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# The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway regulates developmental cerebral-vascular stability via prenylation-dependent signalling pathway

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ABSTRACT

Spontaneous intracranial hemorrhage is a debilitating form of stroke, often leading to death or permanent cognitive impairment. Many of the causative genes and the underlying mechanisms implicated in developmental cerebral-vascular malformations are unknown. Recent in vitro and in vivo studies in mice have shown inhibition of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway to be effective in stabilizing cranial vessels. Using a combination of pharmacological and genetic approaches to specifically inhibit the HMGCR pathway in zebrafish (*Danio rerio*), we demonstrate a requirement for this metabolic pathway in developmental vascular stability. Here we report that inhibition of HMGCR function perturbs cerebral-vascular stability, resulting in progressive dilation of blood vessels, followed by vessel rupture, mimicking cerebral cavernous malformation (CCM)-like lesions in humans and murine models. The hemorrhages in the brain are rescued by prior exogenous supplementation with geranylgeranyl pyrophosphate (GGPP), a 20-carbon metabolite of the HMGCR pathway, required for the membrane localization and activation of Rho GTPases. Consistent with this observation, morpholino-induced depletion of the β-subunit of geranylgeranyltransferase I (GGTase I), an enzyme that facilitates the post-translational transfer of the GGPP moiety to the C-terminus of Rho family of GTPases, mimics the cerebral hemorrhaging induced by the pharmacological and genetic ablation of HMGCR. In embryos with cerebral hemorrhage, the endothelial-specific expression of cdc42, a Rho GTPase involved in the regulation of vascular permeability, was significantly reduced. Taken together, our data reveal a metabolic contribution to the stabilization of nascent cranial vessels, requiring protein geranylgeranylation acting downstream of the HMGCR pathway.



## Introduction

HMGCR catalyzes the rate-limiting step in the conversion of HMGCoA to mevalonate, giving rise to mevalonate-derived molecules including cholesterol and isoprenoids (Goldstein and Brown, 1990). A complex feedback mechanism involving both steroidal and non-steroidal mediated pathways is involved in the regulation and maintenance of HMGCR activity (Brown and Goldstein, 1997; Nakanishi et al., 1988, Omkumar et al., 1994; DeBose-Boyd, 2008). Statins, competitive inhibitors of HMGCR, are pharmaceuticals that bind part of the HMG-binding residues of the enzyme and inhibit its activity (Istvan and Deisenhofer, 2001). In addition to lowering cholesterol levels, statins also curtail the biosynthesis of other lipids, namely isoprenoids, which otherwise serve as essential lipid attachments for the Rho family of GTPases (Bishop and Hall, 2000). Hence, an efficient way to elucidate HMGCR gene function during development is to use statins for which the biochemical mode of action is well characterized. Deficiencies in HMGCR activity during development are implicated in prenylation-dependent germ cell migration delay and misguidance in both *Drosophila* and zebrafish (*Danio rerio*) models (Santos and Lehmann, 2004; Thorpe et al., 2004). A point mutation identified at position 1575 of *hmgcrb*, the maternally-expressed *hmgcr* paralog in zebrafish, results in defective heart-tube formation due to impaired post-translational prenylation of small GTPases (D'amico et al., 2007). In contrast, targeted disruption of *hmgcr* in mice results in early embryonic lethality, which is indicative of the crucial function that HMGCR-mediated metabolism and downstream signalling events play during mammalian development (Ohashi et al., 2003). Presently, the developmental processes that require adequate HMGCR function remains poorly understood.

Pharmacological inhibition of the HMGCR pathway was shown to exert both angiostatic and angiogenic effects in vitro (Lu et al., 2004; Khaidakov et al., 2009; Weis et al., 2002; Kaneta et al., 2003; Li et al., 2002; Acquavella et al., 2009). An outstanding question is whether perturbations of the HMGCR pathway would disrupt the stabilization of nascent vessels and overall vascular morphogenesis in vivo, and,

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if so, what would be the pathophysiological consequences of interference with these processes during a period of rapid angiogenesis. Zebrafish have recently been utilized as a suitable model to dissect the etiology of cerebral cavernous malformation (CCM) (Kleaveland et al., 2009; Hogan et al., 2008; Yoruk et al., 2012). Moreover, recent work in zebrafish demonstrates that proteins involved in the activation and transduction of Rho GTPase-mediated signalling pathways synergistically participate in the development of the vascular network in zebrafish. Deficiencies within this pathway impair the stabilization and maturation of nascent vessels by preventing the association of mural cells with endothelial cells, giving rise to multiple hemorrhages in the brain (Buchner et al., 2007; Liu et al., 2007), along with more pronounced defects in vascular morphogenesis (Epting et al., 2010). It is known that the mevalonate-derived metabolite, geranylgeranylpyrophosphate (GGPP) serves as an end-product for the lipidation and subcellular localization of Rho GTPases to the plasma membrane, which is critical for their activation and function (Bishop and Hall, 2000). Hence, depleted GGPP levels reduce the GTP-binding capacity of Rho GTPases, rendering them cytosolic and inactive. Although the Rho GTPase pathway has been implicated in CCM pathology, there is discrepancy between the mechanisms in mouse and zebrafish. More specifically, studies in mice show that global inhibition of Rho GTPase prenylation via statin-mediated blockage of the HMGCR pathway restores endothelial barrier integrity, thus reversing the vascular permeability dysfunction in mice that are genetically predisposed for CCM (Whitehead et al., 2009). Consistent with this observation, transcript levels of the Rho GTPases, RhoA, Rac and cdc42, were significantly elevated in lesions of a mouse model of CCM (Louvi et al., 2011). Hence, in mice, statin-mediated inhibition of Rho GTPase signalling, through decreased GGPP biosynthesis, is reported to be an effective treatment to attenuate vascular permeability (Li and Whitehead, 2010), whereas in zebrafish, mutation and morpholino induced loss of function of proteins that participate in the activation and transduction of Rho GTPase signalling promotes vascular permeability (Buchner et al., 2007; Liu et al., 2007).

To further investigate this discrepancy, we used two structurally different statin molecules and two morpholino oligonucleotides with non-overlapping sequences to inhibit zebrafish HMGCR function, all of which resulted in grossly dilated vessels, followed by progressive loss of vascular stability in the brain at specific developmental stages during which nascent cranial vessels are more prone to rupture. Prior supplementation with exogenous mevalonate or GGPP, two of the metabolites downstream of the HMGCR pathway, rescued the cerebral hemorrhage phenotype. Furthermore, morpholino-mediated specific disruption of the GGTase I-mediated prenylation pathway also mimicked the cerebral hemorrhages attributed to the inhibition of the HMGCR pathway. Interestingly, in embryos with cerebral hemorrhage, the vascular-specific expression of cdc42, a Rho GTPase implicated in the mediation of endothelial barrier function (Kouklis et al., 2003; Broman et al., 2006; Ramchandran et al., 2008; Spindler et al., 2010) and highly enriched on the vacuole membranes (Eitzen et al., 2001; Isgandarova et al., 2007), was noticeably reduced in the head and trunk vasculature, as early as 24 hpf. Our results highlight a requirement for HMGCR metabolism, through GGPP biosynthesis, to the establishment of cerebral-vascular stability during zebrafish development, likely mediated by CAAX proteins that require geranylgeranylation for their activation.

## Materials and methods

### *Zebrafish husbandry and transgenic lines*

Adult zebrafish were maintained under a constant temperature of 28 °C and a 14 h light: 10 h dark photoperiod in the University of Ottawa Aquatic Care Facility. Embryos were obtained through natural breeding of adult zebrafish and were kept at 28.5 °C in embryo medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L $CaCl_2$, 0.33 mmol/L $MgSO_4$). The double transgenic Tg(*fli1:EGFP*); (*gata-1:DsRed*) zebrafish line was kindly provided by Dr. Beth Roman (University of Pittsburgh, Pennsylvania). The Tg(*fli1:EGFP-cdc42wt*)$^{y48}$ was kindly provided by Dr. Brant Weinstein (National Institute of Child Health and Human Development, Maryland). All experiments were carried out in accordance with a protocol approved by the University of Ottawa Protocol Review Committee and conform to the published guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching.

### *Drug treatment and metabolite rescue experiments*

For pharmacological inhibition of HMGCR, zebrafish embryos (1–2 hours post fertilization; hpf) were treated with Atorvastatin (ATV; Pfizer Inc., Connecticut USA) or Cerivastatin (CVT; Sequoia Research Products Ltd. Pangbourne, UK) in a single-exposure manner in embryo medium. ATV was dissolved in DMSO, whereas CVT was dissolved in sterile/distilled water. All solutions were aliquoted and kept at −20 °C until used. Embryos for the dose-response experiments were placed in final concentrations of 0.3, 0.5 or 1 mg/L ATV; CVT was administered at 0.15 mg/L. For mevalonate rescue experiments, similar numbers of embryos were placed in embryo medium containing 0.5 mg/L ATV and 1, 1.6, 3.2 or 4.4 μmol/L mevalonic acid (Sigma-Aldrich) or DMSO as vehicle control. Each treatment group consisted of more than 70 embryos and all exposure experiments were repeated at least 8 times. GGPP rescue experiments were performed by administering 0.5 mg/L ATV and 0.5, 2 or 4 mg/L GGPP (Sigma-Aldrich) dissolved in DMSO/methanol or DMSO/methanol, as vehicle control.

### *Whole-mount o-Dianisidine (OD) staining and cryosectioning*

Zebrafish embryos at various developmental stages were fixed in 4% PFA/1X Phosphate Buffered Saline Tween-20 (PBST) overnight and subsequently stained for hemoglobin using o-Dianisidine, a sensitive marker of hemoglobin. Briefly, unfixed embryos were dechorionated and stained with a solution of OD (0.6 mg/mL) (Sigma-Aldrich) containing 0.01 M sodium acetate (pH 4.5), 0.65% hydrogen peroxide, and 40% (v/v) ethanol in the dark for 15 min. Treated embryos were visualized using a Nikon NBZ 1500 dissecting microscope with a Nikon DXM 1200 C digital camera and stored in 50% glycerol at 4 °C. For cryosectioning, whole-mount embryos previously stained with OD were equilibrated to 30% sucrose/1X PBST overnight. The next day, the samples were mounted in tissue freezing medium (Triangle Biomedical Sciences). Transverse sections of ~10 μm thickness were cut on a Leica CM1850 standard cryostat (Leica Microsystems) at −20 °C.

### *Confocal microscopy*

For confocal microscopy, 48–52 hpf Tg(*flk1:EGFP;gata1:dsRED*) embryos previously treated with ATV or DMSO were embedded in 1% low melting agarose in embryo medium and tricaine mesylate (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich). A rendered Z-stack, at ~80 μm thickness each, was taken with a Zeiss LSM 510 AxioImager.M1 confocal microscope using an Achroplan 40 × /0.8 W objective with an argon laser (488 nm) and a helium-neon laser (543 nm).

### *Morpholino design, resuspension and microinjection*

A splice-modifying antisense morpholino oligonucleotide against *hmgcrb* pre-mRNA (*hmgcrb* MO), a translation blocking morpholino oligonucleotide targeting the AUG initiation site of