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Mechanisms of microRNA-mediated regulation of angiogenesis

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Role of angiogenesis in stroke and the molecules and proteins involved in the process
3.1. Role of angiogenesis in stroke
3.2. Molecules and proteins involved in angiogenesis after stroke
3.2.1. Endothelial progenitor cells and endothelial cells
3.2.2. Molecules and proteins associated with angiogenesis
3.2.2.1. VEGF and its receptors
3.2.2.2. Angiopoietins and tie receptors
3.2.2.3. Hypoxia-inducible factor 1
3.2.2.4. Other factors in angiogenesis
3.3. Traditional approaches to improve stroke recovery by enhancing angiogenesis
4. MicroRNA-mediated regulation of angiogenesis in oncology and non-brain angiogenesis
4.1. MicroRNAs: biogenesis and mechanisms of action
4.2. Dicer, Drosha and angiogenesis
4.3. Individual microRNAs involved in oncology and non-brain angiogenesis
4.3.1. MiR-15b and -16
4.3.2. MiR-17-92 cluster
4.3.3. MiR-126
4.3.4. MiR-130a
4.3.5. MiR-210
4.3.6. MiR-221 and -222
4.3.7. Let-7f
5. Potential therapeutic options via mediating associated microRNAs after stroke
5.1. Inhibition of associated microRNAs
5.2. Over-expression of associated microRNAs
6. Summary

1. ABSTRACT

Stroke is the second most common cause of death and consumes about 2-4% of total health-care costs worldwide. Although most studies have focused on neuroprotection during the past decades, current therapeutic options are still very limited. Recently scientists have intensified their work on neurorestorative therapies, including angiogenesis, which allow a far greater time window for improving neurological recovery. MicroRNAs have emerged as crucial players, regulating almost every cellular process investigated to date, and evidence of their role in the context of angiogenesis and stroke has been rapidly accumulating. The goal of this review is to summarize the mechanisms of microRNA-mediated regulation of angiogenesis and the implications for a novel molecular approach to enhance neurological recovery after stroke.

2. INTRODUCTION

Stroke is the second most common cause of death (1) and consumes 2-4% of total health-care costs worldwide (2, 3). On average, every 45 seconds someone in the United States has a stroke (4). With the population aging, the burden will increase greatly during the next 20 years, especially in developing countries (5). Advances have occurred in stroke treatment during the past decades, but current therapeutic options are still very limited. Recently scientists have intensified their work on neurorestorative therapies, including angiogenesis, with the aim of improving stroke recovery in a longer time window (6). Angiogenesis, always coupled with neurogenesis, could be interpreted as a natural defense mechanism, helping to restore oxygen and nutrient supplies to the affected brain tissue. It has been demonstrated that

angiogenesis is involved in functional recovery after ischemic stroke and correlates with longer survival (7-12).

MicroRNAs, 19-24 nucleotides, have emerged as key players regulating magnitude of gene expression in a variety of organisms (13). It is estimated that about 3% of human genes encode for microRNAs (14), and approximately 30% of mRNAs are regulated by them (14-17). MicroRNAs have been identified to participate in almost every cellular process investigated to date, and their dysregulation is also observed in different human pathologies involved in tumor, inflammation, and apoptosis (14, 18-23). Accumulated evidence from different laboratories suggests that microRNAs play a crucial role in the regulation of angiogenesis and stroke (22-30). Specific microRNAs have been identified in the angiogenic process (24, 31-64). Because of their distinct mechanisms of action, microRNAs represent a potential therapeutic target; it is possible that angiogenesis may be enhanced for stroke recovery by over-expressing or inhibiting associated microRNAs (24). Despite increasing evidence for the regulatory influence of microRNAs in angiogenesis, individual microRNAs involved in the process and the potential approaches of using microRNAs to enhance angiogenesis after stroke have not been previously evaluated. Therefore, this review summarizes the mechanisms of microRNA-mediated regulation of angiogenesis and implications for a novel molecular approach to enhance neurological recovery after stroke.

3. ROLE OF ANGIOGENESIS IN STROKE AND THE MOLECULES AND PROTEINS INVOLVED IN THE PROCESS

Time is brain for patients suffering stroke. The only approved effective drug, recombinant tissue plasminogen activator, must be administered within 4.5 hours after the onset of stroke in very carefully selected patients (65). Neurorestorative processes, which allow a far greater time window for improving neurological recovery, include angiogenesis, neurogenesis and synaptic plasticity (6). Increasing evidence suggests that angiogenesis, always coupled with neurogenesis, plays a key role in neurological recovery after stroke. Below we will summarize the role of angiogenesis in stroke, associated molecules and proteins, and traditional approaches.

3.1. Role of angiogenesis in stroke

Angiogenesis is a physiological process during development; however, during some pathological events such as stroke, endothelial cells become activated and angiogenesis ensues to provide conduits for blood flow (66). In addition, angiogenic vessels provide neurotrophic support to newly generated neurons. Thus, angiogenesis plays a crucial role in the recovery of blood flow in affected brain tissues. It has been suggested that greater microvessel density in the ischemic border correlates with longer survival in stroke patients (12). In addition, increased spontaneous vascularization during neurological recovery in the site of penumbra has been described (13, 14).

It has been demonstrated that endothelial cells surrounding the infarcted brain area start to proliferate as early as 12–24 hours after stroke, and active angiogenesis takes place in human brain at 3-4 days following ischemic insult (67-70). It still remains unclear how long angiogenesis persists. Hayashi and colleagues reported that vessel proliferation continues more than 21 days following ischemic stroke (68).

3.2. Molecules and proteins involved in angiogenesis after stroke

Several molecules and proteins that change after stroke have been identified as playing a role in the angiogenic process. The following is a summary of the literature to date on molecular mechanisms and proteins associated with angiogenesis after stroke.

3.2.1. Endothelial progenitor cells and endothelial cells

The increase of circulating endothelial progenitor cells after acute ischemic stroke leads to good functional outcome and reduced infarct growth (71), and a lower concentration contributes to severe neurological impairments (72). Circulating endothelial progenitor cells can home to sites of neovascularization and differentiate into endothelial cells (73, 74).

Endothelial cell migration and proliferation involved in angiogenesis are associated with vascular remodeling in penumbra (75, 76). It has been demonstrated that endothelial cells surrounding cortical infarcted brain areas began to proliferate as early as 12 hours in different cerebral ischemic models (67, 68). Furthermore, intact and injured endothelial cells have different effects on neurogenesis (6, 8, 69, 77).

3.2.2. Molecules and proteins associated with angiogenesis

Increasing molecules and proteins have been shown to be involved in the angiogenic process following stroke, including vascular endothelial growth factor (VEGF), angiopoietins and tie receptors, hypoxia-inducible factor 1 (HIF1), and matrix metalloproteinases (MMPs).

3.2.2.1. VEGF and its receptors

VEGF, widely expressed in the normal brain, is the most important mitogen in the process of angiogenesis after stroke. An elevated level of VEGF expression in the infarcted hemisphere was observed as early as 3 hours after ischemic insult and continued up to 3 days (69) or even as much as 7 days following stroke (78, 79). VEGF is capable of promoting cerebral angiogenesis and improving oxygen and nutrient delivery to the affected brain tissue. Thus, VEGF has been shown to be neuroprotective in different animal models (78-83). VEGF has been demonstrated to exist in microvessels in penumbra and special types of cells in brain after stroke (78, 79, 84-87). These data suggest that VEGF is produced and secreted by these cells and binds to its receptors on nearby vascular endothelial cells to directly initiate an angiogenic response (79). The binding of VEGF to its receptors on the surface of endothelial cells activates intracellular tyrosine kinases and triggers multiple downstream signals that promote angiogenesis (76). Increased levels of VEGF have been identified in human brain and serum (88-90).

VEGF binds to its receptors (VEGFR-1 or VEGFR-2) and then leads to receptor dimerization, signal transduction and thus angiogenesis (76). VEGFR-1 expressed in distinct vascular beds increases after ischemic stroke. This indicates its inert "decoy" effects by binding VEGF, subsequently regulating the availability of VEGF for activation of VEGFR-2 (69, 76, 79, 85, 91). VEGFR-2 is expressed on almost all endothelial cells, and mediates the majority of the downstream angiogenic effects of VEGF, including endothelial cell proliferation, invasion, migration and survival (91). Up-regulation of VEGFR-2 follows an ischemic insult (69, 78, 87) for up to 7 days (69).

Notably, a deleterious effect of VEGF in ischemic stroke has been observed (92). Blood vessel growth initiated by VEGF alone stimulates the formation of an immature, leaky vasculature, which may contribute to edema and worsen cerebral injury. Timing, dose and route of administration are likely to be important in dictating the balance of favorable versus detrimental effects associated with VEGF therapy for acute stroke (93).

3.2.2.2. Angiopoietins and tie receptors

The angiopoietin family consists of four members (Ang-1-4). Both Ang-1and Ang-2 levels increased in affected brain tissue after an ischemic insult, up to 28 days (94-97). In concert with VEGF, Ang-2 promotes angiogenesis (98). It blocks the stabilization and maturation function of Ang-1 and induces loosening of endothelial cell/ pericyte contacts, thus allowing vessels to convert into a more plastic state (76). However, Ang-2 leads to endothelial death and vascular regression in the absence of VEGF (98).

Tie-1 and Tie-2 are two receptor tyrosine kinases of angiopoietins. All four angiopoietins have been identified as ligands for Tie-2, which is majorly expressed on endothelial cells. Natural ligands for the Tie-1 receptor have not been found (76). Tie-1 was expressed in ischemic lesion as early as 2 hours after ischemic insult (99). Lin and his colleagues described a biphasic expression pattern of Tie-1 and Tie-2 following an ischemia-reperfusion model. A few hours after the insult they observed an up-regulation of both receptors in capillaries inside the ischemic cortex. The second peak started at 3 days and continued to 7 days after ischemic stroke (100).

3.2.2.3. Hypoxia-inducible factor 1

Hypoxia-inducible factor 1 (HIF1), the first characterized member of the HIF family, is a heterodimer composed of subunits HIF1-alpha and HIF1-beta. Expression of HIF1-alpha may be induced by a number of pathways, and its degradation is highly sensitive to O_2 levels. Intracellular HIF1-alpha, called a master switch for hypoxic gene expression, is experimentally undetectable under normoxic conditions. However, it rapidly accumulates in the cell nucleus and triggers gene expression during hypoxia (101-103). In addition, microRNA profiles of cancer cells revealed that at least a subgroup of hypoxia-regulated microRNAs was induced by HIF, supporting the key role of HIF as a transcription factor for microRNA expression during hypoxia (104).

3.2.2.4. Other factors in angiogenesis

Increasing numbers of molecules and proteins have been identified as being involved in angiogenesis following stroke. MMPs collectively regulate the angiogenic processes of sprout initiation, tube formation and stability, and capillary regression (105, 106). Recently, Lee and his colleagues (107) reported that VEGF can induce MMP-9 activities and focal angiogenesis.

Neuropilins (comprised of NP-1 and NP-2) appear to increase binding of VEGF isoforms to VEGFR-2, but decrease binding to VEGFR-1 (108, 109). Both NP-1 and NP-2 mRNA were up-regulated after ischemic stroke (110, 111).

Placenta growth factor is a ligand of VEGFR-1 that specifically potentiates the angiogenic response to VEGF by activation of VEGFR-1. Its mRNA and protein were both up-regulated in vessels in affected brain tissue with a peak of expression at 3 days after an ischemic insult (111). Moreover, elevated expression of erythropoietin by hypoxia has been identified (112-115).

3.3. Traditional approaches to improve stroke recovery by enhancing angiogenesis

For patients with acute stroke, interventions of proven benefit include management in a stroke care unit, intravenous tissue plasminogen activator within 4.5 hours or aspirin within 48 hours of stroke onset, and decompressive surgery for supratentorial malignant hemispheric cerebral infarction (2, 65). However, time is brain to the patients' functional recovery. Recently scientists have intensified their work on different approaches beyond the hyperacute phase of stroke. Angiogenesis, always coupled with neurogenesis and synaptogenesis, is one of the neurorestorative processes (42). The induction of angiogenesis, primarily in the ischemic boundary zone, enhances oxygen and nutrient supply to the affected tissue. Additionally, the generation of new blood vessels facilitates highly coupled neurorestorative processes which in turn lead to improved functional recovery (76).

There are several methods to enhance angiogenesis for improving stroke recovery, including physical activity, pharmacological approaches and stem cells. Physical training promotes angiogenesis by up-regulating mRNA levels of the angiopoietin family, VEGF and increased phosphorylation of eNOS for improving long-term stroke outcome (116-118). A large variety of agents can also boost angiogenesis after cerebral ischemia, including VEGF, phosphodiesterase type-5 inhibitors, heparin-binding epidermal growth factor-like growth factor, hepatocyte growth factor, fibroblast growth factor-2, adrenomedullin, HMG-COA reductase inhibitors or "statins," kallikreins, erythropoietin, nitric oxide donors, angiotensin 2 type 1 receptor blockade, and granulocyte colony stimulating factor (76).

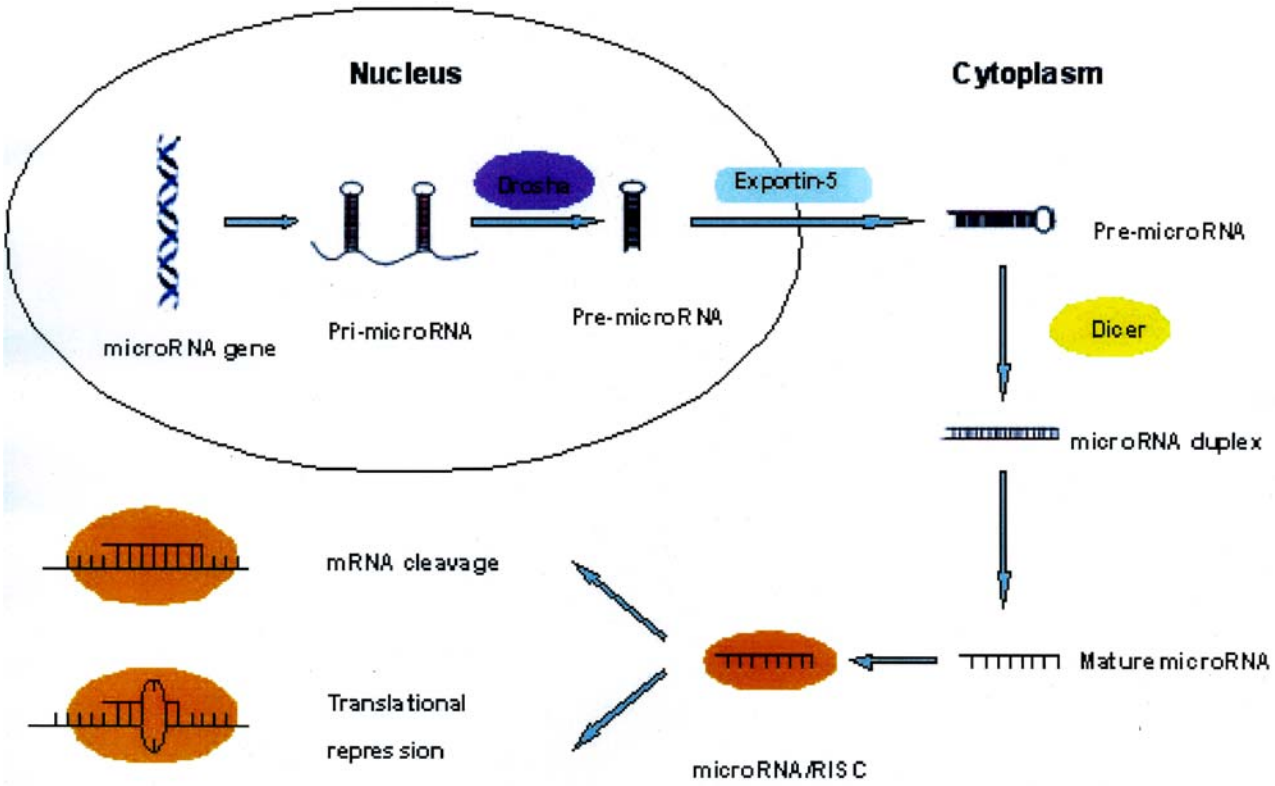

Figure 1. MicroRNAs: biogenesis and mechanisms of action. MicroRNAs are initially transcribed as pri-microRNAs by RNA polymerase 2, before being processed by Drosha to generate pre-microRNAs which are exported from the nucleus into the cytoplasm where they are further processed by Dicer to form the mature microRNA. Mature microRNAs post-transcriptionally regulate target gene expression by forming hybrid complexes with target mRNAs in their 3'UTR, and subsequently repress translation or lead to target mRNA cleavage, depending on the degree of complementarity that exists between the microRNA and its targets.

Recent work has focused on cell transplantation as a therapeutic option following ischemic stroke, and the observed improvement has been attributed to the release of trophic factors, possibly promoting endogenous repair mechanisms, reducing cell death and stimulating neurogenesis and angiogenesis (119). An increased understanding of the biology of stem cells offers potential in the treatments for ischemic stroke. Moreover, transplanted stem cells have been involved in the release of endogenous growth factors stimulating vasculogenesis (120). Cell replacement therapy in ischemic stroke from both clinical and experimental points of view presents considerable variability in outcome. Functional improvement and long-term outcome can be influenced by the properties of stem cell type, the route of cell administration, and time interval following the ischemic insult (119).

4. MICRORNA-MEDIATED REGULATION IN ONCOLOGY AND NON-BRAIN ANGIOGENESIS

Since the 1993 discovery of lin-4 in *C. elegans* (121,122), thousands of microRNA sequences have been noted in animals, plants and even viruses. As this inventory of known microRNAs continues to increase, it is becoming evident that microRNAs regulate a variety of important cellular functions. Accumulated data show an important role of microRNAs in regulating angiogenesis of different pathologic processes such as tumor, inflammation, and apoptosis (14-23).

The expression of microRNAs in endothelial cells has been demonstrated in different labs (123, 124). The highly expressed microRNAs include miR-15b and -16, -20, -21, -23a and -23b, -24, -29a and -29b, -31, -99a, -100, -103, -106, 125a and -125b, -126, -130a, -181a, -191, -221, -222, -320, let-7, let-7b and let-7c (123-125). However, few specific targets and functions involved in angiogenesis have been identified.

4.1. MicroRNAs: biogenesis and mechanisms of action

Mature microRNA is part of a 60-80-nucleotide stem-loop structure contained within the pri-microRNA. The first step in microRNA biogenesis occurs in the nucleus and requires the excision of this hairpin structure by a complex called Microprocessor (Figure 1). This complex contains the RNase 3-like Drosha enzyme and the RNA binding protein DGCR8/Pasha (126). The excised hairpin, now called pre-microRNA, is exported to the cytoplasm by a protein heterodimer consisting of the transport factor Exportin-5 and its cofactor Ran (127). In the cytoplasm, Dicer, an RNase 3 enzyme, excises the pre-microRNA to a 19- to 24-base-pair product. The product is able to be incorporated into the RNA-induced silencing

complex (RISC) (128-134). The RISC, in turn, is capable of using the "seed sequence" of the microRNA to recognize complementary messenger RNA (mRNA) transcripts for degradation or translational silencing (15, 130, 135).

The microRNA is now ready to direct its activities on target mRNA by binding microRNA responsive elements usually located in the 3'UTR of the transcript (Figure 1). This association may result in either cleavage or translational repression of the target mRNA, depending on the degree of base-pairing between the microRNA and the responsive element. Perfect complementarity generally results in target mRNA cleavage, whereas imperfect base-pairing leads to translational repression (136). The interaction between the microRNA and its target mRNA occurs between the 5'UTR of the microRNA to the 3' UTR region of the mRNA by a matching seed element in the microRNA. Utilizing these data, one microRNA can regulate hundreds of genes (137) and, at the same time, one mRNA can be regulated by a number of microRNAs.

4.2. Dicer, Drosha and angiogenesis

Because Dicer and Drosha are essential for microRNA processing, blocking Dicer or Drosha expression might be expected to cause the down-regulation of most microRNAs (138). Studies have shown that Dicer plays a crucial role in angiogenesis *in vivo* and *in vitro*. Reduction of microRNA levels *via* Dicer silencing strongly impacts the functions of endothelial cells *in vitro*, which suggests a key role for microRNAs in angiogenesis (123, 124). Reduction of endothelial microRNAs by cell-specific inactivation of Dicer attenuates postnatal angiogenic responses to a variety of stimuli, including exogenous VEGF, tumors, limb ischemia and wound healing (42). Depletion of Dicer was found to impair the development of capillary-like structures and to show an anti-proliferative effect (139, 140). Furthermore, knockdown of Dicer expression has been shown to cause profound dysregulation of angiogenesis-related genes (140, 141).

In contrast to Dicer, genetic silencing of Drosha expression in endothelial cells with siRNA resulted in a significant reduction in capillary sprouting and tube formation *in vitro*, although the reduction was much smaller than that resulting from genetic silencing of Dicer. Drosha siRNA did not block angiogenesis *in vivo*. The difference between the effects of siRNA-mediated knockdown of Dicer and those of Drosha on capillary sprouting, migration, proliferation and *in vivo* angiogenesis might be due to the involvement of Dicer in other cellular processes, such as regulation of heterochromatin formation, or an alternative Drosha-independent microRNA processing pathway that could compensate for the loss of Drosha (123, 124, 142).

4.3. Individual microRNAs involved in oncology and non-brain angiogenesis

Although a number of studies have emphasized the importance of the microRNA pathway in several aspects of the angiogenic process, the majority do not provide information regarding the functions of specific microRNAs. Studies aimed at elucidating the role of individual microRNAs in the regulation of angiogenesis in oncology and non-brain angiogenesis are increasingly being performed, and most of the examples that illustrate principles of microRNA function in angiogenesis are presented here.

4.3.1. MiR-15b and -16

An association between microRNAs and human cancer was first reported in 2002 when it was shown that miR-15a and miR-16a map to chromosome 13q14, a region deleted at high frequency in chronic lymphocytic leukemia (CLL) (143). MiR-15 and -16 enhance tumor angiogenesis, tumor cell survival, and growth by targeting tumor suppressors (123, 144). Both microRNAs have been shown to induce apoptosis of leukemic cells by targeting the anti-apoptotic protein Bcl-2, to block cell cycle progression and to be frequently down-regulated in chronic lymphocytic leukemia (145, 146).

Although the direct effects of miR-15b and -16 on endothelial cells have not been determined, both microRNAs are down-regulated by hypoxia and regulate the expression of VEGF in a carcinoma cell line (123, 138, 144). In addition, miR-15b and -16 have been shown to control the expression of VEGF, a key pro-angiogenic factor (87). These data indicate that hypoxia-induced reduction of miR-15b and -16 contributes to an increase in VEGF (138).

4.3.2. MiR-17-92 cluster

A potent angiogenesis-promoting activity has been attributed to the miR-17-92 cluster (42, 64, 147, 148-153). In the human genome, the miR-17-92 cluster encodes six microRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) which are tightly grouped within an 800-base-pair region of human chromosome 13.

The levels of miR-17, -18a, and -20a in quiescent endothelial cells were very low, and VEGF induction of these microRNAs suggests that they may regulate the proliferative actions of VEGF. Over-expression of these microRNAs in Dicer-knockdown endothelial cells rescues the defect in cell proliferation and cord formation (42), suggesting that VEGF-induced proliferation and morphogenesis are mediated in part by miR-17-92 cluster activation. MiR-17-92 cluster knockdown partly restores Tsp1 and CTGF expression. Furthermore, transduction of Ras-only cells with a miR-17-92-encoding retrovirus attenuated Tsp1 and CTGF levels. Cells transduced with miR-17-92 cluster, also known as Oncomir-1, formed larger, better-perfused tumors. These findings establish a role for the miR-17-92 cluster in non-cell-autonomous Myc-induced tumor vascular biology (46, 64, 154). When components of this cluster are over-expressed in tumor cells, they specifically target anti-angiogenic proteins containing thrombospondin type 1 repeats such as Tsp1, connective tissue growth factor, and SPARC (64).

4.3.3. MiR-126

The best-characterized endothelial cell-specific microRNA is miR-126 (43, 44, 155). In mammals, it is

encoded by intron 7 of the EGF-like domain 7 (Egfl7) gene also known as VE-statin (156, 157). Indeed, the expression of Egfl7 and miR-126 largely matched that of endothelial cell markers during embryoid body formation, being highly enriched in Flk1-positive vascular progenitors at embryonic day 4 and in mature CD31- expressing endothelial cells at embryonic day 7 (43).

MiR-126 has been identified as enriched in tissues with a high vascular component in both mouse and zebrafish (158, 159). *In vitro*, miR-126 regulates many aspects of endothelial cell biology, including cell migration, organization of the cytoskeleton, capillary network stability and cell survival (43). MiR-126 has an increased expression in endothelial cells, where it sustains pro-angiogenic factor signals through SPRED1 and PI3K regulatory subunit 2 (PI3KR2) repression (43), and its down-regulation in tumor cells contributes to abnormal proliferation (160).

MiR-126 regulates the response of endothelial cells to VEGF (43) and FGF (44), and developmental angiogenesis *in vivo* (44). It was shown that knockdown of miR-126 in zebrafish resulted in a loss of vascular integrity and hemorrhage during embryonic development (43). A targeted deletion of miR-126 (miR-126$^{-/-}$) in mice results in similar effects, including leaky vessels, hemorrhaging, and partial (~40%) embryonic lethality. MiR-126$^{-/-}$ mice that survived to adulthood appeared normal, indicating the important effect of miR-126 in vascular integrity during embryogenesis (44). MiR-126-targeted deletion reduces survival after myocardial infarction, since neovascularization is essential for cardiac repair (44). These findings illustrate that a single microRNA can regulate vascular integrity and angiogenesis, providing a new target for either pro- or anti-angiogenic therapies (161).

4.3.4. MiR-130a

The pro-angiogenic miR-130a is expressed at low levels in quiescent HUVEC and is up-regulated in response to fetal bovine serum (162). MiR-130a is a regulator of the angiogenic phenotype of endothelial cells through its ability to modulate the expression of the anti-angiogenic homeobox proteins GAX (growth arrest homeobox) and HoxA5. MiR-130a antagonizes the inhibitory effect of GAX on endothelial cell proliferation, migration, and tube formation and the inhibitory effects of HoxA5 on tube formation (162). The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link vascular oxygen supply to metabolic demand (163).

4.3.5. MiR-210

MiR-210 is induced by hypoxia in endothelial cells (164). Over-expression of miR-210 in normoxic endothelial cells stimulates the formation of capillary-like structures and VEGF-driven migration, whereas its blockade inhibits the formation of capillary-like structures and decreases the migration in response to VEGF. The modulation of endothelial cell responses to hypoxia is mediated *via* the regulation of the receptor tyrosine-kinase ligand Ephrin-A3 (164). Although the importance of Eph-A2 in the regulation of angiogenesis and VEGF signaling has been reported, little is known yet about the specific role of Eph-A3. However, these data suggest that down-regulation of Eph-A3 is necessary for the miR-210-mediated stimulation of capillary-like formation and endothelial cell chemotaxis in response to VEGF and may contribute to modulating the angiogenic response to ischemia (165).

4.3.6. MiR-221 and -222

MiR-221/-222 over-expression in Dicer-knockdown endothelial cells restored the elevated eNOS protein levels eNOS induced by after Dicer silencing (125). NO synthesized by eNOS is necessary for endothelial cell survival, migration and angiogenesis (166). However, prediction sites for these microRNA were not found in eNOS 3'UTR, suggesting that the regulation of eNOS protein levels by miR-221/222 is likely to be indirect. Collectively, these reports suggest an anti-angiogenic action for these microRNAs, making them possibly a potential tool to block angiogenesis (167). In addition, loss of miR-221 and miR-222 in endothelial cells sustains the proliferative and angiogenic properties of KIT, and regulates CKI p27 (167), increasing cell proliferation and enhancing their metastatic potential (168,169).

4.3.7. Let-7f

Members of the let-7 family are enriched in endothelial cells and are also highly expressed in normal rat carotid arteries (123-125, 154), suggesting that these microRNAs indeed belong to the specific microRNA signature of the vasculature. With regard to angiogenesis, the highly expressed let-7f exert pro-angiogenic effects as evidenced by the blockade of *in vitro* angiogenesis with 2'-O-methyl oligonucleotide inhibitors (123). Additional pro-angiogenic microRNAs include let-7f, as assessed by the blockade of *in vitro* angiogenesis by 2′-O-methyl oligonucleotide inhibitors (123, 139).

Other microRNAs that were implicated in promoting angiogenesis include miR-378 (170), miR-27b, and let-7f (123), while microRNAs may also act *via* interconnected complex networks.

5. POTENTIAL THERAPEUTIC OPTIONS *VIA* MEDIATING ASSOCIATED MICRORNAS AFTER STROKE

To date, there is no direct evidence to show that microRNA-mediated angiogenesis could reduce the infract volume or improve functional outcomes after stroke. However, microRNAs represent an attractive potential therapeutic target since enhancement of angiogenesis has been identified as an effective therapeutic strategy and microRNAs are involved in the angiogenic process. Thus, selective regulation of particular microRNAs targeting angiogenesis is a promising prospect for stroke. The synthesis, maturation and activity of microRNAs can be manipulated with various oligonucleotides that encode the sequences complementary to mature microRNAs (171). Over-expression of microRNAs can be induced by using either synthetic microRNA mimics or chemically modified

oligonucleotides (130). Conversely, microRNAs can be silenced by antisense sequences (synthetic analogues of microRNAs) (172, 173). Such manipulation may control angiogenesis and have potential as a new therapy for ischemic stroke. It is important to seek routes to interfere with microRNAs and to develop these as novel cerebral ischemia therapies.

5.1. Inhibition of associated microRNAs

MicroRNA antisense oligonucleotides provide an effective way to inhibit the activity of a microRNA involved in angiogenesis. The fact that microRNAs bind to their mRNA targets by Watson-Crick base-pairing indicates that a potential effective method of inactivating pathological microRNAs is to use an oligonucleotide complementary to the microRNA that effectively competes with the mRNA target. This method could avoid down-regulation of important targets that promote the stimulation of gene expression (172, 174, 175). A number of groups have shown that vectors expressing microRNA target sites can be used to saturate an endogenous microRNA and prevent it from regulating its natural targets. This technology, which has been described using the terms *decoy*, *sponge*, *eraser*, *antagomir*, *anti-miRs* and *knockdown* (176-180), has utilized a variety of gene delivery systems, including plasmids as well as vectors based on adenoviruses, retroviruses and lentiviruses.

The use of antisense sequences in cultured cells has been successful; however, the key development was chemical modification of microRNA inhibitors for *in vivo* utility. The large body of research undertaken during the development of antisense therapeutics has led to effective strategies for the pharmacological delivery of nucleic acids, facilitating the development of microRNA therapeutics (181). Three different chemical modifications have been carried out to fulfill the inhibition of microRNA function *in vivo*. One class of antisense is conjugated to cholesterol (antagomiR) to facilitate cellular uptake. Other classes use oligonucleotides with locked nucleotide acid (LNA anti-miRs) or 2-O-methoxyethyl phosphorothioate (2- MOE) modification (172,174,175). Studies have revealed that inhibition of miR-17-92 cluster activity is associated with angiogenesis (64, 182).

5.1.2. Over-expression of associated microRNAs

Alternatively, microRNA mimics (double-stranded oligonucleotides designed to simulate the function of endogenous mature microRNAs) may induce target mRNA down-regulation and thereby diminish gene expression (183). For example, over-expression of miR-15 and -16 might be an attractive anti-tumor strategy that could target tumor cell survival and proliferation and block VEGF-mediated angiogenesis (138).

Despite the advantages of microRNA technology, this new therapeutic approach has limitations. A high vector copy number or strong expression of the target-bearing transcript is needed, and this can be difficult to achieve in some cell and tissue types for the cell/tissue specificity (167). In addition, modifying a viral protein can have a negative impact on both the stability of the virus and its ability to infect and replicate in the desired tissue, and some bystander infection of other tissues is difficult to avoid (183). Furthermore, the regulatory actions mediated by microRNAs are complex, and the same microRNA can cause the opposite biological effect depending on the context. Thus, despite the promise of the early studies, we must recognize that our still-limited understanding of microRNA biology and function caution us to move carefully towards a clinical translation of these new strategies.

6. SUMMARY

Time is brain for patients suffering stroke. Angiogenesis, always coupled with neurogenesis and synaptogenesis, leads to improved functional recovery after acute injury, and microRNAs have been suggested to be involved in the regulation of angiogenesis and stroke. In the present review, we have summarized the role of microRNAs in the regulation of angiogenesis and their potential therapeutic function after stroke. Although experimental microRNA therapy results look promising, they must be validated through studies involving different cohorts of patients before they can be introduced into clinical practice.

7. ACKNOWLEDGEMENTS

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Abbreviations: VEGF: vascular endothelial growth factor; HIF1: Hypoxia-inducible factor 1; MMPs: matrix metalloproteinases; GAX: growth arrest homeobox; Egfl7: EGF-like domain 7.

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