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Mutation of *FOXC1* and *PITX2* induces cerebral small-vessel disease

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Patients with cerebral small-vessel disease (CSVD) exhibit perturbed end-artery function and have an increased risk for stroke and age-related cognitive decline. Here, we used targeted genome-wide association (GWA) analysis and defined a CSVD locus adjacent to the forkhead transcription factor *FOXC1*. Moreover, we determined that the linked SNPs influence *FOXC1* transcript levels and demonstrated that patients as young as 1 year of age with altered *FOXC1* function exhibit CSVD. MRI analysis of patients with missense and nonsense mutations as well as *FOXC1*-encompassing segmental duplication and deletion revealed white matter hyperintensities, dilated perivascular spaces, and lacunar infarction. In a zebrafish model, overexpression or morpholino-induced suppression of *foxc1* induced cerebral hemorrhage. Inhibition of *foxc1* perturbed platelet-derived growth factor (Pdgf) signaling, impairing neural crest migration and the recruitment of mural cells, which are essential for vascular stability. GWA analysis also linked the FOXC1-interacting transcription factor *PITX2* to CSVD, and both patients with *PITX2* mutations and murine *Pitx2*$^{-/-}$ mutants displayed brain vascular phenotypes. Together, these results extend the genetic etiology of stroke and demonstrate an increasing developmental basis for human cerebrovascular disease.

Introduction

Stroke is a leading cause of morbidity and mortality, whose prevalence increases dramatically with age. Despite its substantial heritable basis, only a small number of causative genes have so far been identified, generally for severe early-onset phenotypes (cerebral autosomal dominant or recessive arteriopathy with subcortical infarcts and leukoencephalopathy: CADASIL [*NOTCH3*], CARASIL [*HTRA1*], and porencephaly [*COL4A1*]) (1–3). Such cases have revealed important pathways that contribute to stroke, including the roles of Notch and TGF-β signaling. In the same way, the vascular basement membrane's contribution (*COL4A1* and *COL4A2*) (3, 4) to juvenile stroke phenotypes further stimulated investigation of the cellular components (endothelial and mural cells) upon which brain vascular integrity depends. The demonstration that Notch signaling regulates pericyte numbers (5, 6) has in turn provided a mechanistic explanation for disorders such as CADASIL. These examples of juvenile stroke resulting from severe alterations in brain vascular development raise the intriguing possibility that milder changes contribute to late-onset disease and that a larger proportion of strokes have embryonic origins. It is therefore notable that the same genes regulate cerebral structural development and angiogenesis (7) and that the cell populations essential for cerebral vascular homeostasis (pericytes and vascular smooth muscle) are predominantly derived from the neural crest (8, 9).

The increasing prevalence of stroke exerts disproportionately severe effects on the quality of life of affected individuals and their families. Consequently, phenotypes predictive of future stroke merit investigation, with the goal of developing treatments targeting causative pathways and preventing a frequently preterminal disease. One such phenotype is cerebral small-vessel disease (CSVD), which represents a major risk factor for both ischemic and hemorrhagic stroke (10–13). Characterized by perturbed perforating end-artery function, CSVD results in lesions apparent on MRI that encompass white matter hyperintensities (WMHs), dilated perivascular spaces, microbleeds, and lacunar infarcts. These markers of cerebrovascular pathology provide opportunities for gene discovery and for defining the mechanisms that contribute to subsequent stroke.

Our study evaluated the hypothesis that the forkhead box transcription factor *FOXC1*, which patterns multiple organs including the CNS, contributes to CSVD. It was prompted by a higher incidence of self-reported stroke in some of our local pedigrees with *FOXC1* mutations and supported experimentally by: (a) blood-stained hydrocephalus in murine *Foxc1*$^{-/-}$ mutants, (b) related zebrafish *foxc1* morphant phenotypes, and (c) the exten-

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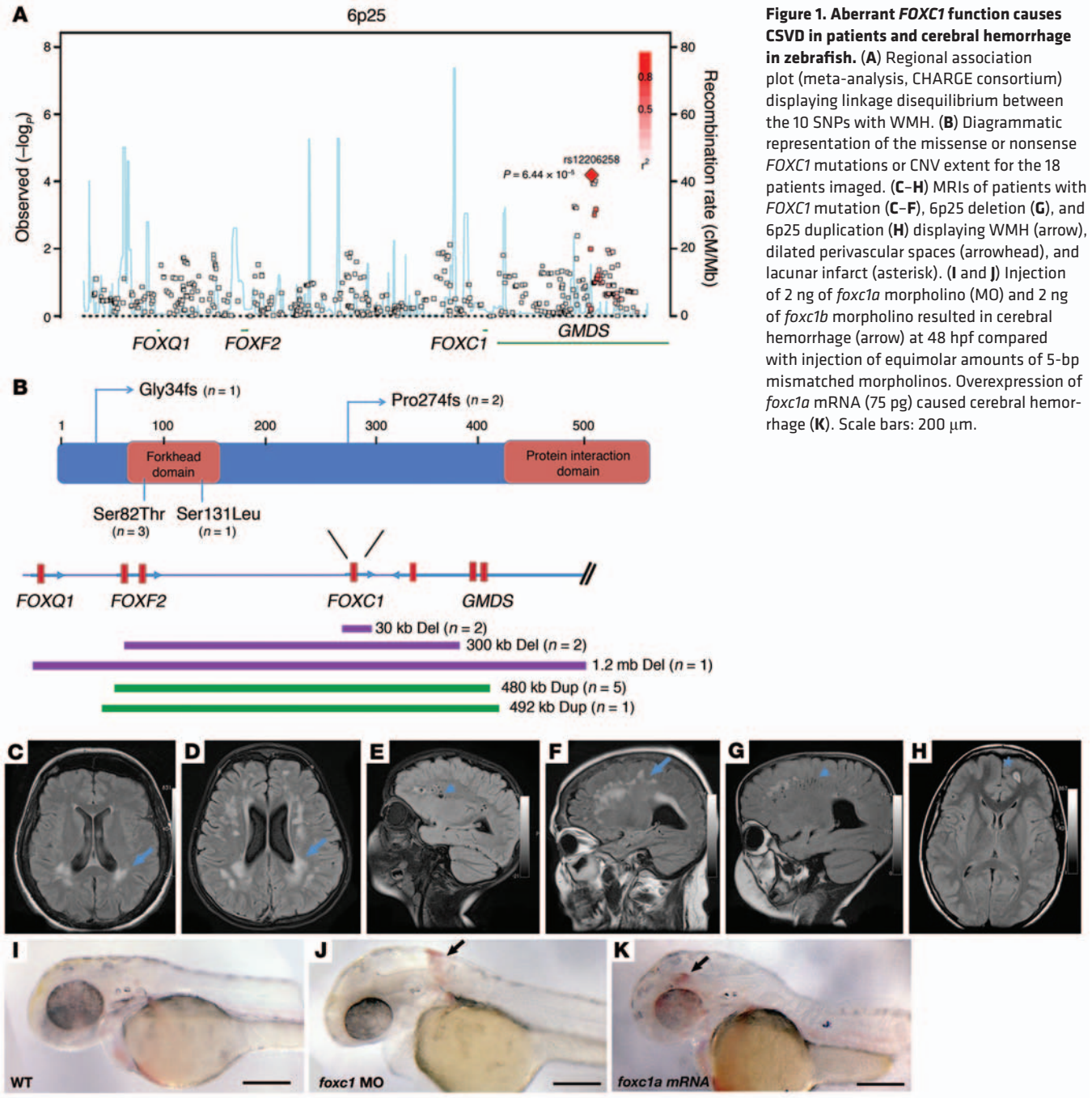

Figure 1. Aberrant *FOXC1* function causes CSVD in patients and cerebral hemorrhage in zebrafish. (**A**) Regional association plot (meta-analysis, CHARGE consortium) displaying linkage disequilibrium between the 10 SNPs with WMH. (**B**) Diagrammatic representation of the missense or nonsense *FOXC1* mutations or CNV extent for the 18 patients imaged. (**C**–**H**) MRIs of patients with *FOXC1* mutation (**C**–**F**), 6p25 deletion (**G**), and 6p25 duplication (**H**) displaying WMH (arrow), dilated perivascular spaces (arrowhead), and lacunar infarct (asterisk). (**I** and **J**) Injection of 2 ng of *foxc1a* morpholino (MO) and 2 ng of *foxc1b* morpholino resulted in cerebral hemorrhage (arrow) at 48 hpf compared with injection of equimolar amounts of 5-bp mismatched morpholinos. Overexpression of *foxc1a* mRNA (75 pg) caused cerebral hemorrhage (**K**). Scale bars: 200 μm.

sive involvement of Foxc1 in vascular development (14–16). The latter encompasses essential roles in arterial specification, angiogenesis regulation, endothelial lymphatic cell sprouting (17), as well as a requirement for Foxc1 in brain pericytes (18). Here, we demonstrate a role for FOXC1 in cerebrovascular disease through targeted genome-wide association (GWA) analysis, MRI of glaucoma patients with *FOXC1*-attributable Axenfeld-Rieger syndrome (ARS), and detailed zebrafish analyses.

Results and Discussion

We first performed a meta-analysis of GWA data for 500 kb encompassing *FOXC1* on 6p25 in 9,361 individuals with brain MRI data from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. This identified 10 WMH-associated SNPs (P = 0.0031–0.048, Bonferroni-corrected) located in an intron of *GMDS*, which catalyzes GDP-mannose metabolism and lies adjacent to *FOXC1* (Figure 1A and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI75109DS1). Analysis of 2 independent expression quantitative trait loci (eQTL) datasets (19, 20) demonstrated that 3 of these WMH-associated SNPs strongly influence *FOXC1* transcript levels (study 1: $P = 2.96 \times 10^{-8}$–5.82×10^{-11}; study 2: P = 0.01–0.008; Supplemental Table 1).

We next assessed whether patients with *FOXC1*-attributable ARS exhibited CSVD. Eighteen patients with either a *FOXC1* mutation or copy number variation (CNV) (comprising missense

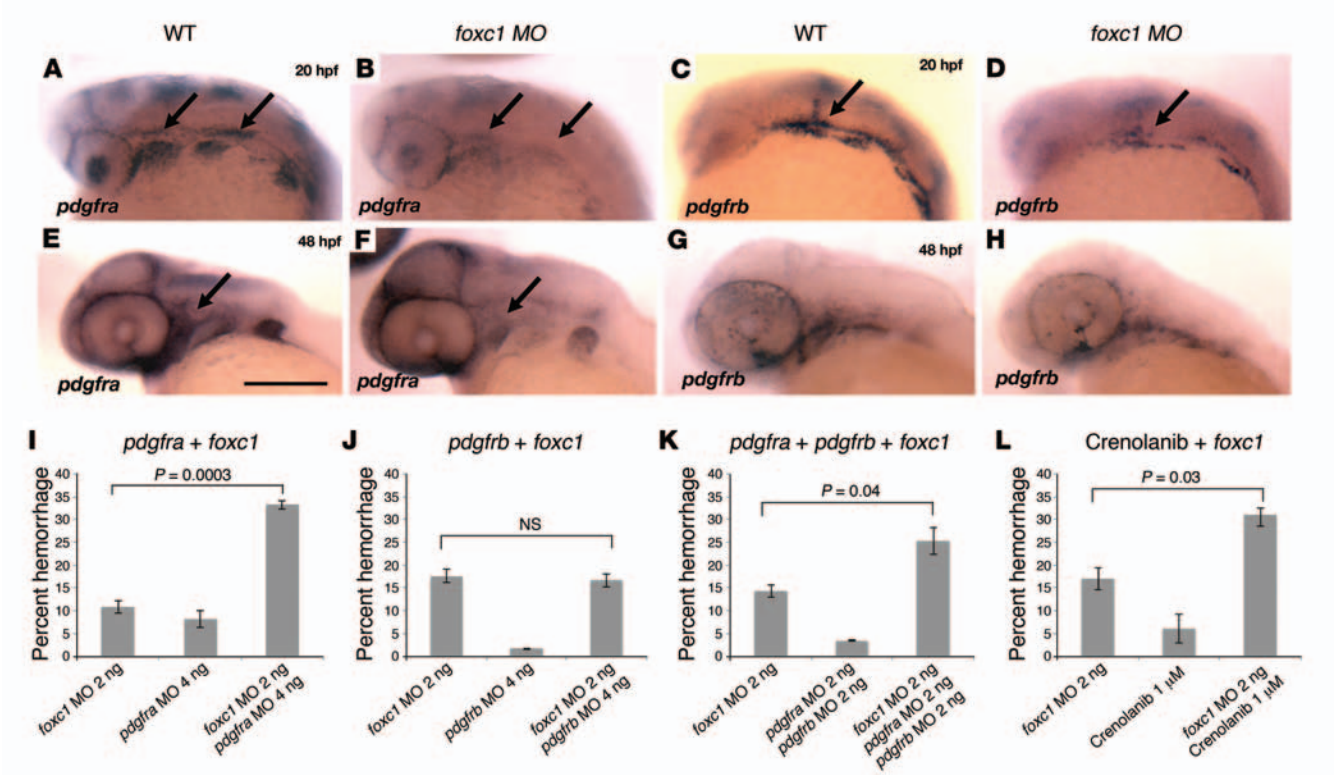

Figure 2. Foxc1 regulates Pdgf signaling. Transcripts for *pdgfra* (Pdgfrα) and *pdgfrb* (Pdgfrβ) were expressed in neural crest cells at 20 hpf (arrows) in WT embryos (**A** and **C**) and were highly downregulated in *foxc1* morphants (**B** and **D**). At 48 hpf, expression of *pdgfra* was observed in major cerebral blood vessels and ventral head mesenchyme (**E**, arrow), with the latter being reduced in *foxc1* morphants (**F**), whereas *pdgfrb* expression was unaltered (**G** and **H**). A genetic interaction was observed between subthreshold inhibition of *foxc1* and *pdgfra* (**I**) or with treatments that affected both Pdgfrα and Pdgfrβ signaling, including dual-morpholino suppression (**K**) and the pan-Pdgfr inhibitor crenolanib (**L**). No interaction was observed using subthreshold inhibition of *foxc1* and *pdgfrb* alone (**J**). Scale bar: 200 μm.

[$n = 4$] or frameshift mutation [$n = 3$], segmental deletion [$n = 5$], or duplication [$n = 6$]) (Figure 1B) were recruited for cerebral MRI (Supplemental Table 2). All 18 displayed CSVD, including WMH, dilated perivascular spaces, and lacunar infarcts (Figure 1, C–H, Supplemental Figure 1, and Supplemental Table 2). These changes, present in patients as young as 1 year of age, reveal a developmental component to CSVD. Cerebrovascular phenotypes were evident with both 6p25 segmental duplication or deletion, demonstrating that deviations from normal *FOXC1* gene dosage result in similar anomalies, as previously observed with *FOXC1*-dependent cerebellar maldevelopment (21), corneal neovascularization (22), and ARS (23, 24). The 7 patients with coding mutations unambiguously demonstrate that impaired *FOXC1* function alone is sufficient to induce CSVD.

Consistent with these data, overexpression of zebrafish *foxc1a* or dual suppression of both paralogs (*foxc1a* and *foxc1b*) results in cerebral hemorrhage (overexpression 32 of 111 [28.8%], suppression 111 of 352 [31.5%]; Figure 1, I–K, and Supplemental Table 3). Notably, acellular perivascular spaces were evident on electron microscopy of *foxc1* morpholino oligonucleotide–treated embryos (*foxc1* morphants) (Supplemental Figure 2). *Foxc1* was expressed in the neural crest, and in morphants, there was aberrant migration of the cerebral neural crest from which most mural cells are derived (Supplemental Figures 3 and 4). Since platelet-derived growth factor (PDGF) signaling regulates neural crest recruitment to the developing vasculature, we evaluated this candidate pathway in *foxc1* morphants. We observed reduced expression of both receptor tyrosine kinases (*pdgfra* and *pdgfrb*) in *foxc1* morphants (Figure 2, A–H), positioning Pdgf signaling genetically downstream of Foxc1. Importantly, the prevalence of cerebral hemorrhage induced by morpholino inhibition of *pdgfra* alone, or pharmacological inhibition of *pdgfra* and *pdgfrb*, synergized with *foxc1* morpholino inhibition (Figure 2, I–L). This is consistent with a model in which Foxc1 regulates vascular stability through the Pdgfrαα homodimer and Pdgfrαβ heterodimer, either by the control of *pdgfra* and *pdgfrb* expression or indirectly as a consequence of aberrant neural crest migration and/or survival.

Since loss of neural crest–specific *Pdgfra* induces murine cerebral hemorrhage and irregular vascular smooth muscle cell coverage (25, 26), we predicted loss of such cells in *foxc1* morphants. In keeping with the aberrant cerebral neural crest migration and increased cell death (Supplemental Figure 4), *foxc1* morphants exhibited reduced numbers of neural crest cells associating with the cerebral vasculature at 32 hours post fertilization (hpf) ($foxc1^{MO}$ 1.6 ± 0.5, WT 2.2 ± 0.4; $P = 0.0008$) (Figure 3, A–C). Consistently, at 4 days post fertilization (dpf), this manifested as reduced numbers of vascular smooth muscle cells ($foxc1^{MO}$ 48 ± 10, WT 61 ± 9; $P = 0.01$) (Figure 3, D–F). In contrast, we found that expression of markers for other vascular components (*col4a1* and *claudin5b*) and endothelial cell numbers in both morphants and murine endothelial–specific $Foxc1^{-/-}$ mutants was unaltered (Supplemental Figure 5). Together, these data support a model of reduced neural crest–derived smooth

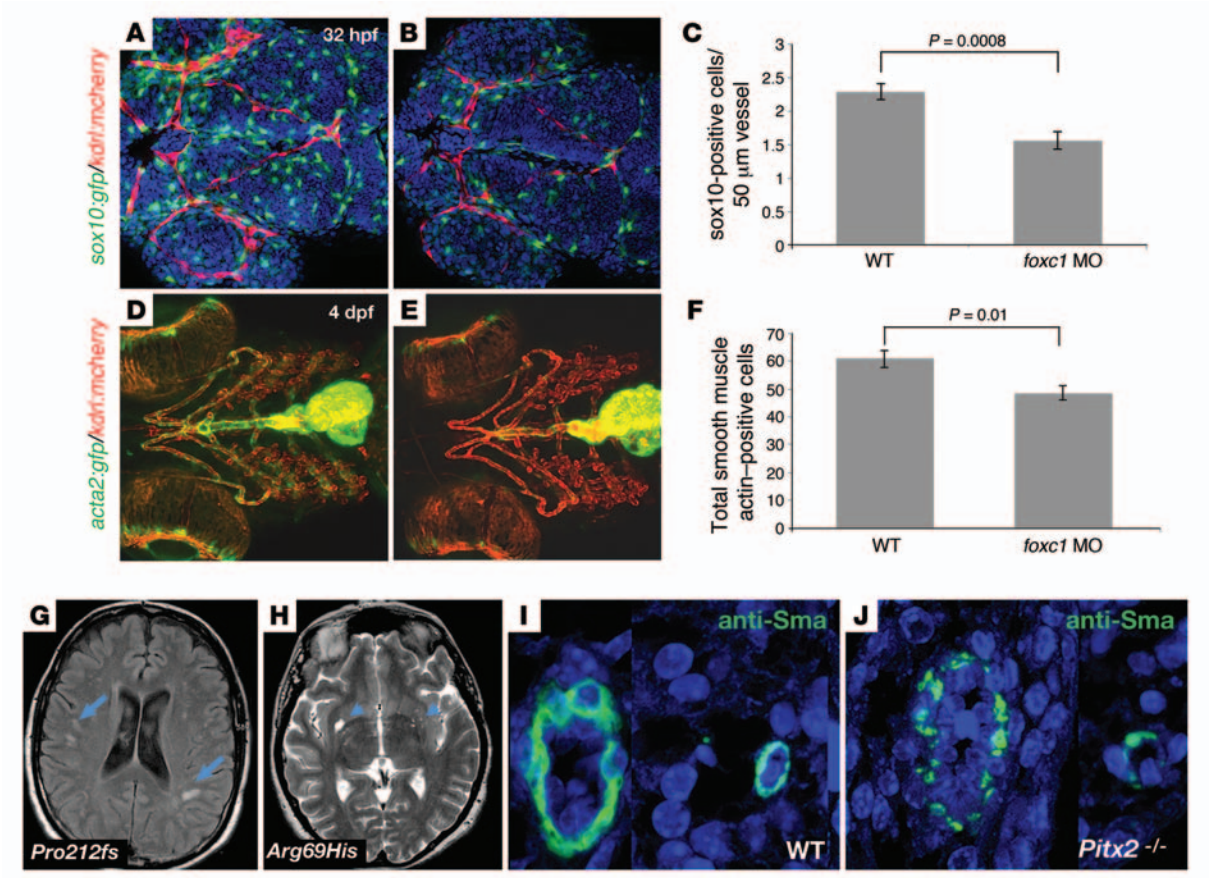

Figure 3. *foxc1* and *PITX2* regulate vascular smooth muscle cell numbers. Number of *sox10*-positive neural crest cells that associated with the cerebral vasculature in WT embryos at 32 hpf (**A**) was significantly reduced in *foxc1* morphants (**B** and **C**). Cerebral vascular mural cells expressed smooth muscle actin by 4 dpf (**D**), with fewer smooth muscle actin–positive cells observed in *foxc1* morphants (**E** and **F**). Patients with *PITX2*-attributable ARS exhibited CSVD: (**G**) WMH, (arrows) and (**H**) dilated perivascular spaces (arrowheads). Compared with WT embryos (**I**), murine *Pitx2*$^{-/-}$ mutants demonstrated reduced and discontinuous smooth muscle actin staining of large and small cerebral vessels (**J**). Original magnification, ×200 (**A** and **B**), ×100 (**D** and **E**), and ×400 (**I** and **J**).

muscle cell coverage, impairing vascular stability (27), as a key component of *foxc1* morphants' hemorrhagic phenotype.

To test the hypothesis that other ocular developmental genes with neural crest roles contribute to CSVD, we analyzed regional GWA data for *PITX2*, a neural crest–expressed, ARS-causing transcription factor that physically interacts with FOXC1. *PITX2* is associated with atrial fibrillation (28) and cardioembolic stroke (29), with the latter attributed to cardiac arrhythmia. Nine SNPs within a 500-kb *PITX2*-encompassing interval were significantly associated with WMH (P = 0.0071–0.022, Bonferroni-corrected; Supplemental Table 4 and Supplemental Figure 6, and MRIs of young *PITX2*-attributable ARS patients revealed CSVD (Figure 3, G and H). Thus, *PITX2* may increase stroke risk independently of atrial fibrillation. Consistent with such a primary alteration to cerebral vasculature, murine *Pitx2*$^{-/-}$ mutants exhibited reduced and discontinuous smooth muscle actin staining of cerebral vessels (Figure 3, I and J) as well as increased cerebral vessel density (*Pitx2*$^{-/-}$ 12.2 ± 0.8, WT 9.3 ± 1.0; P = 0.04).

By demonstrating that aberrant FOXC1 function causes human cerebrovascular disease, this study extends the knowledge of disorders whose genetic etiology remains largely unexplained. Our data from patients and zebrafish models, coupled with another laboratory's detailed analysis of murine *Foxc1* mutants (18), reveal the contribution from altered neural crest function and substantially increase the proportion of strokes known to have developmental origins. Furthermore, the evidence presented here from Foxc1 and Pitx2 implicates other transcription factors with neural crest roles as candidates and thus provides practical opportunities for accelerating the identification of the molecular basis for stroke through integrated human genetic and zebrafish analyses. Our observation of a predominantly mural role for Foxc1 in the cerebral vasculature, which contrasts with an endothelial cell contribution systemically (16), also merits investigation and may correlate with the unique endothelial barrier properties of the cerebral circulation. From a clinical perspective, the substantial interval that elapses between the onset of MRI-detectable features of CSVD and the occurrence of stroke provides a therapeutic window for intervention, and potentially, patients with mutations involving neural crest genes may benefit from common stroke-prevention strategies. Finally, our findings have direct implications for Axenfeld-Rieger syndrome, a glaucoma subtype frequently associated with progressive visual decline despite surgical control of intraocular pressure (30). Evidence of a cerebral vasculopathy raises the possibility that perturbed vascular function contributes to the visual loss that has previously been attributed to optic nerve disease.

Methods

Statistics. GWA data are presented as the $-\log_p$ value, with Bonferroni's correction for the number of independent comparisons determined via the number of linkage disequilibrium blocks. For analysis of cell numbers and genetic interactions, unpaired 2-tailed Student's *t* tests were used to assess significance. Analyses are displayed graphically as the mean ± SEM. All experiments were conducted in triplicate. A *P* value of less than 0.05 was considered statistically significant.

Study approval. Ethical approval was provided by the University of Alberta Health Research Ethics Board, with written informed consent received from all participants prior to their inclusion in the study. Animal experiments were approved by the IACUC of the University of Alberta, the University of Michigan, and Northwestern University.

Further details regarding the methods are available in the Supplemental Methods.

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