ABSTRACT

Roles of Zic2 and Zic3 in early development

The Hedgehog (Hh) signaling pathway plays a crucial role in modulating embryonic development. Malfunctions in the vertebrate Hh pathway involving Sonic Hedgehog Homolog (Shh) have been linked to cancers including basal cell carcinoma and developmental disorders like holoprosencephaly. Zic3, a zinc-finger transcription factor, is hypothesized to activate Shh-mediated Hh signaling. This is based on data demonstrating ZIC3 often binds to GLI consensus motif, and Zic3-depleted embryos express lower levels of Shh. To test this hypothesis, a line of zebrafish embryos carrying a nonsense mutation of Zic3 was examined for morphology of the forebrain and retina. Unexpectedly, no visible defects were found in embryos homozygous for the mutant allele through five days of development, and an expression assay of three Hh pathway genes (Shh, Hhip, and GlI2a) via in situ hybridization in Zic3 mutants showed normal patterns of Hh target expression. These data suggest Zic3 has redundant functions or gene compensation is occurring, in comparison to Zic3-depleted embryos.

In a parallel approach, we are investigating Axl1, a candidate target of Zic2. Axl1 is strongly implicated in the development of neural crest (NC) cells, as is the Zic gene family. Zic2 and Axl1 promote specification of NC cells. Pericytes, a mural cell type important for vascular development, are one of many cell lineages derived from NC cells. Zic2 mutants exhibit hemorrhage indicative of aberrant vasculogenesis, which may be due to aberrant pericyte formation. To test this hypothesis, immunohistochemistry was used to assay expression of Pdgfrα, a gene expressed in pericytes, in Axl1 morpholino-injected embryos. Analysis of the dorso-medial midbrain-hindbrain boundary found smaller quantities of pericytes within Axl1-depleted embryos than in controls, suggesting Axl1 has an impact on migration, differentiation, or a combination of both factors on pericyte development. This study has provided potential insight into Zic gene family function in early development and may enhance understanding of diseases associated with it.

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Roles of Zic2 and Zic3 in early development

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Abstract

The Hedgehog (Hh) signaling pathway plays a crucial role in modulating embryonic development. Malfunctions in the vertebrate Hh pathway involving Sonic Hedgehog Homolog (Shh) have been linked to cancers including basal cell carcinoma and developmental disorders like holoprosencephaly. Zic3, a zinc-finger transcription factor, is hypothesized to activate Shh-mediated Hh signaling. This is based on data demonstrating ZIC3 often binds to GLI consensus motif, and Zic3-depleted embryos express lower levels of Shh. To test this hypothesis, a line of zebrafish embryos carrying a nonsense mutation of Zic3 was examined for morphology of the forebrain and retina. Unexpectedly, no visible defects were found in embryos homozygous for the mutant allele through five days of development, and an expression assay of three Hh pathway genes (Shh, Hhip, and Gli2a) via in situ hybridization in Zic3 mutants showed normal patterns of Hh target expression. These data suggest Zic3 has redundant functions or gene compensation is occurring, in comparison to Zic3-depleted embryos.

In a parallel approach, we are investigating Alx1, a candidate target of Zic2. Alx1 is strongly implicated in the development of neural crest (NC) cells, as is the Zic gene family. Zic2 and Alx1 promote specification of NC cells. Pericytes, a mural cell type important for vascular development, are one of many cell lineages derived from NC cells. Zic2 mutants exhibit hemorrhage indicative of aberrant vasculogenesis, which may be due to aberrant pericyte formation. To test this hypothesis, immunohistochemistry was used to assay expression of Pdgfrb, a gene expressed in pericytes, in Alx1 morpholino-injected embryos. Analysis of the dorso-medial midbrain-hindbrain boundary found smaller quantities of pericytes within Alx1-depleted embryos than in controls, suggesting Alx1 has an impact on migration, differentiation, or a combination of both factors on pericyte development. This study has provided potential insight into Zic gene family function in early development and may enhance understanding of diseases associated with it.
Introduction

Zic3 as a candidate activator of Sonic Hedgehog Homolog-mediated Hedgehog signaling

The Hedgehog (Hh) signaling pathway has been established as an important regulator of embryonic development in both vertebrates and invertebrates. Sonic Hedgehog Homolog (Shh) is the best-characterized member of the vertebrate Hh pathway and is linked to a variety of diseases. Studies have implied there are numerous regulators of Shh, many of which are still unknown (Tucker and Caspary, 2013).

Roles for Shh have been well established in a range of processes, such as left-right (L-R) asymmetry and neural patterning (Ingham and McMahon, 2001). Studies have also linked malfunctions in the pathway to cancers, such as basal cell carcinoma, and developmental disorders like holoprosencephaly (Ming et al., 1998). In the last twenty years, the fundamental molecular basis of the pathway has been characterized.

After translation, SHH protein undergoes autocatalytic processing, producing an N-terminal signaling domain to which cholesterol is added. SHH initiates either autocrine or paracrine signaling, the latter of which requires the Dispatched (DISP) protein and can be short or long range. After reaching the target cell, SHH binds to the Patched-1 (PTCH1) receptor, relieving inhibition of Smoothened (SMO) protein. This leads to activation of GLI transcription factors, modulating transcription of target genes. Hhip, a target gene of the Hh pathway, inhibits Hh signaling by binding and sequestering SHH ligand (Ingham and McMahon, 2001). Zic3 has been proposed to regulate a component of this mechanism (Winata et al., 2013).

Zic3 was classified as a Zinc Finger of the Cerebellum gene due to its conservation of the zinc finger motif found in Zic1 and because of Zic3's expression in the cerebellum of adult mice (Aruga et al., 1996). It has been identified as a critical component of embryonic development in processes such as ensuring fidelity of embryonic patterning, positioning the primitive streak, and initiating gastrulation (Ware et al., 2006; Sutherland et al., 2013). Another member of the Zic family, Zic2α, has been identified by our lab as a modulator of Hh signaling in the forebrain and
retina (Sanek et al., 2009). In a study by Winata et al. (2013), ZIC3 frequently bound to the GLI consensus binding motif in zebrafish embryos when performing a de novo motif search using ChIP-Seq, at both 8 and 24 hours post fertilization (hpf). Additionally, the ZIC3 consensus binding motif was near the GLI motif in half of the ZIC3 binding peaks observed. These data suggest competition, or some allosteric regulation, between ZIC3 and GLI for modulating transcription of target genes. There was some conservation of these sites with humans (Winata et al., 2013). Additionally, a Zic3 microarray expression analysis found four genes integral to the Hh pathway differentially expressed in Zic3-depleted embryos: Shh, Shhb, and Gli2a, differentially expressed at 24 hpf, and Hhip, regulated at 8 hpf. Both Shh and Shhb were upregulated, indicating Zic3 is an activator of Shh-mediated Hh signaling. Morphologically, Zic3 morpholino-depleted embryos exhibited defects in gastrulation and convergent-extension (C-E). A study by Cast et al. (2012) found similar gastrulation and C-E defects, as well as L-R asymmetry defects in zebrafish embryos with loss of function Zic3. Quinn et al. (2012) found evidence suggesting ZIC3 upregulates Hh signaling through activation of Gli3 and Shh.

Synthesizing the literature indicates Zic3 acts as an activator of the Hh pathway involving the SHH ligand in a conserved manner. Another Zic family member, Zic2a, is a known regulator of Shh-mediated Hh signaling (Sanek et al., 2009). Zic3 binds to partially conserved GLI consensus motif sites and regulates genes in the Hh pathway (Winata et al., 2013). In order to further test the hypothesis put forth by Winata et al. that Zic3 is a candidate activator of the Shh-mediated Hh pathway, I used a newly derived mutant zebrafish line that carries a disruptive point mutation in the Zic3 locus. In contrast, Winata et al. used morpholino-mediated (MO) knockdown of Zic3, which may result in a different degree of developmental disruption than that caused by a chromosomal mutation (Bedell et al., 2011; Rossi et al., 2015; Kok et al., 2015).

Zic2 may regulate neural crest cell development through Alx1
Zic2, another member of the Zic gene family, has been implicated in the development of neural crest (NC) cells, a cell type arising from the ectoderm layer that can differentiate into numerous cell types, including melanocytes, craniofacial cartilage and bone, smooth muscle, and glia (Dupin and Sommer, 2012). Defects in neural crest cell development often manifest as craniofacial defects. These defects have been observed in both Zic2-depleted and Zic2 mutant zebrafish models (Tcs1aa et al., 2013; Roberson et al., in preparation).

RNAseq analysis performed in our lab identified Axl as a candidate target of Zic2 based on reduced level of its expression in Zic2a;Zic2b compound homozygous embryos (Roberson et al., in preparation). Axl encodes ALX homeobox 1, a transcription factor (McGonnell et al., 2011). Dee et al., 2012) showed craniofacial defects in Axl-depleted embryos that were similar to those observed in Zic2-depleted and Zic2 mutant models. This evidence suggests that Zic2 is regulates NC cell development through its transcriptional control of Axl expression.

The Grinblat lab has recently generated several Zic2 mutant compound lines, which carries mutant alleles at both the Zic2a and Zic2b loci. A proportion of Zic2a;Zic2b compound homozygotes exhibit hemorrhage indicative of aberrant vasculogenesis (see Fig. 1), which may be due to aberrant formation of pericytes, a mural cell type derived from NC (Etchever et al., 2001). Pericytes are located in microvessels (arterioles and capillaries) throughout the brain, eye, and kidneys (von Tell, et al. 2006). Pericytes, unlike their counterpart mural/smooth muscle cells, are not continuous. They exist as segregated cells embedded in the basement membrane of the vessels (Mandarino, et al., 1993). There are multiple roles for pericytes, including physically supporting vessels, secreting ECM, providing vascular tone, inducing vessel quiescence, supporting the blood brain barrier, promoting contractility to blood vessels, and preventing continued proliferation of endothelial cells once they have covered the vessel (Gaegnel et al., 2009; Armulik et al., 2010; Peppiatt et al., 2006; Benjamin et al., 1998).

During angiogenesis, endothelial cells attract perivascular mural cells via reciprocal signaling. Endothelial cells express Platelet-Derived Growth Factor, PDGF-B, which mural cells
receive via PDGF-Receptor β (PDGF-Rβ), inducing release of angiopoietin1 (Lindahl et al., 1997; Patan et al., 1998). Angiopoietin1 then binds to Tie2 receptors on endothelial cells to promote differentiation (Davis et al., 1996), resulting in two-layered cell formation. Pericytes are highly mobile and migrate along newly formed endothelial tubes (Stratman et al., 2009). Thus, it is plausible that defective pericytes formation is responsible for the hemorrhaging observed in Zic2 mutant embryos.

Methods

Zic3 mutant zebrafish genotyping assay

Zebrafish were maintained and embryos staged according to established protocols (Kimmel et al., 1995; Westerfield, 2000). A Zic3 line of zebrafish (sa13365) containing a C>A nonsense mutation in the second exon was obtained from the Sanger Institute Zebrafish Mutation Project. This line is established in the large zebrafish breeding facility the Grinblat lab maintains. This mutant allele encodes a truncated and likely non-functional ZIC3 protein (see Fig. 2). Zebrafish embryos and adults were genotyped to determine the presence of mutant vs wild type alleles of Zic3 using PCR, restriction enzyme cutting, and MetaPhor gel electrophoresis. Tail clips were obtained from adult fish according to Meeker et al. (2007). The forward primer sequence is 5'-TAAATGCAGTTCGATGCT-3'. The reverse primer has the sequence 5'-AGAGAGCTGGAGTGTTGTA-3'. The 25 μl PCR reaction contained GoTaq Flexi DNA and 5x Green GoTaq Flexi Buffer (Promega), 25 μM MgCl₂, 10 μM dNTPs, 200 nM of each primer, and 1 μl of the cDNA template. Components shared across reactions were initially mixed and then aliquoted to guarantee equivalent reaction conditions. The reaction was run on an Applied Biosystems Veriti Thermal Cycler as follows: denaturation at 95°C for one minute; amplification at 95°C for 20 seconds, 54°C for 30 seconds, and 72°C for 30 seconds (40x). The 30 μl restriction digest reaction used the DdeI restriction enzyme with a recognition sequence of 5'-CTNAG-3' (the proper sequence for a Restriction Fragment Length Polymorphism assay), the PCR reaction,
and CutSmart Buffer (NEB). As before, components shared across reactions were initially mixed and then aliquoted to guarantee equivalent reaction conditions. The reaction was incubated at 37°C for one hour. Only the mutant allele was cut, forming 114 nt and 21 nt bands, while the wild type allele presented with single a 135 nt band.

**Morphological assays of Zic3 mutant embryos**

Embryos were derived from a cross between heterozygous parents. They were examined for morphology of the forebrain and retina. Any observed developmental defects were tested for linkage to Zic3 mutation. To do this, embryos were lysed and genotyped as described above.

Gene expression was assayed via *in situ* hybridization (ISH). ISH was performed on 24-hour old embryos using antisense probes for *Shh*, *Hhip*, and *Gli2a*. Single-color ISH were carried out as previously described (Gillhouse *et al.*, 2004). Although Winata *et al.*, (2013) only noted *Hhip* regulation at 8 hpf, the forebrain and retina are not developed at that stage (Kimmel *et al.*, 1995). However, *Hhip* expression is seen in the forebrain at roughly 24 hpf (Hammond and Whitfield, 2009). *Shh* and *Gli2a* are expressed in the forebrain at 24 hpf (Kur *et al.*, 2011; Karlstrom *et al.*, 1999). *Shh* and *Hhip* produce secreted proteins that diffuse from the forebrain into the developing retina (Sanek *et al.*, 2009). Embryos displaying aberrant expression were genotyped according to the protocol mentioned above.

**Pericyte analysis**

*Tg(pdgfrb: citrine, acta2: mcherry)*, a double-transgenic line of zebrafish obtained from Nathan Lawson, University of Massachusetts-Amherst, were used to visualize pericyte expression, as *pdgfrb* expression is specific to pericytes (Armulik *et al.*, 2011). 1 nl microinjections according to previous methods (TeSlaa *et al.*, 2013) were performed of a 2:1 morpholino-mix of *Alx1l1e2*, designed to target the intron1-exon2 splice acceptor site 5'
CACGCTGAGGAGGTCAGAGAAAT-3’ (0.165 mM) and p53 (0.0825 mM), obtained from Gene Tools.

At five days post-fertilization (dpf), morpholino-injected and control embryos were fixed in 4% formaldehyde in PBS and stained using the following antibodies: Anti-green mouse green fluorescent antibody (1:500 from Millipore) and Alexa Fluor 488 conjugated goat anti-mouse secondary (1:1000). DAPI staining was performed at the time of secondary antibody staining (1:5000 from Invitrogen). Following staining, embryos were mounted with their dorsal side toward the coverslip in VectaShield Antifade Mounting Medium for Fluorescence (Vector Laboratories), and confocal images were taken on with a 40X lens on an Olympus FV1000 with FV10-ASW software (Olympus). The region of interest for pericyte quantification was the dorso-medial midbrain-hindbrain boundary, with pericytes being distinguished from background fluorescence based upon reference images as well as consistent localization through slices. The average number of pericytes was then taken, along with standard deviation.

Results

Zic3 mutants have no morphological defects or aberrant Hh-target expression

No embryos, observed for five days post-fertilization, produced by the Zic3 heterozygotes displayed any abnormalities (78/78). PCR confirmed there were mutants present among those scored.

Expression of all three genes analyzed, Shh (47/47), Hhip (37/37), and Gli2a (28/31) were normal (See Fig. 3). 3/31 Gli2a embryos displayed an aberrant expression pattern (see Fig. 3B), but PCR confirmed this expression was unlinked to Zic3.

Alx1-depleted embryos have fewer pericytes in the dorso-medial midbrain-hindbrain

Analysis of embryos at the dorso-medial midbrain-hindbrain boundary showed a significantly lower number of pericytes in Alx1-depleted embryos than in controls (See Fig. 4 and
5). All Alx1-depleted embryos appeared to have significantly smaller brains than controls, but this defect was not quantified.

Discussion

Zic3 may have redundant function or undergo gene compensation when nonfunctional

One probable explanation for normal development of Zic3 mutants is that Zic3 functions redundantly with other Zic gene family members. Zic redundancy has been suggested in other vertebrate models (Inoue et al., 2007), and overlapping Zic expression patterns have been observed in zebrafish (Grinblat and Sive, 2001). Alternatively, a compensatory mechanism may be activated in Zic3 mutants, which is not activated by morpholino-mediated knockdown. Rossi et al. (2015) has recently shown this to be the case in a line of egfl7 mutant zebrafish compared to their morphant counterpart. Both possibilities suggest defects seen as a consequence of Zic3 depletions are not solely due to Zic3 mutations, but rather reflect functional requirement for several Zic genes. Double mutant models including Zic3 with other Zics should be created in order to test these hypotheses.

Alx1 may be involved in differentiation, migration, or both aspects of pericyte development

Although the data are preliminary, the stark difference in the quantity of pericytes between the Alx1 morphants and controls suggests three possible interpretations. First, Alx1 could be modulating the expression of genes involved in pericyte differentiation, such as FoxD3 and Sox10, thus directly promoting pericyte formation. The second possibility is that Alx1 interferes with the proper development of vasculature such that the reciprocal signaling occurring between endothelial cells and pericytes is disrupted, thus causing improper migration of pericytes. Under this scenario, reduction in pericytes in Alx1 (and Zic2) mutant vertebrates should be accompanied by severely disrupted vasculature structure and integrity, perhaps across the entire body. Finally,
it is possible both factors are involved in Alx1's role in pericyte development, with similar implications as the latter interpretation.

In order to determine if there is differentiation failure, in situ hybridization with pdgfrb probes should be done on Alx1MO embryos at 3 dpf to see pericyte development at an earlier stage. There also should be studies conducted to determine if migration is affected using a fli1a transgenic, exclusively expressed on endothelial cells (Brown et al., 2000). Finally, experiments should be done to further strengthen the link between Zic2 and Alx1. Zic2 mutants should undergo the same immunohistochemistry and confocal imaging protocol as the Alx1 morphants, to determine if there are similar differences in pericyte numbers. Further, Alx1 should be overexpressed Zic2 mutants, to see if it rescues the hemorrhage.

In conclusion, this study has shed light on roles of the Zic gene family in early development, specifically with regards to their relationship to the Hh pathway as well as pericyte development, and has opened new avenues of research.

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References


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**Figures**

**Figure 1:** (A-B): *Zic3* compound mutants at 48 hpf A: Eye displaying coloboma B: Eye displaying coloboma and hemorrhage (cr. Laura Roberson, unpublished)

**Figure 2:** *Zic3* Mutant Allele Schematic
Figure 3: (A-D): mRNA expression of embryos following *in situ* at 24 hpf. A. *Gli2a* normal expression (28/31) B. *Gli2a* aberrant expression, unlinked to *Zic3* (3/31) C. *Hhip* normal expression (37/37) D. *Shh* normal expression (47/47)
Figure 4: (A-B): Confocal images of Tg(pdgfrb:citrine; acta2:mcherry) embryos at 5 dpf with pericytes showing at GFP, in DAPI background A. Control embryo B. Alx1/MO-injected embryo C. 5 dpf control embryo depicting region of interest (red box): Dorso-Medial Midbrain-Hindbrain Boundary

Figure 5: Pericyte quantification at dorso-medial midbrain-hindbrain boundary done from confocal images of Tg(pdgfrb:citrine; acta2:mcherry) embryos at 5 dpf with pericytes showing at GFP