

## REVIEW ARTICLE

## Zebrafish models of cerebrovascular disease

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Perturbations in cerebral blood flow and abnormalities in blood vessel structure are the hallmarks of cerebrovascular disease. While there are many genetic and environmental factors that affect these entities through a heterogeneous group of disease processes, the ultimate final pathologic insult in humans is defined as a stroke, or damage to brain parenchyma. In the case of ischemic stroke, blood fails to reach its target destination whereas in hemorrhagic stroke, extravasation of blood occurs outside of the blood vessel lumen, resulting in direct damage to brain parenchyma. As these acute events can be neurologically devastating, if not fatal, development of novel therapeutics are urgently needed. The zebrafish (*Danio rerio*) is an attractive model for the study of cerebrovascular disease because of its morphological and physiological similarity to human cerebral vasculature, its ability to be genetically manipulated, and its fecundity allowing for large-scale, phenotype-based screens.

*Journal of Cerebral Blood Flow & Metabolism* (2014) **34**, 571–577; doi:10.1038/jcbfm.2014.27; published online 12 February 2014

**Keywords:** aneurysm; arteriovenous malformation; cavernous malformation; moyamoya; stroke; zebrafish

## ZEBRAFISH CHARACTERISTICS

## Vasculature

Drug discovery involves a stepwise series of processes that typically begin with biochemical and cellular assays to screen for agents of potential value, which are later validated in animal models, and ultimately in human subjects.<sup>1</sup> The process is costly, resource intensive, and time consuming. The use of zebrafish (*Danio rerio*) models in this process has been used effectively to identify new drugs, discover new indications for already FDA-approved drugs, and to better understand the mechanisms for various human diseases.<sup>2,3</sup> The advantages of using zebrafish to model human diseases are numerous, and relate to their ability to study all developmental stages, coupled with a scale that is not possible in other vertebrate systems.<sup>2,4</sup> For cerebrovascular disease in particular, the zebrafish offers the unique advantage of their embryo stage being optically transparent, making it possible to study the functional and morphological changes in cerebral blood vessels in a living organism. The study of cerebral vasculature can be further highlighted with the use of transgenic zebrafish, such as Tg(flk1:GFP) that express green fluorescent protein in their endothelium and can be readily visualized with the use of epifluorescence microscopy.<sup>5</sup> While fluorescent vasculature is not a direct measurement of cerebral blood flow, it does represent an *in vivo* means to assess vascular structures with a high level of resolution, capable of rapid phenotyping of thousands of individual subjects. Other methods of physiologic assessment include microangiography<sup>6,7</sup> and laser-scanning velocimetry<sup>8</sup>, which can be used to further characterize the qualitative and quantitative changes in cerebral blood flow. Furthermore, zebrafish embryos are able to undergo live imaging<sup>9,10</sup>, allowing for real-time visualization of angiogenesis and vasculogenesis. In addition, the dimensions of blood vessels can be measured in any axis following fixation in resin with an extremely high degree of accuracy.<sup>11</sup> These techniques allow for spatial and temporal resolution of alterations in hemodynamics and blood vessel structure, which are useful tools in the study of cerebrovascular disease.

## Genetics

For zebrafish models of disease to be pertinent to the pathophysiology of humans, they must share genetic underpinnings, not just merely a common phenotype. Although the zebrafish appears as a relatively simple organism, comparison of the two genomes has demonstrated a high degree of conservation in genes implicated in processes ranging from oncogenesis to angiogenesis.<sup>12–14</sup> There is also a high degree of conservation between humans and zebrafish with respect to drug responses, indicative of a high degree of amino-acid sequence identified at protein-active sites where many drugs bind.<sup>15</sup> Although the genetic sequence of zebrafish is highly conserved with humans, it is more readily manipulated for the purposes of experimentation. Several tools exist in the armamentarium of the zebrafish biologist that can be used to dissect pathophysiologic pathways. Broadly speaking, the use of 'forward' genetic screens can be used where chemicals with both known and unknown functions, along with other mutagens, can be administered to zebrafish and their phenotype can be characterized.<sup>16</sup> Conversely, 'reverse' genetic screens can be performed where the gene of interest is manipulated precisely with one of the several methods and then the phenotype is observed. Examples of this include morpholino oligonucleotide knockdown<sup>17,18</sup>, transcription activator-like effector nucleases<sup>19,20</sup>, zinc-finger nucleases<sup>21,22</sup>, and the clustered, regularly interspaced, short palindromic repeats (CRISPR)–CRISPR-associated (Cas) systems<sup>23</sup>, each of which can induce targeted genetic modifications in zebrafish embryos.

## Phenotype Screening

The size of the zebrafish facilitates a large experimental scale size that is not possible with other vertebrates. Adult zebrafish pairs can generate up to 300 embryos at each mating, allowing for experiments with thousands of organisms at a time. Because the zebrafish embryo is much smaller than 1 mm in diameter, experiments can be performed in 96 or 384-well plates. Most small molecules readily diffuse into zebrafish, requiring only a dilution of

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Received 11 November 2013; revised 27 December 2013; accepted 7 January 2014; published online 12 February 2014

the drug into the fish water to effectively 'treat' organisms. Tens of thousands of known and unknown compounds are commercially available, including libraries specifically generated with FDA-approved compounds.<sup>2</sup> The zebrafish's microscopic size, high fecundity, and ease of drug administration, coupled with ease of phenotype recognition, make it well suited for high-throughput screening.<sup>24</sup>

## ZEBRAFISH STROKE MODELS

### Cerebral Amyloid Angiopathy

Cerebral amyloid angiopathy is a cause of potentially fatal lobar intracranial hemorrhage, particularly in the elderly.<sup>25</sup> Histologically, the diagnosis is characterized by deposition of amyloid peptides around brain vessels, resulting in fibrinoid necrosis, microaneurysm development, and ultimately, vascular rupture.<sup>26</sup> Effective therapeutics do not currently exist that have been shown to alter the natural history of the disease and the exact pathogenesis of the condition remains elusive. One of the leading hypothesis underlying cerebral amyloid angiopathy-related hemorrhage is that beta amyloid deposition leads to endothelial dysfunction (early senescence).<sup>27</sup> Emerging work in a zebrafish model has been used to explore the pathogenic mechanisms of the disease.<sup>28</sup> In these experiments, beta amyloid peptide was administered via diffusion in fish water and a senescence phenotype was assessed by measuring beta-galactosidase activity and the cyclin-dependent kinase inhibitor p21 expression (*in situ* hybridization in whole-mount zebrafish embryos). This work demonstrated that amyloid deposition has activity related to the senescence of the endothelium, producing progressive alterations of microvessel morphology and function. Importantly, it also highlights the utility of the zebrafish model in the investigation of this disease. Future studies are anticipated to further elucidate the effects of beta amyloid administration in the endothelium. Given that small peptides can diffuse into zebrafish, small molecule screens to identify novel therapeutic targets may be indicated in cerebral amyloid angiopathy, Alzheimer disease, and other disorders of amyloid deposition.

### Cerebral Arteriovenous Malformation

Arteriovenous malformations (AVMs) of the brain are vascular anomalies of children and adults who carry a high risk of hemorrhage, about 2% to 4% per year over the patient's lifetime.<sup>29</sup> The primary pathological phenotypic character of AVMs is a direct communication between arteries and veins without an intervening capillary bed. Treatment of these lesions, either with surgery, endovascular embolization, or external beam radiation (stereotactic radiosurgery), carries significant risks, especially when lesions are large or located in eloquent brain areas. No specific medical therapies currently exist and their pathogenesis is incompletely understood. Despite a robust understanding of vasculogenesis and angiogenesis<sup>30–39</sup>, the mechanisms behind the formation of discrete AVMs are not well known. It is thought that abnormalities in blood vessel formation and segregation during embryonic development are thought to be responsible, although *de novo* and recurrent lesions have been seen in adult life and dysfunctional angiogenic processes have also been implicated.<sup>40–42</sup>

Arteriovenous malformations occur sporadically or much more rarely, in the context of a hereditary syndrome. One such syndrome in humans, hereditary hemorrhagic telangiectasia type 2 (OMIM phenotype ID 600376), is caused by a mutation in the gene encoding activin receptor-like kinase 1, a type 1 transforming growth factor beta receptor in the BMP signaling pathway.<sup>43</sup> This known mutation provides an opportunity to study the pathogenic mechanisms of AVMs in vertebrate models.<sup>44,45</sup> Another syndromic form of cerebral AVM development, such as

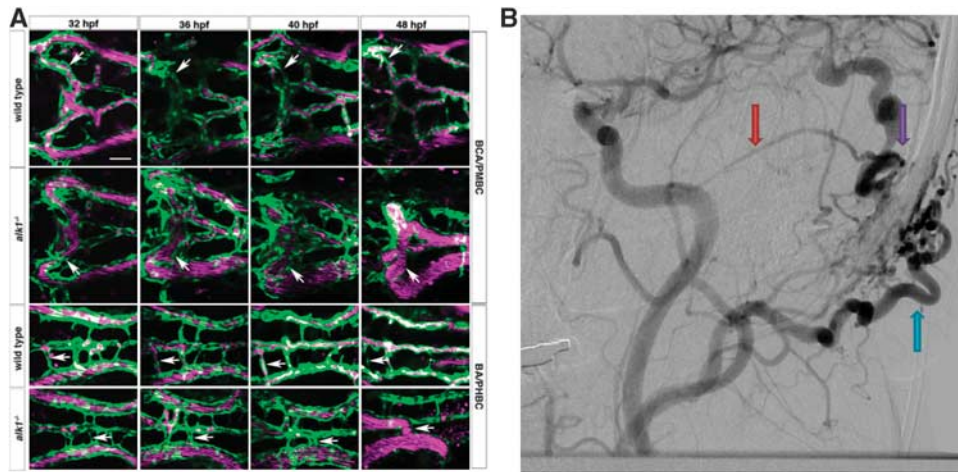
CM-AVM (OMIM phenotype ID 608354), is caused by a mutation in *RASA1*,<sup>46,47</sup> a gene that has been fully sequenced in the zebrafish. Even though only a small percentage of AVMs are thought to be related to these different Mendelian patterns of inheritance,<sup>48–50</sup> evidence does exist that that single nucleotide polymorphisms in genes such as *alk1* occur in many sporadic occurring AVMs.<sup>51,52</sup> Therefore, further dissection of mechanistic pathways leading to AVM development may be applicable to AVMs that occur in the sporadic setting, as well as in cases of Mendelian inheritance.

Several mammalian models of cerebral AVMs exist, but are limited in their applicability for drug discovery. For example, cerebral AVMs have been created in swine, but this method requires a surgical intervention to generate a lesion that mimics the human condition.<sup>53</sup> Other animal models, including mice, have been used, although they require angiogenic stimulation with vascular endothelial growth factor, in addition to genetic manipulation to generate lesions.<sup>54–56</sup> In comparison, zebrafish are an attractive model in that their endothelium is visualized easily with fluorescent proteins and their entire cranial circulation can be observed *in vivo*. By manipulating gene expression, AVMs can be generated in the cranial circulation of zebrafish, recapitulating the human disease with a high level of fidelity.<sup>44,45</sup> (Figure 1) Beyond the appearance of the abnormal blood vessels, zebrafish models of cerebral AVM also demonstrate systemic manifestations of the accompanying pathophysiologic hemodynamic response that is seen in humans, such as in high output cardiac failure in the pediatric population.<sup>46,57,58</sup> The recent announcement from the National Institute of Neurological Disorders and Stroke halting enrollment in the ARUBA trial because of the procedural risk associated with any form of interventional treatment emphasizes the urgent need to find effective medical therapies for these lesions.<sup>59</sup> Zebrafish models show promise in not only accelerating the discovery of pathogenic mechanisms, but also in the discovery of effective, targeted therapeutics through high-throughput screening.

### Cerebral Aneurysm

Aneurysms are lesions of the cerebral vasculature that have a typical phenotypic characteristic of an outpouching of a blood vessel wall as a result of an inherent weakness. As aneurysms enlarge, their propensity for catastrophic rupture increases.<sup>60</sup> Treatment of these lesions is optimally performed prior to an hemorrhagic event, either with microsurgical obliteration or endovascular techniques.<sup>61</sup> Their pathogenesis is generally thought to result from the interaction between genetic and epigenetic factors (such as cigarette smoking and hypertension).<sup>62,63</sup> Evidence for the genetic aspects of aneurysm pathogenesis is well established from powerful population based studies, such as the familial intracranial aneurysm study.<sup>64</sup> In addition, informative studies, known as genome-wide linkage studies, have been performed on rare families that are affected with aneurysms.<sup>65,66</sup> What is known from these studies is that there are many genetic susceptibilities implicated in the development of familial aneurysms.<sup>67</sup> Furthermore, cerebral aneurysms can occur in the setting of autosomal dominant polycystic kidney disease (OMIM phenotype ID 601313), a hereditary condition caused by a mutation in the *PKD1* gene. This syndrome has effectively been modeled in the zebrafish, where knockdown of *PKD1* orthologs resulted in a distinct phenotype, related to deficiencies in extracellular matrix integrity.<sup>68</sup>

The understanding of aneurysm development is best examined experimentally where *in vivo* imaging and genetic analysis can be performed. Multiple types of organisms have been utilized in the study of aneurysms, including the rabbit<sup>69,70</sup>, the mouse<sup>71,72</sup> and the dog.<sup>73</sup> While the size of these organisms allows for detailed physiologic study, it also inherently prohibits their use in high-throughput-type experiments. The embryonic zebrafish is an ideal



**Figure 1.** Phenotype comparison of zebrafish and human arteriovenous malformations (AVM). **(A)** In wild-type embryos (row 1), transient connections between the basal communicating artery (BCA) and primordial midbrain channel (PMBC) carry blood at 32 hpf but regress by 48 hpf (white arrows). In *alk1* mutants (row two), one or both of these bilateral connections may be retained, forming an abnormal BCA-to-PMBC arteriovenous connection (white arrows). More posteriorly, lumenized connections drain the basilar artery (BA) to the primordial hindbrain channel (PHBC) in wild-type embryos at early times, but almost all regress by 48 hpf (row 3, white arrows). In *alk1* mutants, one or more of these connections may be retained, forming a BA-to-PHBC AVM (row 4, arrows). This model resembles the human condition, seen in a digital subtraction cerebral angiogram. **(B)** In human AVMs, arterial branches (red arrows) connect directly to the venous circulation (blue arrows) through a high-flow fistula (purple arrow). One theory for AVM development is that they represent the abnormal persistence of normal transient developmental connections. Scale bars, 50  $\mu$ m. Zebrafish images are two-dimensional confocal projections of *Tg(kdrl:GFP)<sup>la116</sup>; Tg(gata1:dsRed)<sup>sd2</sup>* embryos, dorsal views, anterior leftwards. Endothelial cells are green; erythrocytes are magenta. Human digital subtraction angiogram is a lateral projection carotid artery injection in the late arterial phase. (Figure and legend modified from Corti P *et al.*<sup>44</sup> Distributed under the terms of the Creative Commons Attribution (CC-BY) License).

organism for the study of aneurysmal disease. It is completely transparent allowing for visualization of its cranial vasculature, is readily genetically modified, and has a genetic homology that is strikingly similar to humans.<sup>12</sup> Angiography is readily performed allowing for determination of hemodynamic variables that are key to aneurysm pathogenesis, such as blood flow rate and wall shear stress.<sup>74,75</sup> In this way, the zebrafish approximates the human condition and allows for its manipulation in unparalleled ways.

#### Cerebral Cavernous Malformations

Cerebral cavernous malformations, also known as cavernous angiomas or cavernomas, are one of the few causes of stroke known to reliably be caused by one of at least three genetic mutations.<sup>76</sup> Histologically, they consist of enlarged capillary cavities (low flow, low pressure) without any intervening brain parenchyma. These lesions can result in seizure and/or hemorrhage, and therefore, the treatment is typically recommended for when they are symptomatic.<sup>77,78</sup> Currently, no therapeutics exist outside the realm surgery<sup>79,80</sup> or focused external beam radiation (stereotactic radiosurgery).<sup>81–83</sup> Treatment becomes challenging, carries significant risk, or can even be considered impossible for lesions situated deep within critical brain structures. In addition, lesion multiplicity also complicates treatment suitability and is more commonly seen with familial cases.<sup>84</sup>

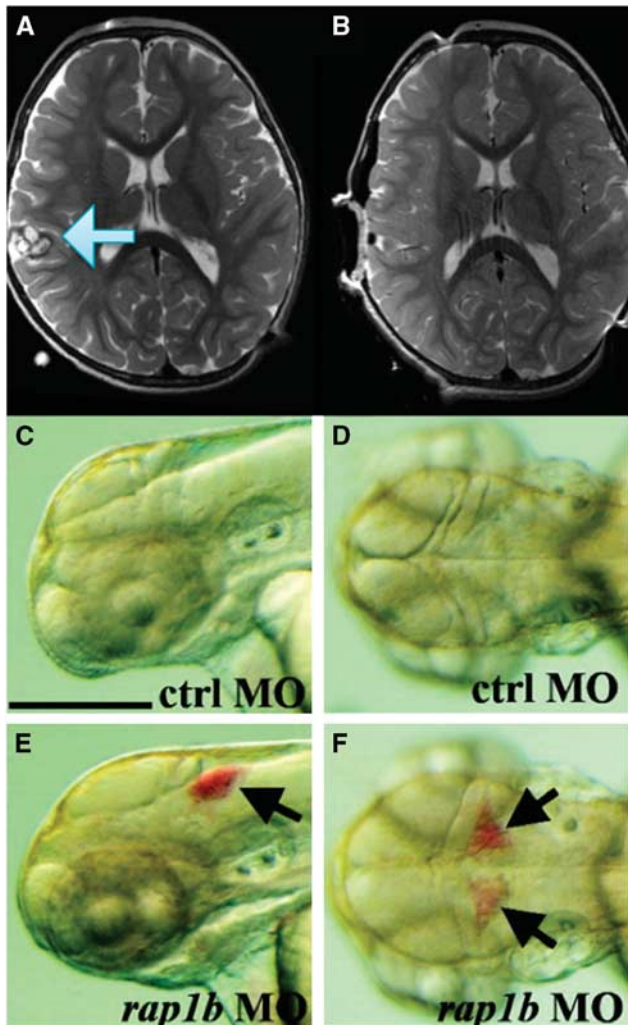
While both Mendelian inheritance and sporadic cases exist, mutations in *CCM1*, *CCM2*, or *CCM3* can be identified the majority of these lesions.<sup>85–87</sup> A growing understanding of the function of their gene products has allowed for progress toward elucidating the basic mechanisms of disease pathogenesis.<sup>88–92</sup> However, a more complete understanding of the biochemical and cellular processes that lead to the disease phenotype are necessary, and require the context of an *in vivo* assay. The zebrafish continues to be used as a model organism given its optical transparency of the embryonic stage and its ability to be genetically manipulated.

The zebrafish exome shares a striking similarity with humans, and the orthologs of the three genes (*CCM1*, *CCM2*, and *CCM3*) responsible for cavernous malformations have been identified (*santa*, *valentine*, and *pcdc10*, respectively).<sup>93–95</sup> These mutations can be introduced in zebrafish either through genome editing or with the use of morpholino knockdown technology. Loss of these gene products results in impaired cardiovascular development, specifically a characteristic dilated heart phenotype.<sup>96</sup> In addition, zebrafish with loss of function in these genes also develop a vascular phenotype, in addition to their cardiac developmental abnormalities. Looking at the cerebral vasculature, the zebrafish develop thin-walled vessels that are prone to hemorrhage, reminiscent of what is seen in pathologic human lesions.<sup>95,97</sup> (Figure 2) The experience with CCM modeling in zebrafish is one of the leading examples of how cerebrovascular disease can be studied in animal models.

#### Moyamoya Disease

Moyamoya disease is a life-threatening cerebrovascular disease that predisposes patients to both ischemic and hemorrhagic stroke.<sup>98</sup> The key angiographic feature that defines the condition is progressive stenosis of the intracranial internal carotid arteries and their branches. The classic configuration of their intracranial circulation is described as a 'puff of smoke', with network of abnormally dilated collateral vessels that attempt to compensate for the lack of blood flow through the normal conduits in the circle of Willis. A variety of direct and indirect surgical revascularization procedures are used to treat the condition, each with varying rates of success.<sup>99–101</sup> No pharmacologic therapy has been shown to alter the natural history of the disease. Although several susceptibility loci have been identified<sup>102,103</sup>, the pathogenesis of the condition remains elusive.

Zebrafish have proven to be valuable tools in this condition by allowing further investigation of gene function. Following



**Figure 2.** Phenotype comparison of zebrafish and cerebral cavernous malformation. In an magnetic resonance imaging of a human (A), a cerebral cavernous malformation is apparent in the right frontal lobe (blue arrow). These lesions can result in catastrophic hemorrhage and/or seizure activity. Treatment with surgery is effective, and complete resection can be achieved (B) if lesions are in accessible areas. When lesions are deep or multiple, surgical treatment may not be indicated, underscoring the need to develop novel therapeutics. To understand the molecular mechanisms in the CCM pathway, zebrafish models of the disease have been generated using morpholino technology. Compared with control organisms (C and D), morphant knockdowns of *rap1b* (E and F), a gene that encodes a Ras GTPase effector protein for CCM1/Krit1, demonstrate disrupted endothelial junctions, resulting in intracranial hemorrhage (black arrows), similar to human lesions. Bars, 250  $\mu$ M. (Figure modified from Gore A V *et al.*<sup>95</sup> Distributed under the terms of the Creative Commons Attribution (CC-BY) License).

genome-wide linkage analysis of affected families, a candidate gene *RNF213* has been identified and subsequently knocked down in a zebrafish model.<sup>104</sup> The phenotype generated by morpholino oligonucleotide injection was abnormal vessel sprouting and irregular vessel diameter, supporting the role of *RNF213* in vascular development and stability. In another study, a rare x-linked moyamoya syndrome was found to be caused by Xq28 deletions (removing *MTCP1/MTCP1NB* and *BRCC3*). In a functional study of *BRCC3*, morphant zebrafish were generated. Knockdown of this gene resulted in angiogenesis defects, which were also rescued by

endothelium specific expression of *BRCC3*.<sup>105</sup> These studies demonstrate the utility of zebrafish to serve in experiments of gene function, allowing for the visualization of abnormalities in the cranial vasculature *in vivo*. By establishing a model phenotype based on gene mutations found in humans, dissection of corresponding signal cascades can be performed. Establishment of these novel morphants and mutant lines also facilitates their integration into high-throughput screening platforms in search of small molecules that rescue the disease phenotype. Certain limitations of embryonic zebrafish in the study of arterial disease and vascular malformations must be acknowledged, and are centered on the apparent lack of pericytes and smooth muscle cells in the very young embryo, which are known to contribute to disease pathogenesis in humans.<sup>106</sup>

#### Ischemic Stroke

Ischemic stroke, resulting from cerebral vascular occlusion, is a major cause of death and disability worldwide. With the exception of tissue plasminogen activator, there are no targeted medical therapies available, highlighting the need for accelerated drug discovery. The use of zebrafish in the study of ischemic stroke lags behind hemorrhagic stroke, and only relatively few preliminary research efforts have been published on the subject. A notable study describes the establishment of a zebrafish model of hypoxic-ischemic injury<sup>107</sup>, with a follow-up report from the same group describing the neuroprotective effects of a zinc chelator using the same model.<sup>108</sup> Furthermore, certain hereditary forms of ischemic stroke in humans, such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy have been modeled in the zebrafish.<sup>109</sup> This model, generated by mutations in the *notch3* gene, has a phenotype in the fish typified by enlargement of vessels in the telencephalon and fin, disorganization of the normal stereotyped arrangement of vessels in the fin, and gaps in the arterial wall.

Even though limited work has been performed with ischemic stroke models, zebrafish represent a vast platform to investigate gene function. In particular, genes that control the expression of ion transport channels are central importance in understanding the pathophysiologic sequelae that follows an initial ischemic insult.<sup>110</sup> For example, the cation-chloride co-transporter NKCC1 (sodium-potassium-chloride co-transporter 1) is one of the key ion channels that contributes to the development of cytotoxic and ionic edema following ischemia.<sup>110–114</sup> This ion channel is well described in the zebrafish and has been shown to be important in the regulation of endolymph volume in the otic vesicle and swim bladder volume.<sup>115</sup> Even though an inhibitor of this ion channel is available in humans (bumetanide), it is limited by low blood–brain barrier penetration<sup>116</sup> and lack of specificity at high concentrations.<sup>117</sup> The zebrafish represents an opportunity to discover another more selective NKCC1 inhibitor with better blood–brain barrier penetration, with potential as therapy to preempt post-ischemic cytotoxic and ionic edema. Several other channels implicated in dysregulation of the neuroglial unit following ischemic stroke, such as the *N*-methyl-D-aspartate receptor and acid-sensing ion channel, have been described in the zebrafish.<sup>118,119</sup>

In addition to mitigating the secondary effects from ischemic stroke, such as hemorrhagic transformation and cerebral edema formation, an effort to understand the mechanisms of post-stroke recovery has the potential to uncover novel therapeutic targets. Specifically, a focus on adult neurogenesis and the migration of regenerating neurons in the post-injury recovery period is an ongoing area of research.<sup>120–122</sup> These processes are being studied in the zebrafish, using adult fish as model organisms.<sup>123–125</sup> Further refinements in experimental injury will allow for the study of brain recovery from injury, whether it is from stroke or trauma.

In any zebrafish model of cerebrovascular disease, it should be noted that many physiologic aspects of the cerebral circulation in zebrafish are not yet well known. Factors that have proven important in human ischemic stroke, such as collateral circulation<sup>126</sup> and autoregulatory capacity<sup>127</sup>, are yet to be evaluated in the zebrafish.

## SUMMARY

Zebrafish have been used as model organisms in the investigation of both hemorrhagic and ischemic stroke. They have been shown to be useful not only in the investigation of gene function, but also as a high-throughput drug discovery screening platform. With the conservation of many molecular mechanisms of disease among vertebrates, zebrafish experiments are poised to result in a better understanding and new therapeutics for human cerebrovascular disease.

## DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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