuperscript{30}. Briefly, cells were grown to sub-confluency (approximately 80%) before culturing in exosome-free medium for 24 hrs. Then, culture medium was centrifuged subsequently for 15 min at 1,500xg, 30 min at 10,000xg, and 60 min at 100,000xg. Peletted exosomes were resuspended in basal medium, pelleted again and finally resuspended in appropriate medium. Exosomes from 1x10\textsuperscript{6} cells were resuspended in 150 µl medium.

Sucrose gradient analysis
Exosomes were resuspended in 250 µl 2.5M sucrose, 20 mM TRIS HCl pH 7.4 and floated into a linear sucrose gradient (2.0-0.25M sucrose, 20 mM Tris-HCl, pH 7.4) for 16 hrs at 190,000xg. Gradient fractions (250 µl) were collected and analyzed by immunoblotting.

Immunoblotting
Exosome samples were diluted 1:1 in Exosome Sample Buffer (5% SDS, 9M urea, 10 mM EDTA, 120 mM Tris-HCl, pH 6.8, 2.5% beta-mercaptoethanol) and heated (95°C, 5 min). After SDS-PAGE, proteins were blotted onto immobilon polyvinyl membranes (P.V.D.F.; Millipore, Bedford, NY, USA), blocked for 1 hr with 5% non-fat dried milk (ELK; Campina, Amersfoort, The Netherlands) in TBS-Tween (TBS, 0.2%(v/v) Tween-20) and incubated with rabbit-anti-Flottilin-1 (1:500; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), goat-anti-PECAM-1 (1:1,000; Santa Cruz Biotechnologies) mouse-anti-ATM (1:500, Santa Cruz Biotechnologies), or mouse-anti-β-actin (1:20,000; Sigma) in TBS-T with 5% NFDM. As secondary antibodies 1:2,000 diluted affinity-purified swine-anti-rabbit, rabbit-anti-mouse, or donkey-anti-goat coupled to horseradish peroxidase (Dako, Glostrup, Denmark) were used. Antigen-antibody reactions were visualized with enhanced chemiluminescence according to manufacturer’s guidelines (Chemiluminescent Peroxidase Substrate, Sigma) and imaged using a GelDoc XR+ system (Bio-Rad, Hercules, CA, USA).

Electron microscopy
Transmission electron microscopy was performed as described\textsuperscript{31}. Briefly, carbon-coated Formvar filmed grids were placed on 5 µl exosome suspension for 20 min and washed three times with 0.15% glycine in PBS, and once with 0.1% BSA in PBS. Vesicles were fixed in 1% glutaraldehyde in PBS for 5 min and washed twice with PBS. After washing with distilled water, grids were placed on a drop of ice-cold 1.8% methylcellulose (25 Ctp)/0.4% Uranyl acetate for 5 minutes and air-dried. Exosomes were visualized using a FEI Tecnai 12 (FEI, Hillsboro, OR, USA) transmission electron microscope.
Exosome labelling
Exosomes were labelled with PKH67 (Sigma) as described. Exosomes from 150 million cells were resuspended in 180 µl PBS with 20 µl of 1:50 diluted PKH67 (in Diluent C). After 3 min incubation at RT, 3.8 ml exosome-free medium was added to terminate the labelling reaction and exosomes were harvested and washed twice with PBS by centrifugation (100,000xg for 1 hr). Exosomes were resuspended in 9.6 ml basal medium, and 250 µl was added to a subconfluent layer of HMEC cells and incubated for 2 hrs at 37°C. Cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at RT and stained with DAPI. After embedding (Vectashield, Vector Labs, Burlingame, CA, USA), cells were analyzed using an Olympus CX60 microscope (Olympus, Tokyo, Japan).

Matrigel angiogenesis assay
Angiogenic capacity of HMEC-1 cells was tested by seeding 10,000 cells (in 10 µl basal medium) onto 10 µl solidified Matrigel (ECMatrix, Millipore) in an Angiogenesis µ-slide (Ibidi). Fifty µl of test medium (with/without exosomes) was added, following incubation at 37°C for 18 hrs. Images were recorded using an Olympus CX60 microscope, converted to black-and-white and analyzed using Angioquant software. Tube length increase (relative to basal medium) was regarded a measure for angiogenesis.

Migration assay
EC migration was assessed by scratching a confluent layer of HMEC-1 cells in a 24-well plate using a 20-200 µl pipette tip. Loose cells were removed by a PBS wash and 200 µl test medium (with/without exosomes) was added followed by incubation at 37°C. Images were recorded at t=0 and t=6 hrs, after which wound area reduction was determined using ImageProPlus software (version 3.0.00.00, Media Cybernetics).

Sprouting assay
Angiogenic sprouting was determined by seeding HMEC-1 cells onto Cytodex beads (Sigma), and embedding them in a 1:1:4 mixture of basal medium:test medium:growth factor-reduced-matrigel (Becton Dickinson, Franklin Lake, NJ, USA). Solidified gels were overlaid with basal medium and incubated at 37°C, 5% CO₂ for 72 hrs. Images were recorded and sprout lengths were determined using angioquant software.

Transfections
Anti-, pre-miR-214 and non-targeting control RNAs (Life Technologies) were transfected with siPORT NeoFX (Life Technologies) using 100 nM oligonucleotide concentrations according to manufacturer’s guidelines. After 16 hrs, medium was refreshed. After 72 hrs, cells were passaged and exosomes were harvested another 72 hrs later. ATM and control siRNA (Promega) transfections were performed using 50 nM oligos at t=0 in migration assays. Knock-down at t=6 was confirmed by qRT-PCR.

Quantitative PCR
Total RNA was isolated using the MirVana RNA Isolation Kit (Ambion). MiR-214 and RNU19 levels were determined using TaqMan MiRNA Assays (Life Technologies) by quantitative
RT-PCR amplification in a MyIQ single-color real-time PCR system (Bio-Rad). Ct values were extracted and ΔΔCt values were calculated to determine relative abundances.

**In vivo matrigel plug assay**

Cell infiltration and blood vessel formation in vivo were analyzed by subcutaneous injection of 200 µl growth factor-reduced Matrigel containing exosomes from 10^7 cells into C57Bl6 mice (one plug/mouse). After 2 weeks, plugs were excised (n=7 [no exosomes], 6 [control-miR-214-exosomes], 5 [anti-miR-214-exosomes]) and embedded in paraffin. Six µm sections were cut (3 depths, 0.7 mm intervals). Cell infiltration was determined by counting nuclei in >6 hematoxilin/eosin stained high power fields. Endothelial cells were stained for CD31 and relative CD31+ cell amounts were determined by counting of CD31 positive cells in at least 1,000 DAPI-stained nuclei. Blood vessel surfaces were determined by measuring surfaces of red blood cell-containing areas in HE-stained sections. All analyses were performed using ImageJ. Animal experiments were performed with permission of the local animal experiments ethical committee.

**Gene expression analysis**

Total RNA was isolated after migration assays (t=6 hrs) using the MirVana RNA isolation kit (Ambion) and quantified using a ND1000 spectrophotometer (Nanodrop Technologies). RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and RNA 6000 Nano LabChip kit, accepting RNA integrity numbers of >9.0. Samples were labelled using the Agilent Low RNA Input Linear Amplification Kit Plus (5188-5340) according to manufacturer’s guidelines. Briefly, 500 ng RNA was amplified, reverse transcribed using T7-polymerase and labelled with Cy3 or Cy5. Dye incorporation was measured using a ND1000 spectrophotometer. Subsequently, cRNA was hybridized using the Agilent Gene Expression Hybridization Kit. Cy3 and Cy5 labelled cRNA were mixed 1:1 (825 ng each), fragmented (60°C, 30 min) and hybridized on Agilent Hybridization Chamber Gasket Slides (G2534-60011) while rotating (65°C, 17 hrs). Slides were scanned using a Microarray Scanner (Agilent). Image analysis was performed using feature extraction software version 9.5 (Agilent), applying the GE2-v5_95 protocol with default settings. Data pre-processing and analysis was performed using the R-Bioconductor package Limma, using robust Edwards background correction. Within-array and between-array normalization was performed using loess and scale standardization. Significantly differentially expressed transcripts (p<0.05) from recipient anti-miR-214 and recipient control-miR-214 cells compared to control-miR-214-cells were selected.

**Gene ontology and Pathway enrichment analysis**

Gene Ontology Term-for-Term analysis was performed using Ontologizer. P-values for significant overrepresentation of Gene ontology terms in identified gene sets as compared to human genes was calculated using a modified Fisher exact test (one-tailed) applying Benjamini-Hochberg multiple testing correction, with the significance threshold at p=0.05.

**Senescence assay**

Sub-confluent layers of HMEC-1 were incubated with 100 µl medium with or without exosomes. After 6 hrs, cells were fixed and stained using the senescence cells histochemical
staining kit (Sigma) according to manufacturer’s guidelines. Images were recorded and the percentage of positive cells was determined.

**Luciferase Reporter Assay**

HEK293 cells were co-transfected with 100 ng pRL-ATM-3’UTR\(^{36}\) (kind gift from Hailiang Hu, UCLA, Los Angeles) and 50 nM Pre-miR-214 or NT oligonucleotides (both from Ambion) using Lipofectamine 2000 (Invitrogen). pMIR-REPORT (Firefly luciferase, Promega) was co-transfected to normalize Renilla expression levels expressed from pRL-ATM-3’UTR. After 24 hrs cells were lysed (Reporter Lysis Buffer; Promega), and luciferase activity was measured using a luminometer (Lumat LB9507; EG&G Berthold).

**Statistics**

Data were normalized to means of each experiment with the reference condition set at 1. Normal distribution was tested using the Shapiro-Wilk test and statistical significance was determined using (paired) Student’s t-test or analysis of variance (ANOVA) with Student-Newman-Keuls posthoc correction where appropriate. Alpha for all tests was 0.05, all values are expressed as mean +/-SD. Blood vessel surfaces were \(10^6\) log transformed to obtain a Gaussian distribution.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


EZH2-REGULATED DAB2IP IS A MEDULLOBLASTOMA TUMOR SUPPRESSOR AND A POSITIVE MARKER FOR SURVIVAL


ABSTRACT

Purpose
Medulloblastoma is the most common malignant brain tumor in children. Despite recent improvements, the molecular mechanisms driving medulloblastoma are not fully understood and further elucidation could provide cues to improve outcome prediction and therapeutic approaches.

Experimental Design
Here, we performed a meta-analysis of mouse and human medulloblastoma gene expression datasets, in order to identify potential medulloblastoma tumor suppressor genes.

Results
We identified DAB2IP, a member of the RAS-GTPase activating protein family (RAS GAP), and demonstrated that DAB2IP expression is repressed in medulloblastoma by EZH2-induced trimethylation. Moreover, we observed that reduced DAB2IP expression correlates significantly with a poor overall survival of medulloblastoma patients, independent of metastatic stage. Finally, we demonstrated that ectopic DAB2IP expression enhances stress-induced apoptosis in medulloblastoma cells and that reduced expression of DAB2IP in medulloblastoma cells conveys resistance to irradiation-induced cell death.

Conclusion
These results suggest that repression of DAB2IP may at least partly protect medulloblastoma cells from apoptotic cell death. Moreover, DAB2IP may represent a molecular marker to distinguish medulloblastoma patients at high risk from those with a longer survival prognosis.
INTRODUCTION

Brain tumors are the most common form of solid tumors in children of which medulloblastoma is the most frequent malignant variant, accounting for 20% of cases\(^1\). Treatment modalities consist of surgery, radiotherapy and chemotherapy and result in a 5-year survival rate of 40% in high risk patients and 80-90% in low risk patients\(^2\). Approximately 30% of patients however remain incurable and current intensive treatment protocols cause significant adverse long term effects\(^3\). Medulloblastomas comprise four subtypes: WNT, SHH, Group 3 and Group 4 which differ regarding histology and clinical outcome\(^4\) and are believed to derive from the deregulation of various signalling pathways in brain development, such as the WNT-pathway and sonic hedgehog (SHH) signalling pathways. Over activation of these pathways leads to a loss of cell cycle control and a dysfunctional apoptosis program, allowing for continued growth and tumorigenesis, predominantly in the cerebellum\(^5\).

DAB2IP - disabled homolog 2-interacting protein, located at chromosome 9q33.1-q33.3 - is a member of the RAS-GTPase activating protein family (RAS GAP) that inactivates RAS by promoting conversion of GTP into GDP\(^6\). DAB2IP acts as a putative tumor suppressor gene and is down-regulated by epigenetic modification in multiple aggressive cancers. In prostate cancer DAB2IP expression was shown to be repressed by promoter methylation and histone modification\(^7\), while in breast cancer\(^8\), lung cancer\(^9\), and gastrointestinal tumors\(^10\), aberrant promoter hypermethylation was shown to down-regulate DAB2IP. Moreover, it was shown in prostate cancer that down-regulation of DAB2IP expression results in resistance to ionizing radiation\(^11\), it initiates epithelial-to-mesenchymal transition\(^12\) and promotes tumor growth and metastasis\(^13\). In addition, DAB2IP is involved in TNF\(\alpha\)-induced apoptosis in prostate cancer cells by suppressing the ASK1-JNK and PI3-AKT pathway\(^14\), and in endothelial cells via the ASK1-JNK pathway\(^15\).

Apoptosis is a programmed variant of cell death common to all human cells. Defects in the apoptosis program result in an imbalance in the rate of cell proliferation and the rate of cell death thereby contributing to tumor growth and treatment resistance. Essential steps in the apoptotic mechanism are inactivated in medulloblastoma cells, resulting in resistance to apoptosis. A recent in vivo study showed that cerebellar stem cells can give rise to medulloblastomas when having acquired an impaired apoptosis mechanism\(^16\). Consequently, many of the apoptosis mediators are generally considered tumor suppressors\(^17\).

Here, we describe a comprehensive meta-analysis of gene expression studies of mouse and human medulloblastoma\(^18-23\) identifying multiple medulloblastoma tumor suppressor candidates, including DAB2IP. We found DAB2IP expression to be strongly down-regulated in human medulloblastoma cells and in primary human medulloblastoma tissues. We demonstrate that DAB2IP down-regulation is - at least partially - caused by EZH2-mediated repression through histone methylation, conveying apoptosis resistance in immortalized neural precursor and medulloblastoma cells. Furthermore, we show that DAB2IP expression correlates significantly with the overall survival of medulloblastoma patients, independent of metastatic stage.
RESULTS

Meta-analysis of candidate tumor suppressor genes in medulloblastoma

In order to identify candidate tumor suppressor genes in medulloblastoma, we determined transcripts that were down-regulated in medulloblastoma as compared to normal cerebellar precursor cells in seven data sets comparing gene expression of normal cerebellar precursor cells and medulloblastoma in mice. We used mouse studies since that allowed for a comparison between medulloblastoma cells and proliferating progenitor cells. All data sets employed mouse models that mimic the SHH-medulloblastoma subgroup (Supplemental Table S1A, GSE9299, GSE2426, GSE7212, GSE11859, GSE6463)\(^{18-22}\). This analysis yielded 56 genes that were significantly down-regulated (>2-fold) in at least five out of the seven mouse medulloblastoma datasets. Subsequently, to increase the relevance of our screen for human medulloblastoma, we compared the expression of the significantly down-regulated mouse genes to a human medulloblastoma gene expression dataset (GSE10327)\(^23\) and an independent gene expression dataset of normal human cerebellum (GSE3526)\(^{24}\). We established that 37 genes out of our set of 56 genes were significantly down-regulated in both mouse and human medulloblastoma (Supplemental Table S2A). In addition, we compared genes that were significantly down-regulated in both mouse and human medulloblastoma to a composed list of designated tumor suppressor genes. This resulted in two candidate gene sets, one set of 37 highly deregulated genes that have not before been designated as tumor suppressors (Fig. 1A and Supplemental Table S2A), and one set of 26 genes that have been reported as a tumor suppressor before – mostly in cancers other than medulloblastoma (Fig. 1B and Supplemental Table S2B).

DAB2IP is down-regulated in medulloblastoma and is associated with poor clinical outcome

We focused on the down-regulated medulloblastoma genes with designated tumor suppressor function (Fig. 1B) and analyzed by literature search to which of these genes a pro-apoptotic function could be attributed. This resulted in the emergence of DAB2IP as a potential pro-apoptotic tumor suppressor gene in medulloblastoma, since DAB2IP is the third most differentially expressed transcript in our analysis and literature search suggests its function in apoptosis regulation\(^{14, 15, 25}\). To confirm that DAB2IP is down-regulated in medulloblastoma, we first analyzed the DAB2IP expression levels in medulloblastoma cells. qRT-PCR revealed that DAB2IP mRNA expression was down-regulated in medulloblastoma cells D283-med, D556-med, Daoy and in primary human medulloblastoma cells (Fig. 2A), as compared to normal human cerebellar tissue. This was confirmed on protein level by DAB2IP Western blot analysis (Fig. 2B). In order to determine the correlation of DAB2IP down-regulation to clinical outcome, DAB2IP expression was evaluated in mRNA expression datasets of human medulloblastoma\(^{23, 26}\), and was correlated to survival (Fig. 2C). The clinical characteristics of the tumors/patients used in the survival analysis are summarized in Table 1. Kaplan-Meier analysis demonstrates that low DAB2IP mRNA expression correlates significantly with a poor prognosis, as measured by a lower overall survival probability (p=0.010). Multivariate Cox proportional-hazards analysis shows that DAB2IP expression can
predict prognosis (Hazard ratio: 3.0, CI 95% = 1.1 – 8.6, p = 0.036), independently of clinical variables such as age, metastatic stage and histology. Medulloblastoma is known to comprise four subtypes: WNT, SHH, Group 3 and Group 4 which differ regarding histology, molecular biology, genetics and clinical outcome. However, we did not find a significant correlation between DAB2IP expression and any specific subtype in our patient series (Supplemental Fig. S1). We also investigated the relation between DAB2IP expression and metastatic stage in our patient group. However we did not find a significant correlation (Fig. 2D). Finally, to further analyze the effects of DAB2IP expression and metastatic stage on prognosis, patients were stratified based on metastatic stage (Fig. 2D, Supplemental Fig. S2A and S2B). Again Kaplan-Meier analysis demonstrated that low DAB2IP expression correlated with a poor prognosis (p=0.055) both in the metastatic patient and non-metastatic patient groups.

**DAB2IP and EZH2 are inversely expressed in medulloblastoma**

Previously it was described that DAB2IP expression is epigenetically suppressed by EZH2, a member of the polycomb complex and a histone methylating enzyme. Therefore we determined the EZH2 expression levels in medulloblastoma cells and tissues and compared
Figure 2. DAB2IP is down-regulated in medulloblastoma and is associated with poor clinical outcome. (A) RNA, extracted from various medulloblastoma cell lines (D283-med, D556-med, Daoy) and non-neoplastic brain cell lines (left panel), and from two primary human medulloblastoma samples and one normal human cerebellum tissue sample (right panel) were analyzed by qRT-PCR for expression levels of DAB2IP. The data were normalized to the levels of GAPDH mRNA in each sample. Error bars indicate s.d. *p<0.05, ***p<0.001, t-test. (B) Protein expression analysis of DAB2IP in various medulloblastoma and non-neoplastic brain cell lines. Numbers indicate relative DAB2IP protein expression normalized against β-Actin expression. (C) Kaplan-Meier analysis shows that individuals with medulloblastoma that have lower expression of DAB2IP have a significantly lower overall survival probability (p= 0.010). Cut-off for high and low expression is based on maximum likelihood. (D) Scatterplot of DAB2IP mRNA expression levels (arbitrary units) in patients based on metastatic stage M0/M1/M2-3-4 shows that average DAB2IP expression does not correlate with metastatic stage in medulloblastoma. (left panel). Kaplan-Meier survival curves of (C) further stratified based on metastatic stage (right panel).
these to DAB2IP expression. First, DAB2IP and EZH2 expression levels were evaluated in the mRNA expression dataset of 62 human medulloblastoma (9 WNT, 15 SHH, 11 Group 3 and 27 Group 4)23 and nine normal cerebellum samples24. As expected the DAB2IP mRNA levels were significantly down-regulated in the medulloblastoma samples as compared to normal human cerebellum (Fig. 3A). In contrast, EZH2 mRNA levels were significantly up-regulated in the medulloblastoma samples as compared to normal human cerebellum (Fig. 3A). There was no significant difference in the expression of DAB2IP between the four subgroups of medulloblastoma, while the expression of EZH2 demonstrated an increasing trend from the WNT and SHH subgroups to Group 3 and 4 (Fig. 3A). In addition we compared DAB2IP and EZH2 expression in individual samples and found a negative correlation between DAB2IP and EZH2 mRNA expression (Supplemental Fig. S3A). This negative correlation was also found on protein level (Supplemental Fig. S3B). In parallel to the DAB2IP expression analysis in medulloblastoma cell lines and primary tissues (Fig. 2A), EZH2 expression analysis was performed using the same samples. This demonstrated an increased EZH2 expression in medulloblastoma cells as compared to normal cerebellum (Fig. 3B), again correlating

Table 1: Patient/tumor characteristics of medulloblastoma series used for survival analysis.

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<th></th>
<th>DAB2IP low, M0</th>
<th>DAB2IP low, M+</th>
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Figure 3. DAB2IP and EZH2 are inversely expressed in medulloblastoma. (A) Microarray analysis of 62 pediatric/human medulloblastoma tissues (9 WNT, 15 SHH, 11 Group 3 and 27 Group 4) and nine normal human cerebellum tissues. (A) Scatterplot of DAB2IP expression, bar represents mean (left panel). Scatterplot of EZH2 expression, bar represents mean (right panel). (B) RNA extracted from various medulloblastoma cell lines (D283-med, D556-med, Daoy) and non-neoplastic brain cell lines (left panel), and two primary human medulloblastoma samples and non-neoplastic brain samples (right panel) was analyzed by qRT-PCR for expression levels of EZH2. The data was normalized to the levels of GAPDH mRNA in each sample. Error bars indicate s.d. *p<0.05, ***p<0.001, t-test. (C) Representative immunohistochemical staining for EZH2 (left) and DAB2IP (right) in human medulloblastoma tissues. Left and right panels belong to the same tumor sample. Tumor samples are from different medulloblastoma subgroups. Bottom panels depict tumor samples with adjacent normal cerebellar tissue that stains negatively for EZH2 and positively for DAB2IP - opposite to the tumor samples. Scale bar = 100 µm.
inversely with DAB2IP expression in these samples. Finally, immunohistochemical analysis on tissue microarrays, composed of 276 pediatric medulloblastoma tissues from 87 patients, showed that EZH2 expression was significantly overexpressed in 30 out of 87 samples. In contrast, DAB2IP protein expression was not detectable in medulloblastoma samples (Fig. 3C). However, in one patient sample that included adjacent normal cerebellar tissue, the adjacent tissue stained negatively for EZH2 and positively for DAB2IP (Fig. 3C, bottom panel). The clinical characteristics of the tumors/patients are summarized in Table 2.

**Epigenetic modulation of DAB2IP expression in medulloblastoma**

In order to determine whether DAB2IP expression in medulloblastoma is regulated by EZH2-mediated epigenetic histone modulation we transfected medulloblastoma cells with siRNAs directed against EZH2 (siEZH2). At 96 h after transfection we observed significantly reduced EZH2 levels by Western blot in medulloblastoma cells transfected with siEZH2 (Fig. 4A). In addition, EZH2-mediated histone 3 methylation at lysine 27 was reduced and DAB2IP protein levels increased in the siEZH2 transfected medulloblastoma cells. We also analyzed lysates from medulloblastoma cells treated with the S-adenosylhomocysteine hydrolase inhibitor DZNep, a potent inhibitor of EZH2 histone methyltransferase activity.**28-30** Again a reduction in EZH2 protein levels was observed with a delayed increase in DAB2IP levels (Fig. 4B). Besides histone methylation DAB2IP expression can be altered by histone acetylation and promoter DNA hypermethylation. It was previously shown that in various cancer types DAB2IP expression could be restored by treatment with the DNA hypomethylation agent 5-aza-2′-deoxycytidine (DAC). In the medulloblastoma cells used here we could not detect an increase in DAB2IP expression following DAC treatment (Fig. 4C). However, additional treatment with the histone deacetylase inhibitor trichostatin A (TSA) did significantly increase DAB2IP expression (Fig. 4D). This suggests that histone modifications may play a more significant role in suppressing DAB2IP expression in medulloblastoma, as was described also in prostate cancer.

**DAB2IP promotes stress-induced apoptosis in medulloblastoma**

To assess the functional role of DAB2IP in medulloblastoma, we examined the effects of DAB2IP modulation in human medulloblastoma cells and mouse neuronal precursor cells. Since DAB2IP was reported to enhance TNFα-induced apoptotic cell death in endothelial cells and prostate cancer cells, we analyzed the effect of DAB2IP overexpression on TNFα-induced apoptotic cell death in medulloblastoma cells. Lentiviral vectors encoding for DAB2IP or LacZ were used to stably transduce Daoy medulloblastoma cells. Treatment with TNFα (100 ng/ml) resulted in a 2-fold increase in caspase activity in Daoy cells overexpressing DAB2IP, as compared to LacZ control cells. This increase was neutralized by simultaneous treatment with the caspase inhibitor z-VAD (20 μM) (Fig. 5A). In addition we measured the cell proliferation rate of Daoy cells overexpressing DAB2IP or LacZ control. Daoy-DAB2IP cells showed a lower proliferation rate as compared to their controls. This result was confirmed in D283-med cells (data not shown). Treatment with a low dose of TNFα (10 ng/ml) - to induce mild cellular stress - impaired proliferation of the DAB2IP-overexpressing cells even further, whereas the proliferation rate of control cells was not significantly inhibited (Fig. 5B). Furthermore, DAB2IP overexpression also inhibited anchorage independent growth following
Table 2: Patient/tumor characteristics of medulloblastoma series on tissue micro array (TMA).

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Treatment with a low dose of TNFα (10 ng/ml) in Daoy cells (Supplemental Fig. S4). Since ionizing radiation (IR) is an important treatment modality in medulloblastoma and down-regulation of DAB2IP gene expression was related to resistance to IR in prostate cancer cells11, we studied the effect of DAB2IP modulation on the clonogenic growth of medulloblastoma cells after IR. Daoy-DAB2IP and Daoy-LacZ cells were irradiated with doses of 0, 1, 2, 3, 4 and 5 Gy. DAB2IP overexpression showed an IR dose-dependent reduction in clonogenic
Figure 4. Epigenetic modulation of DAB2IP expression in medulloblastoma cells. (A) D556-med and D283-med protein expression analysis of DAB2IP and EZH2 and H3K27me3 at 48 and 96 h after transfection with EZH2 siRNA, or non-related siRNAs. β-Actin expression was used as normalization control. (B) D556-med and D283-med protein expression analysis of DAB2IP and EZH2 at 48 and 96 h after treatment with DZNep. (C) Effect of 5-aza-2′-deoxycytidine (DAC) treatment on DAB2IP mRNA expression in D556-med cells. (D) Effect of combined DAC and trichostatin-A (DAC-TSA) treatment on DAB2IP mRNA expression in D556-med cells. Error bars indicate s.d. *p<0.05, ***p<0.001, t-test.

survival as compared to LacZ control cells (Fig. 5C). Finally, in order to further study the effect of DAB2IP on TNFα-induced apoptosis, we used a shDAB2IP construct to transiently knock down DAB2IP expression in C17.2 murine cerebellar progenitor cells immortalized by v-myc. Moderate knock down of DAB2IP was confirmed by Western blot analysis. DAB2IP knock down in these immortalized neural precursor cells significantly reduced TNFα-induced caspase activation (Fig. 5D), suggesting that DAB2IP has a pro-apoptotic function in these stressed neural precursor cells. In the absence of TNFα induced stress, caspase activation was similar as was observed for DAB2IP knock down and C17.2 shCTRL cells (Fig. 5D).

DISCUSSION

We performed a meta-analysis of publicly available medulloblastoma gene expression datasets in order to identify potential medulloblastoma tumor suppressor genes. We identified DAB2IP to be strongly down-regulated in medulloblastoma cells and in primary medulloblastoma tissues. Reduced DAB2IP expression was shown to correlate significantly with poor overall survival of medulloblastoma patients, independent of clinical variables.
Figure 5. DAB2IP promotes stress-induced apoptosis in medulloblastoma cells. (A) DAB2IP overexpression increased caspase activation in Daoy cells treated with TNFα 6 h after treatment. Overexpression of DAB2IP in Daoy cells was confirmed by Western blot. (B) Acumen proliferation assay of Daoy cells overexpressing DAB2IP following treatment with or without a low dose of TNFα. (C) Colony formation of DAB2IP overexpressing Daoy cells, following exposure to increasing doses of IR. (D) DAB2IP knock down decreased caspase activation in C17.2 neural precursor cells treated with TNFα 6 h after treatment. Knockdown of DAB2IP in C17.2 cells was confirmed by Western blot. Error bars indicate s.d. *p<0.05, ***p<0.001, t test.
such as age, metastatic stage and histology. Moreover DAB2IP was shown to be regulated by histone modifications, including histone acetylation, and histone methylation by the polycomb group member EZH2. Finally, we demonstrated that ectopic DAB2IP expression enhances stress-induced apoptosis in medulloblastoma cells, and that reduced expression of DAB2IP in medulloblastoma conveys resistance to irradiation-induced cell death.

Medulloblastomas are known to comprise four subtypes: WNT, SHH, Group 3 and Group 4 which differ regarding histology, molecular biology, genetics and clinical outcome. The studies used in our meta-analysis all employed mouse models that mimic SHH-subgroup medulloblastoma (Supplemental Table S1A). While this article was being revised, the first MYC-based medulloblastoma mouse models that mimic Group 3 were generated. The medulloblastomas generated by one of these MYC models demonstrate a reduced DAB2IP expression in line with our results (Supplemental Fig. S5A). However the medulloblastomas generated by the other model do not show differential DAB2IP expression compared to the control tissue used in that study (Supplemental Fig. S5B). Recently a mouse model that mimics WNT subgroup medulloblastoma was also described. However the small number (three) of samples and the strong variation in their DAB2IP expression preclude making any meaningful comments on DAB2IP expression in this model (Supplemental Fig. S5C). Finally, our human medulloblastoma dataset demonstrates that DAB2IP expression is significantly reduced in all subgroups (Fig. 3A, Supplemental Fig. S1).

To increase the likelihood of identifying tumor suppressors in medulloblastoma we compared the deregulated mRNA transcripts with a composed list of tumor suppressor genes. This list was established by interrogating publicly available gene ontology databases that included transcripts that have been tied to a tumor suppressor function. A number of well-known tumor suppressors in the context of medulloblastoma such as PTCH1, SUFU, APC, AXIN and TP53 are not included in our candidate gene sets. This is likely to be caused by our stringent threshold and the various medulloblastoma mouse models used to gather the datasets. For instance, PTCH1 is down-regulated >2-fold in two out of seven medulloblastoma mouse model datasets, thereby not reaching our cut-off of deregulation in at least three out of seven datasets. However in two additional datasets PTCH1 is down-regulated ~1.8 fold. Another well known tumor suppressor, TP53, is down-regulated >2-fold in three out of seven different mouse models datasets. However, TP53 is not differentially expressed between the human medulloblastoma and normal cerebellum dataset, supporting reports that TP53 down-regulating mutations in sporadic human medulloblastoma are not very common. In addition, transcripts can have inactivating mutations without being down-regulated at mRNA level, and are therefore not detected using mRNA expression analysis. Similar observations have also been reported for PTCH1, AXIN2 and TP53 in various subgroups of medulloblastoma.

It was previously shown that DAB2IP expression can be silenced by promoter methylation and histone modification. Other reported mechanisms for DAB2IP inactivation include one case of a translocation that disrupts DAB2IP expression in acute myeloid leukemia, and a single nucleotide polymorphism in the DAB2IP gene in aggressive prostate cancer. LOH at DAB2IP was demonstrated in 20% of cases in hepatocellular carcinoma. In addition, a common sequence variant within DAB2IP associates with the risk of abdominal aortic aneurysm and recently a genetic variant of DAB2IP was also shown to be an independent risk factor for early...
onset of lung cancer\textsuperscript{44}. However, as far as we know in medulloblastoma no such mechanisms of DAB2IP deregulation have been reported yet. Here we show a significant negative correlation between overexpressed EZH2 and down-regulated DAB2IP in human medulloblastoma samples. Moreover, we show that DAB2IP suppression in medulloblastoma cells can be at least partly reversed by EZH2 inhibition. Treatment with the DNA hypomethylation agent 5-aza-2'-deoxycytidine (DAC) did not affect DAB2IP expression in medulloblastoma cells. However, additional treatment with the histone deacetylase inhibitor trichostatin A (TSA) did significantly increase DAB2IP expression. This may suggest that histone modifications play a significant role in suppressing DAB2IP expression in medulloblastoma cells, however, we do not rule out that DAB2IP expression in medulloblastoma cells may also be impaired by additional mechanisms. Other transcripts in our list of potential medulloblastoma tumor suppressor genes may also be EZH2 targets, this is however only confirmed for CDH1\textsuperscript{47}.

We found DAB2IP mRNA expression was reduced in medulloblastoma cell lines and primary tissues. DAB2IP protein levels were also reduced in medulloblastoma cell lines. Immunohistochemistry on the medulloblastoma tissue micro array did not reveal DAB2IP expression in these samples. However, this does not necessarily mean that no protein is present at all. It may well be that small traces of DAB2IP are present in some of the medulloblastoma tissue samples, although undetectable by us in these experiments. Another explanation may be that post-transcriptional events account for the discrepancy between mRNA and protein levels.

Recently, two publications have related the loss of DAB2IP expression to increased epithelial-to-mesenchymal transition (EMT) and metastasis in prostate cancer. Xie et al. show that DAB2IP knock down increases nuclear $\beta$-catenin accumulation and trans-activation of target genes involved in EMT by inhibiting GSK3$\beta$, indicating an inhibitory function of DAB2IP in WNT/$\beta$-catenin signalling\textsuperscript{12}. In the context of medulloblastoma this seems paradoxical as metastasis is uncommon in the subgroup of human medulloblastoma in which WNT signalling is active\textsuperscript{4}. In our patient series, average DAB2IP expression was slightly higher in the group of WNT associated medulloblastomas (Fig. 3A), however this difference was non-significant. It would be of interest to determine the role of DAB2IP in WNT signalling in the context of medulloblastoma, since a major role in medulloblastoma oncogenesis has been attributed to WNT/$\beta$-catenin\textsuperscript{18}. Furthermore, it was reported that loss of DAB2IP expression induces the activation of Ras and NF-\kappa B in prostate cancer, where Ras is presumed to play an essential role in primary tumor growth and NF-\kappa B drives prostate cancer metastasis\textsuperscript{13}. Therefore, we investigated the relation between DAB2IP expression and metastatic stage. However, we were unable to demonstrate a significant correlation between DAB2IP expression and medulloblastoma metastases (Fig. 2E).

In a study of mammalian brain development high levels of DAB2IP expression was found in the developing cerebellum, particularly in Purkinje cell precursors\textsuperscript{49}. Although there is no evidence that medulloblastoma arises directly from Purkinje cells, these cells play an important part in the development of the normal cerebellum. Purkinje cells generate SHH that projects on granule neuron precursor cells and stimulate their proliferation, before the granule neuron precursor cells migrate deeper into the forming cerebellum and differentiate further. A subgroup of medulloblastoma is believed to be derived form granule neuron
precursor cells that fail to stop proliferating. We showed that DAB2IP knock down in C17.2 neural precursor cells significantly reduced TNFα-induced caspase activation suggesting that DAB2IP has a pro-apoptotic function in stressed neural precursor cells. However since C17.2 cells are immortalized by overexpression of v-myc the role of DAB2IP in altering apoptosis responses needs to be established in normal neural precursor cells.

We found a significant association between poor overall survival in medulloblastoma patients and reduced DAB2IP mRNA levels. Interestingly, no significant difference in DAB2IP expression was observed between the group of WNT associated medulloblastoma - which has the best prognosis - and Group 3 medulloblastoma - which has the worse prognosis. This association was observed in metastatic as well as in non-metastatic medulloblastoma patients, albeit at near significant levels. These results demonstrate that DAB2IP expression is a prognostic marker for medulloblastoma outcome and suggest that patients with non-metastatic medulloblastoma with low DAB2IP expression may benefit from more aggressive treatment strategies. Recent studies have shown that medulloblastoma is a heterogeneous disease with diverse treatment outcome. Currently staging for treatment is based on clinical parameters such as age, extent of surgical resection, presence of metastases and histological classification. Various studies have suggested that this risk stratification could be improved by including molecular determinants. However, it remains to be investigated to what extent DAB2IP could contribute to the sub-classification of medulloblastoma and whether it can aid as a prognostic factor in clinical practice.

In conclusion, we identified DAB2IP as a potential anti-apoptotic tumor suppressor in medulloblastoma. Further research in its use as a potentially important prognostic factor and/or therapeutic target may contribute to improvements in the future treatment of medulloblastoma patients.

**MATERIALS AND METHODS**

*Detailed protocols are in the Supplementary Data.*

**Biologic samples**

Original data on tumor samples from two retrospective studies were used for this study. Survival analysis was based on 108 cases for which expression and survival data was available. Patient and tumor characteristics are presented in Table 1. In brief, all samples were snap frozen in the institutional pathology departments immediately upon arrival. All samples were reviewed by experienced neuropathologists and examined for tumor content. Total RNA was extracted using Trizol (Invitrogen). Gene expression profiles were obtained by Affymetrix HG-U133 Plus 2.0 arrays. Gene expression data were normalized using the GCRMA procedure. Informed consent and detailed methods are described elsewhere.

Immunohistochemistry was performed on a largely independent medulloblastoma tissue micro array (TMA) cohort with tumors from 87 patients obtained from the files of the Department of Neuropathology of the Academic Medical Center (University of Amsterdam). Subgroup information was obtained by immunohistochemistry using antibodies for the subgroup-specific protein markers β-catenin (WNT), DKK1 (WNT), SFRP1 (SHH), NPR3 (Group 3), and KCNA1 (Group 4). Information on gender, age at diagnosis, histology, metastatic stage at diagnosis...
and survival are presented in Table 2. The mean follow-up time of survivors in the TMA cohort was 6.2 years (range 0.1 - 19.4 years). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. MB1 and MB2 primary human medulloblastoma tissues were obtained from surgical specimens after informed consent and approval by the Medical Ethical Committee of the VU University Medical Center.

**Survival analysis**

Overall survival was calculated from the time of diagnosis to the patient’s last follow-up or death. Survival of patients was analyzed using Kaplan–Meier survival curves, and the log-rank test was used to examine the statistical significance. P-values <0.05 were considered significant. Prognostic impact of covariates on survival was evaluated on the basis of hazard ratios from Cox’s proportional hazards regression model. Multivariate Cox’s proportional hazards regression models were used to estimate effects of additional the covariates age, metastatic stage and histology.

**Cells**

Human D283-med, D556-med (Dr. Darrell Bigner, Duke University), Daoy (ATCC) and C17.2 murine cerebellar progenitor cells immortalized by v-myc were cultured in DMEM containing 10% FBS and antibiotics.

**Acumen proliferation assay**

Cells were plated in 96-well plates (Greiner), fixed at 24, 48, 72 and 96 h after plating using formaldehyde, stained with DAPI and subsequently signal intensity was measured using an Acumen eX3 apparatus (TTP LabTech).

**Apoptosis assay**

Cells were plated in white opaque 96-well plates (Greiner) and treated with TNFα (Invitrogen). After 6 h of treatment, caspase activity was measured using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer’s instructions. Fluorescence and luminescence read-out was performed using a Tecan Infinite F200 Microplate Reader (Tecan Trading AG, Switzerland).

**Clonogenic assay**

Daoy cells were plated in six-well plates at a density of 500-1,000 cells per well depending on the used dose of irradiation or TNFα concentration. Subsequently cells were treated with increasing doses of irradiation or TNFα (10 ng/ml). After 10-14 days of culturing to allow colony formation, the colonies were fixed with 3.7% formaldehyde in PBS and stained with Giemsa solution. Groups consisting of 50 cells or more were defined as a colony. The colony counts using light microscopy were performed independently by at least two investigators.

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REFERENCES


50. Ellison DW, Kocak M, Dalton J, et al. Definition of disease-risk stratification groups in childhood medulloblastoma using combined


DISCUSSION
DISCUSSION

The aim of this thesis was to investigate epigenetic and miRNA signaling in the regulation of gene expression in endothelial cells and GBM cells during angiogenesis. Deregulated miRNA expression is involved in virtually all biological processes, including proliferation, cell cycle regulation, apoptosis, invasion and stem cell behavior. It is also becoming clear that deregulated miRNA expression is a common feature of human diseases and several miRNA-expression profiling studies have identified miRNAs that are up- or down-regulated in GBM. In addition, miRNAs play a part in endothelial cell biology and tumor angiogenesis.

To screen for differentially expressed miRNAs in GBM angiogenesis a co-culture model was used. Primary microvascular endothelial cells isolated from human brain (HBMVECs) were cultured in the presence or absence of human U87 GBM cells to mimic GBM endothelial cells. A point of concern is how well this model mimics “true” GBM endothelial cells in vivo. The use of a single GBM cell line is unlikely to recapitulate the heterogeneous character of the GBMs in vivo. It has also been demonstrated that the culturing of GBM cells induces significant genetic changes when compared with primary GBM samples. Moreover, a cell culture system does not resemble the natural tumor environment well. Similar remarks can be made for the endothelial cell part of the equation. GBM endothelial cells are likely to consist of a heterogeneous group whose behavior may not be well replicated by the use of a single primary cell culture. In addition, different types of endothelial cells used in in vitro experiments differ substantially genetically and it is likely that culturing steps also induce marked genetic changes in primary endothelial cell cultures. Unfortunately, genuine alternative options to this co-culture model are limited. Obviously, endothelial cells isolated from blood vessels dissected from patient glioma samples could be used as we did in follow-up analysis to confirm the differential expression of specific miRNAs that were identified by the initial screen. However, a major obstacle for the use of isolated tumor endothelial cells in screens or follow-up experiments is the difficulty in acquiring a sufficiently large group of endothelial cells that is not contaminated by other cell types present in the microenvironment, such as tumor cells themselves, as these will generate a false differential signal. The recent discovery that part of the GBM vasculature is made up of cells that are derived from tumor cells adds another layer of complexity to this problem. Moreover, with regard to screening for differentially expressed miRNAs, the inter- and intra-tumour heterogeneity of GBMs would likely require a large number of samples to be pooled to identify a group of miRNAs that is significantly deregulated. As an alternative to an in vitro co-culture model, it has been suggested that in vivo intracranial tumor models may better replicate the interaction between tumor cells, immunological cells, endothelial cells and extracellular matrix. However, in such a model the aforementioned problems in isolating and obtaining the endothelial cells would still apply. In addition, the selection and isolation of a control group of endothelial cells in an in vivo setting is equally difficult, whereas acquiring such cells is straightforward in the in vitro setting.

The discovery of miRNAs as key regulators of gene expression and the observation that miRNAs are involved in the pathophysiology of human tumors has opened the prospect of using miRNAs as novel biomarkers or even as potential therapeutic targets in cancer.
Theoretically, miRNA treatment can be based on either reintroduction of significantly down-regulated miRNAs with tumor suppressive properties, or reduction of overexpressed oncogenic miRNAs. However, before doing so, a number of potential pitfalls need to be addressed. Unmodified RNA oligonucleotides have low in vivo stability as they undergo rapid RNAse-mediated degradation and renal excretion\textsuperscript{16, 17}. To tackle this problem various chemical modifications have been developed. For example, conjugation of oligonucleotides to cholesterol to enhance stability and cellular uptake\textsuperscript{18}. The use of locked nucleotides (LNA)\textsuperscript{19, 20} and conjugation of 2′-O-methoxyethyl phosphorothioate (2′-MOE)\textsuperscript{21} have both been demonstrated to increase resistance to nucleases. Furthermore, encasing of miRNA-mimics in nanoparticles to form micelle-like-structures may offer protection from hostile environments\textsuperscript{22}. Another problem is target specificity. Since a single miRNA may target many different mRNAs, and since miRNAs have been shown to induce opposing effects depending e.g. on cell type, miRNA therapy is likely to have substantial off-target effects. Consequently, a major challenge will be to ensure tumor-specific delivery. Targeted delivery to specific tissues may be achieved by binding tumor-specific ligands to nanoparticles which can be directed to tumor cells via active or passive targeting\textsuperscript{22, 23}. Delivery of miRNA-based therapy to brain tumors is further complicated by the relative impermeability of the blood brain barrier. Systemically delivered anti-miRs do not effectively cross the blood brain barrier to alter target miRNA expression\textsuperscript{24} while the effectiveness of nanoparticles\textsuperscript{25, 26} and immunoliposomes\textsuperscript{25} to deliver miRNA-therapeutics across the blood brain barrier remains to be determined.

We investigated miRNA signaling in GBM angiogenesis and demonstrated mechanisms by which two specific miRNAs are involved in angiogenic signaling in GBM. However, at this moment our results provide insufficient grounds to consider miRNA-based anti-angiogenic therapy as a fruitful option for GBM treatment. Apart from the abovementioned issues regarding target specificity and delivery, it also needs to be demonstrated that modifying the expression levels of specific miRNAs will have an effect on GBM angiogenesis in an in vivo model. Ideally, such an experiment would involve an intracranial GBM tumor model and using targeted miRNA therapy e.g. based on integrin $\alpha_\text{v} \beta_3$ targeted nanoparticles\textsuperscript{27, 28}. Alternatively, as an initial proof of principle, a model based on a transfected tumor cell line could be used. Another matter of debate is the efficacy of anti-angiogenic therapy in GBM in general. The historic results of anti-angiogenic treatment for GBM in clinical studies have been disappointing\textsuperscript{29-31} until the discovery that bevacizumab, a monoclonal antibody that inhibits VEGF, improved response and progression free survival in patients with recurrent GBM\textsuperscript{32-34}. However, it is probable that in GBM the anti-permeability effect of bevacizumab is responsible for the clinical response rather than its anti-angiogenic effects. Bevacizumab treatment is associated with a significant reduction of edema\textsuperscript{33} and it is postulated that this results in reduced interstitial pressure and increased delivery of chemotherapeutics to the tumor. Moreover, preliminary data from a large phase III trial suggest that patients with newly diagnosed GBM do not benefit from the addition of bevacizumab to chemoradiation therapy\textsuperscript{35}. In addition, an increase in tumor cell migration and metastasis was observed in preclinical studies of anti-angiogenic GBM treatment\textsuperscript{36, 37}. Therefore, to employ a non-specific, indirect molecular mechanism (miRNAs) to target a biologic process (angiogenesis) whose effects on GBM progression are subject to debate, seems premature.
We demonstrated that treatment with the EZH2 inhibitor 3-Deazaneplanocin A (DZNep) reduces blood vessel formation \textit{in vivo} using a subcutaneous GBM mouse model. In conjunction with a decreased number of blood vessels, tumor growth in the treatment group was lower. Since we also demonstrated (in chapter 3) that EZH2 promotes GBM cell proliferation and has been associated with tumor progression in other types of cancer as well, the inhibition of blood vessel formation by EZH2 inhibition may be caused in part by suppressed tumor growth. In support of our hypothesis that EZH2 also directly affects angiogenesis we performed \textit{in silico} analysis and various functional angiogenesis assays. We demonstrated that EZH2 expression in GBM is associated with a pro-angiogenic signature of the GBM cells themselves as amongst others the transcripts of VEGF-A and HIF-1α were upregulated in association with increased EZH2 expression in a dataset containing multiple primary GBM samples. In addition to analyzing EZH2 function in primary tumor samples and in a GBM mouse model \textit{in vivo}, we also demonstrated that inhibition of EZH2 in endothelial cells caused reduced migration, invasion and tubule formation \textit{in vitro}. This suggests that EZH2 plays multiple roles in GBM angiogenesis, both in GBM cells and in the GBM vasculature. Altogether the results in chapters 3 and 4 suggest a model of regulation in GBM where loss of miR-101 in GBM cells results in overexpression of EZH2 and increased proliferation and secretion of growth factors by GBM cells. Subsequently, the secreted growth factors act to inhibit miR-101 in surrounding endothelial cells, which results in an overexpression of EZH2 and angiogenic stimulus in these cells.

The increased expression of EZH2 in GBM may be transcriptional or post-transcriptional. MiR-101 has been identified as an important post-transcriptional mediator of EZH2. However, in various other tumors, EZH2 can also be regulated by miR-25, miR-26, miR-30d, miR-98, miR-124, miR-137, miR-138, and miR-214\textsuperscript{18-41}. Of these, miR-124, miR-137 and miR-138 have decreased expression in GBM\textsuperscript{44,45} while miR-26 and miR-214 are expressed in endothelial cells. However, whether these miRNAs regulate EZH2 in GBM or endothelial cells has not yet been established. The downregulation of miR-101 can be the result of genomic loss of the miR-101 loci which has been observed in approximately 20% of GBM cases based on data from The Cancer Genome Anatomy (TCGA) project\textsuperscript{46}. However, other mechanisms of miR-101 repression may also apply. We demonstrated a role for the growth factor VEGF\textsuperscript{47}. Others have subsequently demonstrated that in the context of bladder cancer the pro-angiogenic growth factor bFGF was also capable to repress miR-101 by inducing the overexpression of the JmjC domain histone H3 demethylase NDY1. Intriguingly, NDY1 subsequently synergizes with EZH2 to cause chromatin modifications that silence EZH2 inhibitor miR-101, creating a feed forward loop for EZH2 expression\textsuperscript{48}. As a transcriptional mechanism, Lu et al. have shown that in ovarian cancer endothelial cells VEGF can also directly increase EZH2 promoter activity\textsuperscript{49}. Further research is warranted to investigate the different modes of EZH2 regulation in different types of endothelial cells and whether these mechanisms are mutually exclusive.

In our experiments we used DZNep, a S-adenosylhomocysteine hydrolase inhibitor, as a pharmacological agent to inhibit EZH2. It was shown that DZNep induces apoptotic cell death in cancer cells but not in normal cells\textsuperscript{50}. In addition, it was demonstrated that DZNep inhibits EZH2 expression and associated histone3 lysine27 tri-methylation in various cancer cell lines. However, DZNep is not a specific inhibitor of histone methylation and was
shown to inhibit activating histone marks as well. Moreover, DZNep may inhibit reactions carried out by many methyltransferases. Consequently, DZNep may theoretically also cause activation of epigenetically silenced oncogenes or reactivate epigenetically silenced tumor suppressors. Furthermore, it was shown that DZNep-induced changes were not heritable and treated cells returned to their “ground” states within 24 hours after drug removal. Even more detrimental for its potential clinical use in GBM patients is the observation done in a pharmacokinetic study performed in mice that DZNep was not detected in the brain after intravenous injection, suggesting that DZNep is unable to cross the blood brain barrier effectively. The future development of compounds that are able to inhibit only specific histone marks would be clinically more valuable. However, the aim of our studies was to gain more insight into the role that epigenetic signaling plays in the angiogenesis program in GBM and not the development of a feasible therapeutic intervention.

The inability of DZNep to cross the blood brain barrier also led us to use a subcutaneous GBM mouse model in our in vivo experiments. Obviously this raises concerns whether the effects of in vivo EZH2 inhibition on GBM growth and angiogenesis observed in our studies reflect the true clinical response because this model does not reflect the biological properties of patient tumors and the tumors established do not reflect the heterogeneity of human tumors. Unlike GBM, these subcutaneous tumors are characterized by a well-demarcated mass and are perfused by leaky vessels making them much more accessible to systemically administered drugs. Also, as mentioned previously, the culturing of GBM cells induces significant genetic changes, and tumors derived from these cells fail to recapitulate the full range of phenotypic and genomic traits seen in GBM. Even more, it has been demonstrated that significant differences in gene expression profiles occur between subcutaneous and intracranial grown tumors. In retrospect, it would have been more informative to study the effects of EZH2 knock-down in a truly diffuse infiltrative and angiogenic intracranial glioma model. In support of our findings it was recently demonstrated that EZH2 inhibition also decreases tumor growth in an intracranial glioma model.

In chapter 5 we show that miR-125b is involved in GBM endothelial cell function by targeting Myc-associated zinc finger protein (MAZ), a transcription factor that modulates VEGF expression. Several studies have identified targets for miR-125b that suggest a tumor suppressive function, however, in other studies it was reported that miR-125b inhibits tumor suppressor genes. This apparent contradiction does not only pertain to differences in the tumor types used by the various studies. Also in the context of GBM there is conflicting evidence whether miR-125b promotes or inhibits proliferation. We investigated the role of miR-125b in GBM-associated endothelial cells and demonstrated an anti-angiogenic function due to its inhibition of MAZ, a VEGF transcription factor. Other studies confirmed the anti-angiogenic function of miR-125b in various tumors and demonstrated that miR-125b confers its anti-angiogenic effects through the repression of several targets including HIF-1α, PIGF and VE-cadherin. We demonstrated that VEGF and glioblastoma-conditioned medium reduce the expression of miR-125b in GBM endothelial cells. However, the mechanism by which VEGF and glioblastoma-conditioned medium exert their effect on miR-125b is still unknown. In the context of prostate cancer and hepatocellular cancer it was recently demonstrated that miR-125b is repressed by chromatin modifications modulated by EZH2. This suggests a similar mechanism of
regulation may apply as previously discussed for miR-101. However, in a different study of miRNA regulation by Polycomb group complexes, miR-125b was not implicated.

Cells may communicate and exchange information by various mechanisms, including secretion of growth factors and cytokines and cell-to-cell interaction via adhesion molecules. Recently, exosomes - or microvesicles - have been identified as an important mechanism of cellular communication as well. Microvesicles are released by several cell types including progenitor cells and tumor cells and can affect the activity of target cells by transferring a variety of bioactive molecules including membrane receptors, bioactive lipids, nucleic acids including mRNA and miRNA and predominantly proteins. By delivering specific subsets of mRNAs and miRNAs, microvesicles may also induce epigenetic changes in target cells. In chapter 6 we show that endothelial cell-derived exosomes stimulate the angiogenic program in neighboring endothelial cells. We further demonstrate that the depletion of miR-214 in these microvesicles results in suppression of the angiogenic program in recipient endothelial cells. We focused on the role of miR-214 in microvesicle-mediated cross-talk, however, it should be stressed that the biological responses observed are the result of the combined actions of the various microvesicle constituents including proteins and mRNA. Opposed to our findings, other studies in various types of cancer, have demonstrated that downregulation of miR-214 may also result in a pro-angiogenic response through the upregulation of several target proteins. In addition, decreased miR-214 also resulted in increased EZH2 expression in various cancers, although an effect on angiogenesis was not reported. This suggests that the effect of miR-214 deregulation may be tumor-dependent.

Besides endothelial cells, GBM cells also release microvesicles containing mRNA, miRNA and angiogenic proteins that are taken up by cells in the tumor microenvironment, such as endothelial cells. These GBM-derived microvesicles are enriched in angiogenic proteins and stimulated tubule formation by endothelial cells. It was not reported whether specific miRNAs have a function in the observed pro-angiogenic response. However, in other studies performed in cancer cells and peripheral blood mononuclear cells, miRNAs were identified in microvesicles that were capable of inducing a pro-angiogenic response in endothelial cells. In addition to angiogenesis, microvesicles have also been shown to influence a plethora of other cellular processes such as immune responses, drug resistance and invasiveness and metastasis.

In several chapters of this thesis we used (publicly available) mRNA or miRNA gene expression (microarray) datasets. For example, in chapter 3 GBM microarray data were used to analyze the relationship between EZH2 expression and proliferation, migration, invasion and angiogenesis and in chapter 7 we used medulloblastoma microarray data to identify potential tumor suppressor genes. Microarray technology is based on hybridization between a target mRNA or miRNA and a matching probe on the microarray and allows the simultaneous analysis of very large numbers of mRNAs or miRNAs. However, there are some constraints to this technique that need to be taken into account. To start with, there are statistical restraints to testing a very large number of expression values on a single sample. There is a famous analogy in clinical medicine that states that taking 20 lab values from a perfectly normal blood sample will certainly produce two or three false positive results; in a typical microarray experiment about 40,000 expression values are measured.
from a single tissue sample. Also, effects of background noise and data normalization need to be considered. In addition, the relation between a probe and a binding mRNA is not unambiguous, mRNAs may cross-bind to probes that were designed to detect a different mRNA. Obviously, poor quality of the mRNA sample will also produce useless results. Nevertheless, microarray analysis is the contemporary method to screen for the effect of a certain trait on mRNA or miRNA expression.

One of the potential medulloblastoma tumor suppressor genes identified (in chapter 7) was DAB2IP, a RAS-GTPase that can be epigenetically suppressed by EZH2. We subsequently investigated the role of EZH2 and DAB2IP as prognostic markers in medulloblastoma. It was shown that reduced DAB2IP expression correlates significantly with poor overall survival of medulloblastoma. Similar to the case in GBM, we found that EZH2 expression was significantly upregulated in medulloblastoma cells as compared to non-neoplastic brain. Accordingly, we hypothesized that an analogous mechanism of EZH2 overexpression - via miR-101-provoked downregulation - could apply in medulloblastoma. However, in medulloblastoma there is no evidence of differential miR-101 expression, nor other reports of miRNA-induced deregulation of EZH2. This suggests different mechanisms may be responsible for overexpression of EZH2 in GBM and medulloblastoma (such as genomic gain33). Similarly, although DAB2IP is repressed by EZH2-induced histone modifications in medulloblastoma, we found no evidence of DAB2IP inhibition, nor of a negative correlation between EZH2 and DAB2IP expression levels in the GBM mRNA expression dataset that we employed in our analysis of EZH2 function in GBM (in chapter 3). This suggests that, in addition to the regulation of EZH2, also the downstream actions of EZH2 overexpression differ between these two tumor types and may even differ between subgroups of these tumors.

Although we did not further investigate the function of DAB2IP in angiogenic signaling, it was previously reported that DAB2IP regulates endothelial cell apoptosis in vitro and that inhibition of DAB2IP leads to enhanced angiogenesis in vivo, associated with increased VEGF signaling14.

Numerous issues concerning the role of epigenetics in GBM and GBM angiogenesis remain unsolved. Although the consequences of epigenetic changes are started to be unveiled it is largely unclear what causes epigenetic and post-transcriptional changes and to which degree environmental and genetic factors are involved in producing these changes. With regard to epigenetic treatment strategies, target specificity is another important issue that is not resolved sufficiently so far. As reported previously, miRNAs can target several proteins that may have conflicting roles in different tissue types. Therefore, a specific miRNA by no means represents a “silver bullet” in cancer treatment.

We argued before that the use of miRNA-based anti-angiogenic therapy in GBM treatment seems premature until target specificity and delivery issues are resolved and that an important next step would be to demonstrate that modifying the expression levels of specific miRNAs will have an effect on GBM angiogenesis in an in vivo model. Another interesting direction would be to further investigate the suitability of using miRNAs as biomarkers in GBM. There is a strong rationale for using miRNAs as biomarkers since they can be detected in several bodily fluids including cerebrospinal fluid (CSF) and blood samples, thus potentially providing a relatively non-invasive biomarker. In addition, miRNAs have increased stability compared to mRNAs and various studies have already reported miRNAs differentially expressed in GBM.
However, thus far these studies have provided somewhat mixed, or even conflicting, results. Moreover, these results are often based on miRNA expression in tissue samples. Therefore, to confirm the suitability of using miRNAs as a biomarker, it is now important to establish robust, sensitive and specific associations between (groups of) miRNAs and glioma types, prognosis or treatment responses. Subsequently, it would be of major interest to learn if such miRNA-based GBM fingerprints can also be derived from blood or CSF samples. In addition, it is important to compare miRNA-based biomarkers with existing or potential alternatives such as a classification on the basis of mRNA expression, exosome-based biomarkers or simply neuroradiologic imaging and a clinical picture.

Although it is not likely that epigenetic therapy will be the final remedy to GBM, epigenetic therapy may provide novel targets for treatment and new biomarkers for patient stratification in the future, and in doing so, may contribute to improving GBM treatment.

REFERENCES

11. van B, Jr., Griffioen AW. In silico analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles. Biochim Biophys Acta 2005;1755:121-134.


50. Miranda TB, Cortez CC, Yoo CB, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Mol Cancer Ther 2009;8:1579-1588.


