Promoting Angiogenesis and Neuroregeneration in a Vascular Dementia Model via HIF-1 Transcriptional Repressor PRMT1

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Background

Vascular Dementia (VaD) is the second most common cause of dementia and accounts for up to 20 percent of diagnosed cases in the USA (Rohn, 2014). VaD is characterized by the loss of cognitive function due to a variety of cerebrovascular or cardiovascular conditions that lead to ischemic, hemorrhagic, or hypoperfusive brain lesions (Roman, 2003; Choi et al., 2016). One key feature of VaD that contributes to its pathophysiology is hypoxia, or reduced oxygen supply to tissues.

Risk factors associated with VaD include increasing age, history of heart attack, stroke, high cholesterol, and blood pressure (Duron and Hanon, 2008). Diagnosis of VaD relies on numerous criteria such as cognitive loss, vascular brain lesions identified by imaging techniques, and the ability to rule out alternative causes of the dementia (Tang et al., 2004; Pantoni and Inzitari, 1993; Roman, 2003). However, the diagnosis of VaD is often difficult due to its heterogeneous nature and co-existence with other neurodegenerative disorders like Alzheimer's disease (Marcelo and Bix, 2015). Currently, no treatments options exist for VaD, although known therapeutic candidates like dextromethorphan have been shown to alleviate oxidative effects in rat models of VaD (Xu et al., 2016; Li and Zhang, 2015).

The following proposal identifies a therapeutic avenue that may work to restore functional brain vasculature, and promote neural tissue regeneration in VaD patients. A previously identified protein is known to be involved in regulating the expression of an angiogenesis-promoting factor. Hence, it is postulated that targeted silencing/knockdown (KD) of this protein in brain endothelial cells (ECs) may provide a means to induce angiogenesis and the repair or regrowth of nervous tissues in a murine VaD model (Figure 1). This research may further elucidate the role of angiogenesis and brain ECs in promoting nervous tissue regeneration and may represent a way to alleviate cognitive deficits associated with VaD.

Linking Angiogenesis and Brain Neurogenesis/Gliogenesis

Blood vessels (BVs) are critical for delivering nutrients and oxygen to tissues throughout the body efficiently and in a regulated manner. BV abnormalities can cause serious complications like those involved in the pathophysiology of VaD. Multiple studies suggest that BV angiogenesis, or the generation of new vessels from pre-existing ones, is linked with nervous tissue outgrowth and maintenance (Carmeliet, 2000; Vasudevan and Bhide, 2008; Tam and Watts, 2010; Eichmann and Thomas, 2013). Both the nervous system and BVs appear to closely associate with one another at the anatomic and cellular level, and recent evidence indicates that they share similar guidance cues during development (Tam and Watts, 2010; Carmeliet and Tessier-Lavigne, 2005). The coevolution of these systems strengthens the role of BVs in establishing a suitable microenvironment for neural cell proliferation and survival. As a result, studies focusing on pro-angiogenic factors may represent a means

to treat or reverse detrimental effects associated with VaD by reducing hypoxia-associated disease progression.

Compensatory angiogenesis can occur in VaD models, but these BVs are often unable to cope with hypoxic conditions (Marcelo and Bix, 2015). Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) can be examined to assist endogenous cellular mechanisms that respond to hypoxia. VEGF is a protein that is known to play a crucial role in angiogenesis by promoting EC proliferation, migration, and tube formation (Miyamoto et al., 2014). VEGF has also been shown to play a key role in brain angiogenesis and vasculature organization (Raab et al., 2004). Importantly, ECs secrete soluble factors that are known to induce neural stem cell proliferation (Shen et al., 2004; Schanzer et al., 2004). Hence, EC outgrowth and vessel regeneration may lead to neuroregeneration indirectly. Alternatively, VEGF expression also directly influences neuron and glial cell growth, survival, and axonal outgrowth, suggesting its involvement in neural regeneration (Carmeliet and Storkebaum, 2002). One example of VEGF-mediated neural guidance is seen in its ability to bind to neuropilin-1 and neuropilin-2 (Crivellato, 2011). Neuropilin receptors assist axonal outgrowth during development by responding to guidance cues. VEGF's ability to bind to neuropilin receptors further supports its role in neurogenesis. Other secreted molecular signals such as Ephrins, Netrins, and Slits also guide both vascular and neuronal development (Eichmann et al., 2005). Together, EC-directed guidance and VEGF signaling highlight the potential role of vasculature in neural repair or regrowth.

In order to induce angiogenesis in target ECs, direct and indirect methods can be studied. Previous findings indicate hypoxia-inducible factor-1 (HIF-1) is able to upregulate Vegf transcription under hypoxic conditions (Forsythe et al., 1996). HIF-1 is a heterodimeric protein of the basic helix-loop-helix family composed of two subunits, HIF-1α and HIF-1β (Wang et al., 1995). It is a transcriptional activator of genes involved in cellular responses to changes in blood oxygen (Semenza, 2001). A recent study has implicated the involvement of arginine methylation in transcriptional repression of Hif-1, specifically by protein arginine N-methyltransferase 1 (PRMT1) (Lafleur et al., 2014). Here, the authors demonstrated that KD of Prmt1 significantly upregulated transcription of Hif-1 and Vegf. PRMT1 is responsible for the majority of arginine methylation that occurs in cells (~85%) and facilitates the role of this post-translational modification in transcriptional activation, repression, and more (Bedford and Clarke, 2009). Given this knowledge, targeted silencing to reduce Prmt1 expression in brain ECs may represent a viable method to moderately stimulate angiogenesis via intermediary activation of Hif-1 and Vegf. Next, ECs must be able to signal to surrounding neural cells to promote neuroregeneration. This is possible through the production and secretion of neurogenic or gliogenic factors. In this proposal, two EC-secreted neurogenic/gliogenic factors will be discussed.

ECs secrete artemin and neurotrophin-3 (NT-3), which both act to guide axonal outgrowth (Honma et al., 2002; Kuruvilla et al., 2004). Artemin is a glial cell line-derived neurotrophic factor whose expression promotes the survival of sensory and sympathetic neurons (Baloh et al., 1998). Meanwhile, NT-3 is a neurotrophic factor that binds to receptor tyrosine kinases of the trk family and is known to trigger events such as neuronal differentiation and neurite fasciculation in cortical precursor cells (Segal and Greenberg, 1996). NT-3 also plays a role in mediating the survival and proliferation of oligodendrocytes (Heinrich et al., 1999), the central nervous system-myelinating glial cell. The proposed roles of artemin and NT-3 represent ways by which ECs can direct outgrowth of precursor neural cells. ECs may also secrete VEGF leading to neural outgrowth in adjacent tissues. Bocci et al. (2001) indicate that placental ECs may use VEGF for autocrine signaling. Given VEGF's involvement in cell-cell signaling, it is possible that it may also act on neural cells. Thus, low-level induction of Vegf via Prmt1 KD may initiate angiogenesis and neuroregeneration in brain-injured VaD patients.

Assessing the Therapeutic Potential of Prmt1 Targeting In Vitro

Traditionally, the first step towards establishing a novel therapeutic for any disease involves the identification of a target. When doing this, many remain

mindful of our current inability to reliably overexpress genes in humans. As a consequence, targets that can be knocked-down (KD) are commonly chosen. In this proposal, Prmt1 KD can be examined in VaD. After target identification, one may then test its therapeutic potential via in vitro and in vivo studies.

To investigate if Prmt1 KD can induce EC proliferation, migration, and tube formation in vitro, HUVEC and HMEC-1 human EC lines can be employed. Prmt1 KD may be achieved through Dicer-substrate small interfering RNA (DsiRNA) specific for the Prmt1 gene and a scramble negative control (NC) duplex. In conjunction with DsiRNA, Prmt1-specific antisense oligonucleotides (ASOs) can also be examined. These studies will assess the angiogenic potential of Prmt1 KD. If angiogenic potential is confirmed, in vivo experiments utilizing a murine VaD model should be pursued to examine neuroregenerative effects.

To provide a baseline for further functional studies, basal Prmt1, Hif-1, and Vegf transcription, as well as protein expression, should be confirmed in HUVEC and HMEC-1 cell lines by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and western blot. Gene expression Taqman assays, along with Hrpt1 and Gapdh reference genes can be used for normalization. Based on primary literature research, antibodies for western blot and immunocytochemistry should be determined. In order to assess transcript levels, RNA from each cell line must be extracted and reverse transcribed into cDNA. Data can then be normalized to controls using the 2-ΔΔct method. Basal protein levels can be determined by western blot using enhanced chemiluminescence for visualization with beta-actin or vinculin as loading controls. If sufficient expression of the target (Prmt1) is observed, KD of Prmt1 at the transcript and protein level should be confirmed. To do this, cells must first be transfected with anti-Prmt1 DsiRNA, NC duplex, or select Prmt1 ASOs. A lipofectamine transfection reagent control should also be included. Forty-eight hours post-transfection, RNA and protein may be extracted and RT-qPCR and western blot should then be employed to assess target expression. If sufficient gene KD is achieved (>80% transcript silencing), upregulation of Hif-1 and Vegf should be tested in silenced samples (RT-qPCR and western blot). Subsequently, in vitro proliferation, migration, and tube formation assays can be conducted. If target KD is not observed using DsiRNA/NC duplex or ASOs, alternative siRNA or ASO formulations may be pursued. Additionally, other putative PRMT1 targets may be tested through KD as well.

EC proliferation in vitro can be measured in Prmt1 silenced cells using a CellTiter 96 AQueous One Solution Cell Proliferation Assay. This colorimetric assay works by measuring the reduction of an MTS tetrazolium compound to a media-soluble colored product. Absorbance can be read in cells transfected with appropriate treatments and controls seventy-two hours following incubation with MTS reagent. Cells should be visualized prior to incubation with the reagent to avoid cell clustering as this will significantly influence the way the cells utilize the reagent. If target KD increases EC proliferation, migration can be assessed. However, if there is minimal to no effect, alternate assays, including the MTT assay, or alternate cells, such as the human brain microvascular endothelial cell line ACBRI 376, should be considered. Additionally, caspase-3/7, -8, and -9 activities can be measured to rule out apoptosis as a player in reduced proliferation counts in treated cells. This can be done using Caspase-Glo 3/7, 8, or 9 Assays, which generate a luminescent signal from caspase cleavage of a proluminescent substrate added to cells. Results of the caspase assay(s) are consequently normalized to MTS data to determine caspase-3/7, -8, or -9 activities per cell. DMSO can be used as a positive control for apoptosis. Prmt1 silenced EC proliferation and apoptosis can be compared under normal and hypoxic (1% O2) conditions to see if the treatment (Prmt1 KD) can suppress hypoxia-induced EC apoptosis. CellEvent Caspase-3/7 Flow Cytometry Assays can also be employed using hypoxic cells should the previous assays reveal inconclusive results. Considering the importance of hypoxia in VaD, these critical tests may reveal more about the role of PRMT1 in ECs, and subsequently its role in the pathophysiology of VaD.

If effective Prmt1 KD does increase EC proliferation and does not activate caspase-dependent apoptosis, migration assays can be performed. In vitro scratch/wound assays are commonly used

to assess cell migration as described by Liang et al. (2007). ECs can be plated and transfected with appropriate treatments and controls, and green fluorescent protein may be used as a transfection marker. A scratch is then made across the plate and periodic measurements of cell movement are made for up to 24 hours. If Prmt1 KD increases EC migration as indicated by faster scratch closure compared to controls, a tube formation assay can be conducted as a final confirmation of the former assays. The tube formation assay is a comprehensive in vitro experiment involving cell migration, proliferation, invasion, and more (Arnaoutova and Kleinman, 2010). However, if Prmt1 KD does not appear to influence migration of the aforementioned EC lines, another EC migration assay (EMD Millipore) can be tested.

Ultimately, Prmt1 KD should upregulate Hif-1 and Vegf in ECs. This should drive EC proliferation, migration, and tube formation indicative of angiogenesis, and play a role in driving subsequent neural outgrowth in vivo. Direct introduction of VEGF in vitro can also support the role of this mechanism in EC proliferation, migration, and tube formation.

Prmt1 and its Effects on Neurogenesis/Gliogenesis in a Murine Model of Vascular Dementia

Following studies in cultured cells, in vivo delivery of anti-Prmt1 DsiRNA should be considered to assess its therapeutic potential. If Prmt1 KD is successful, lipoprotein-assisted delivery can be employed to selectively deliver DsiRNA to brain ECs. Kuwahara et al. (2011) show intravenous (IV) delivery of lipoprotein-siRNA complexes to brain capillary ECs via receptor-mediated uptake mechanisms. Similar techniques would facilitate the study of Prmt1 KD on brain vasculature and neural tissue regeneration in a murine VaD model. To expand the feasibility of this research, direct overexpression of Vegf in vivo can also be accomplished using an adeno-associated viral (AAV) vector containing human Vegf cDNA. However, for this proposal, only lipoprotein-mediated delivery in vivo will be considered.

To begin in vivo experiments, a control experiment utilizing C57BL/6 mice should be conducted to identify any adverse effects of PRMT1 KD in vivo. Following this, C57BL/6 mice of similar gender and age should then be subjected to total bilateral carotid artery occlusion (BCCO) and isoflurane-induced hypotension to generate a VaD model as previously published (Wang, 2014). The mice can then be separated into groups based on average body size. Lipoprotein is then isolated from the sera of untreated (WT-C57BL/6), Prmt1 silenced (Prmt1KD-C57BL/6), and NC (scramble) duplex (Sc-C57BL/6) mice by similar means as described (Kuwahara et al., 2011). A single dose of DsiRNA- and NC-lipoprotein complexes can be introduced intravenously by tail vein injection into respective animal groups. Using this VaD model, the effects of Prmt1 KD on murine angiogenesis and neuroregeneration (neurogenesis/gliogenesis) can be investigated.

In order to determine if target KD does in fact provide any therapeutic benefit via stimulating active cell division in vivo, bromodeoxyuridine (BrdU) labeling can be examined. BrdU is an analog of thymidine and is incorporated into newly synthesized DNA of actively dividing cells (Lehner et al., 2011). Double-fluorescent immunolabeling using markers for ECs, neurons, astrocytes, mature oligodendrocytes, and oligodendrial precursor cells may be employed (Yang et al., 2006). Target DsiRNA can be administered to WT-C57BL/6, Prmt1KD-C57BL/6, and Sc-C57BL/6 mice by IV injection for a set time frame following dosage optimization. After BrdU incorporation by IV injection, coronal hippocampal brain sections should be prepared for staining (Pan et al., 2013). Respective brain sections are then incubated with BrdU antibody in combination with one of the following antibodies against vWF (ECs), neuron-specific nuclear protein (neurons), GFAP (astrocytes), chondroitin sulfate proteoglycan (oligodendrial precursors), or adenomatous polyposis coli (mature oligodendrocytes) (Yang et al., 2006). Hippocampal brain sections that stain positive for BrdU and other markers are lastly quantified by fluorescence microscopy seeing as these are among the most sensitive regions of the brain to ischemic events and hypoxia (Hossmann, 1999). If Prmt1-silenced tissues exhibit elevated EC and neural cell

presence compared to control tissues, Prmt1 KD is sufficient to induce either or both neurogenesis and gliogenesis in vivo. Some brain sections can also be stored in RNAlater solution for examining Prmt1, Vegf, and Hif1 expression via RT-qPCR to confirm target KD. This will ensure any beneficial effects noted are a result of Prmt1 silencing. Lack of EC or neural cell proliferation with sufficient target KD may indicate that Prmt1 is an undesirable target. However, if the model tissues lack target KD and therapeutic effect, alternative methods of delivery can be considered. Recent evidence supports the use of polysorbate 80-directed nanoparticles for systemic delivery of siRNA to brain microvascular ECs (Wang et al., 2005).

In addition to assessing actively dividing cells, proliferation and caspase-mediated apoptosis can also be determined in coronal brain sections by Ki67 and caspase-3 (Cas3) staining for immunohistochemical analysis. Resulting sections can then be compared with BrdU-labeling to identify cells that may be undergoing apoptosis or are actively proliferating. If elevated levels of EC or neural cell populations stain positive for Ki67, BrdU, and their specific markers in PRMT1-silenced sections compared to the negative control, this form of treatment likely works to stimulate murine neuroregeneration. However, cells positive for Cas3 are initiating or undergoing apoptosis following treatment, indicating that the siRNA treatment may induce tissue cytotoxicity. Results from the BrdU and IHC experiments and the behavioural tests may further support the use of Prmt1 as a potential therapeutic target for VaD.

There are a few concerns pertaining to the upregulation of VEGF. The primary concern is VEGF's role in cancer, as VEGF is known to be overexpressed in various malignancies and its expression appears to be correlated with cancer progression (Costache et al., 2015; Luo et al., 2016). Moreover, targeting players that indirectly promote VEGF expression may not prove to be the optimal method of stimulating EC and neural cell outgrowth. With regards to this concern, direct overexpression of VEGF in vivo may be tested. This can be accomplished by widespread introduction of an AAV-VEGF vector through intrathalamic convection-enhanced delivery (Barua et al., 2013). If VEGF knockout studies are requested, VEGF floxed mice (C57BL/6) under the control of a tamoxifen-inducible Cre recombinase can be utilized (Gerber et al., 1999; Hayashi and McMahon, 2002). In addition to target expression challenges, BrdU labeling may be another experimental concern as (Lehner et al., 2011) proposed that BrdU incorporation represses neuronal and oligodendrial differentiation in vitro. This could potentially interfere with the visualization of any treatment-derived neural cell differentiation in this proposal. Lastly, additional studies such as a Morris Water Maze (MWM) test and the Rotarod test can be utilized to examine if spatial learning and memory deficits are suppressed by Prmt1 KD while still retaining cerebellar (motor coordination) function (Vorhees and Williams, 2006). The MWM test has some caveats, however. D'Hooge and De Deyn (2001) indicated that hippocampal neurons were damaged in response to ischemia (similar to BCCO-hypotension VaD model above), and claimed this may have been partially responsible for any observed MWM deficits. Another caveat of the MWM is that mice are also not the best swimmers (Whishaw, 1995). To account for these problems, alternate tests such as T-mazes can be utilized to assess memory, although T-mazes similarly have their own caveats. These include the inability to determine if rodents are using spatial or non-spatial cues to navigate their surroundings, or if experimenter involvement influences test subject behaviour (Shoji et al., 2012). The Rotarod performance test would enable experimenters to examine cerebellar function in the VaD model with and without treatment (Shiotsuki et al., 2010). This would provide more insight into emerging research indicating a link between cerebellar dysfunction and VaD (Sui and Zhang, 2012).

VaD is a progressive disease that results in cognitive impairment due to ischemic, hemorrhagic, or hypoperfusive events. No current treatment options exist although pre-existing therapies utilized to treat other conditions are being explored. In this proposal, PRMT1 was discussed as a previously identified protein involved in arginine methylation. Its role in Hif-1 and Vegf induction is also known. Characterizing Prmt1 KD in vitro and in vivo may benefit our understanding of ECs and angiogenesis in promoting nervous tissue regeneration follow-

ing ischemic or hemorrhagic brain damage. Given the current state of knowledge in DsiRNA delivery and tissue imaging studies, some neuroregenerative events may not be captured in the proposed experiments. Alternative methods of delivery and functional studies can also be employed to further improve our knowledge on the role of brain EC Vegf induction and subsequent neural cell outgrowth. Ultimately, this is the only study proposed that examines Prmt1 KD as a potential therapeutic avenue to treat brain injury resulting from VaD progression. As a result, it will provide novel insight on the cryptic disease that is VaD.

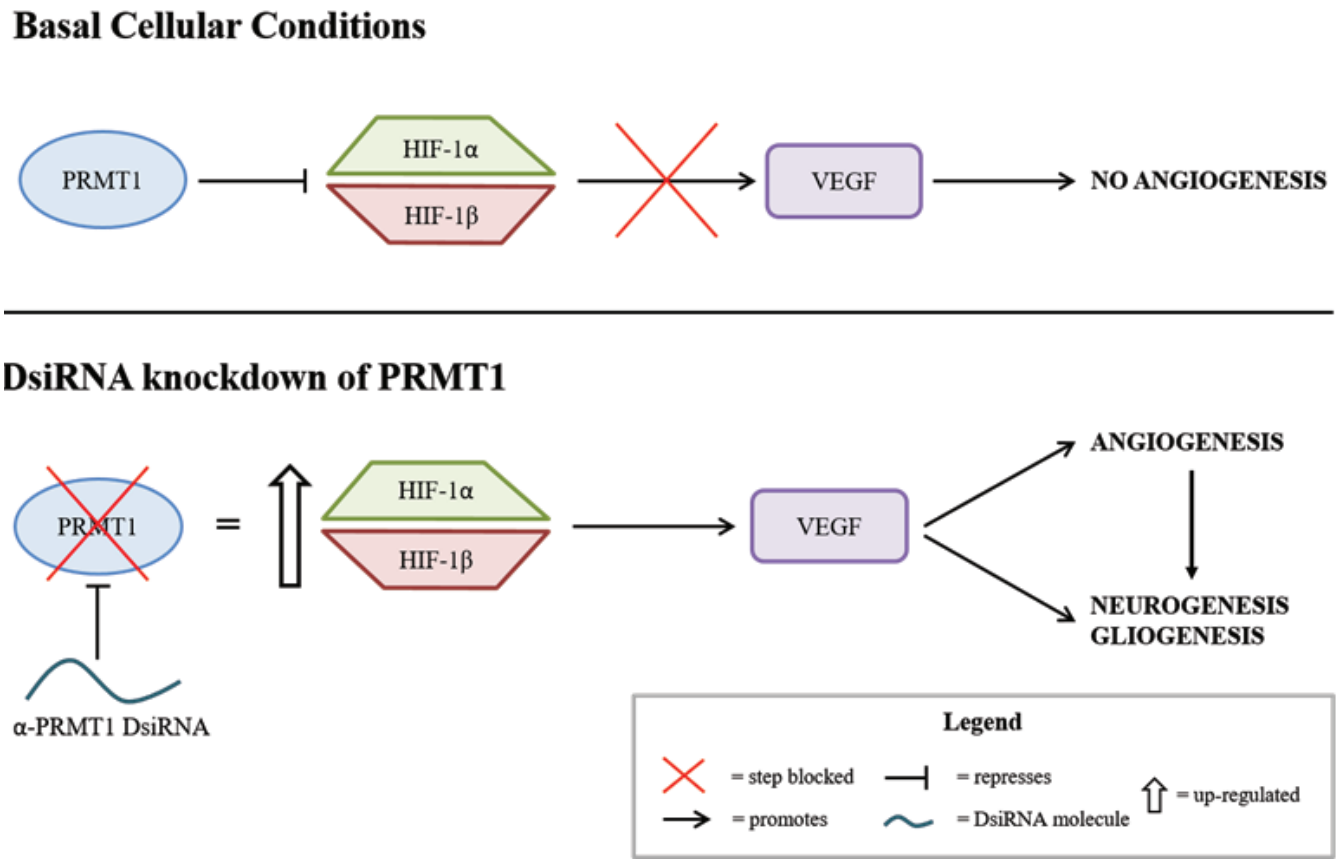

Fig. 1. Hypothesized therapeutic avenue for treating hypoxia-induced brain injury in Vascular Dementia patients. PRMT1 repression of VEGF inhibits aberrant angiogenesis and neural repair/regrowth under normoxic conditions (top frame). PRMT1 silencing (bottom frame) in brain endothelial cells may promote angiogenesis and neuro-/gliogenesis in patients suffering from hemorrhagic or ischemic events like those observed in Vascular Dementia. Both VEGF and angiogenesis may directly lead to neuroregenerative effects.

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