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Planar cell polarity proteins differentially regulate extracellular matrix organization and assembly during zebrafish gastrulation

Michael R. Dohn [a,1], Nathan A. Mundell [a,1], Leah M. Sawyer [a], Julie A. Dunlap [a], Jason R. Jessen [a,b,*]

[a] Division of Genetic Medicine/Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA
[b] Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN 37232, USA

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ABSTRACT

Zebrafish gastrulation cell movements occur in the context of dynamic changes in extracellular matrix (ECM) organization and require the concerted action of planar cell polarity (PCP) proteins that regulate cell elongation and mediolateral alignment. Data obtained using *Xenopus laevis* gastrulae have shown that integrin–fibronectin interactions underlie the formation of polarized cell protrusions necessary for PCP and have implicated PCP proteins themselves as regulators of ECM. By contrast, the relationship between establishment of PCP and ECM assembly/remodeling during zebrafish gastrulation is unclear. We previously showed that zebrafish embryos carrying a null mutation in the four-pass transmembrane PCP protein *vang-like 2* (*vangl2*) exhibit increased matrix metalloproteinase activity and decreased immunolabeling of fibronectin. These data implicated for the first time a core PCP protein in the regulation of pericellular proteolysis of ECM substrates and raised the question of whether other zebrafish PCP proteins also impact ECM organization. In *Drosophila melanogaster*, the cytoplasmic PCP protein Prickle binds Van Gogh and regulates its function. Here we report that similar to *vangl2*, loss of zebrafish *prickle1a* decreases fibronectin protein levels in gastrula embryos. We further show that Prickle1a physically binds Vangl2 and regulates both the subcellular distribution and total protein level of Vangl2. These data suggest that the ability of Prickle1a to impact fibronectin organization is at least partly due to effects on Vangl2. In contrast to loss of either Vangl2 or Prickle1a function, we find that *glypican4* (a Wnt co-receptor) and *frizzled7* mutant gastrula embryos with disrupted non-canonical Wnt signaling exhibit the opposite phenotype, namely increased fibronectin assembly. Our data show that *glypican4* mutants do not have decreased proteolysis of ECM substrates, but instead have increased cell surface cadherin protein expression and increased intercellular adhesion. These data indicate that Wnt/Glypican4/Frizzled signaling regulates ECM assembly through effects on cadherin-mediated cell cohesion. Together, our results demonstrate that zebrafish Vangl2/Prickle1a and non-canonical Wnt/Frizzled signaling have opposing effects on ECM organization underlying PCP and gastrulation cell movements.

Introduction

Several morphogenetic cell movements operate during gastrulation to shape tissues into an embryo with recognizable anterior–posterior and dorsal–ventral body axes (Keller, 2005; Solnica-Krezel, 2005). Here, establishment of planar cell polarity (PCP) is required for polarized cell behaviors including directed migration and mediolateral intercalation (Keller, 2002; Jessen and Solnica-Krezel, 2005). Genes regulating PCP were first described in *Drosophila melanogaster* where they influence cell polarity in epithelial tissues such as the wing (Goodrich and Strutt, 2011). Vertebrate homologs of fly PCP genes regulate changes in gastrula cell morphology and orientation in relation to the dorsal embryonic midline (Jessen et al., 2002). Loss of function of the zebrafish (*Danio rerio*) PCP proteins Vang-like 2 (Vangl2) and Prickle1a disrupts cell polarity resulting in misshapen embryos that are shorter and broader than wild type (Solnica-Krezel et al., 1996; Sepich et al., 2000; Jessen et al., 2002; Carreira-Barbosa et al., 2003; Veeman et al., 2003). Other proteins required for PCP during zebrafish gastrulation include non-canonical Wnts and their co-receptor Glypican4, Frizzled receptors, and downstream components Disheveled and Rho family small GTPases (Jessen and Solnica-Krezel, 2005). Similar to the fly wing epithelium, it is thought that zebrafish Vangl2 and Prickle1a function to oppose or antagonize Frizzled/Disheveled signaling and thereby polarize cell behaviors (Roszko et al., 2009). However, molecular mechanisms underlying

* Corresponding author. Current address: Middle Tennessee State University, Department of Biology, P.O. Box 60, Murfreesboro, TN 37132, USA.
E-mail address: jason.jessen@mtsu.edu (J.R. Jessen).
[1] These authors contributed equally to this work.

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the establishment of PCP during gastrulation are unclear as are the functional relationships between Vangl2, Prickle1a, and components of the Wnt signaling pathway.

Gastrulation movements occur in the context of a dynamic extracellular matrix (ECM) network that is also capable of affecting PCP and morphogenesis and data suggest that PCP proteins themselves regulate ECM organization (Marsden and DeSimone, 2001; Goto et al., 2005; Dzamba et al., 2009; Latimer and Jessen, 2010). Regulation of ECM organization during gastrulation could influence a variety of polarized cell behaviors requiring cell–matrix interactions such as membrane protrusive activity (Davidson et al., 2006; Coyle et al., 2008). Knockdown of fibronectin expression causes PCP-like gastrulation phenotypes characterized by shortened and broadened embryos (Davidson et al., 2006; Latimer and Jessen, 2010). Work from *Xenopus laevis* has demonstrated that interactions between fibronectin and the α5β1 integrin receptor are essential regulators of polarized cell behaviors during gastrulation (Marsden and DeSimone, 2003). Notably, integrin activation suppresses inappropriate membrane protrusive activity at anterior–posterior cell domains thus contributing to cell polarization (Davidson et al., 2006). This indicates that proper cell–matrix interactions are essential for PCP. The concept that PCP proteins themselves could influence matrix organization was established when it was shown that ectopic expression of frog Van Gogh, Prickle, or Frizzled disrupts fibronectin fibril assembly on mesodermal tissue surfaces (Goto et al., 2005). The ability of these three disparate PCP proteins to cause apparently similar ECM phenotypes suggests that in frog, PCP proteins cooperate to affect polarized matrix assembly. Further work demonstrated that frog animal caps injected with dominant-negative Wnt11 fail to assemble fibronectin fibrils (Dzamba et al., 2009). This phenotype was rescued by co-injection with a Disheveled construct (minus the DIX domain) indicating that downstream non-canonical Wnt signaling is required for fibrillogenesis (Dzamba et al., 2009). Here, it was proposed that Wnt11 signaling affects fibronectin assembly by increasing cadherin adhesive activity and subsequently mechanical tension between integrins and fibronectin (Dzamba et al., 2009).

In contrast to frog, it is unclear how ECM assembly and remodeling during zebrafish gastrulation influence PCP. It is known that a fibronectin-containing ECM network is first detected near mid-gastrulation and separates epiblast tissue from the underlying hypoblast (Latimer and Jessen, 2010). As gastrulation proceeds, fibrillar fibronectin is assembled adjacent to mesodermal cells at both ectodermal and endodermal tissue interfaces (Latimer and Jessen, 2010). Notably, the time frame of increased ECM assembly during zebrafish gastrulation correlates with the onset of ectodermal and mesodermal PCP and increased directed cell migration (Jessen et al., 2002; Sepich et al., 2005). How might zebrafish PCP proteins regulate ECM assembly and remodeling during gastrulation? Our previous in vivo data implicated membrane type-1 matrix metalloproteinase (Mmp14) in the regulation of PCP and demonstrated a strong genetic interaction between zebrafish *mmp14* and *glypican4* (Coyle et al., 2008). Mmp14 is a member of the metzincin superfamily of zinc endopeptidases and is capable of cleaving a variety of ECM and non-ECM protein substrates (Sato et al., 1994; Zucker et al., 2003). ECM proteolysis by human MMP14 results in α5β1 integrin-mediated endocytosis and turnover of extracellular fibronectin (Shi and Sottile, 2011). MMP14 is activated intracellularly and endocytic and recycling pathways tightly regulate its expression at the cell surface (Remacle et al., 2003; Steffen et al., 2008). Therefore, regulation of cell surface Mmp14 proteolytic activity and ECM remodeling might provide a mechanism whereby migrating zebrafish gastrula cells influence the formation of polarized membrane protrusions. Indeed, utilizing migratory human cancer cells as a model, we determined that loss of VANGL2 function disrupts endocytosis of cell surface MMP14 causing increased pericellular proteolysis and invasion of ECM substrates (Cantrell and Jessen, 2010; Williams et al., 2012a, 2012b). We subsequently showed that zebrafish *vangl2*-null mutant embryos exhibit increased Mmp14 proteolytic activity and decreased immunolabeling for fibronectin in gastrula-stage embryos (Williams et al., 2012a). Together, our in vitro and in vivo findings support the hypothesis that Vangl2-dependent regulation of proteolysis and ECM organization is required for PCP.

The goal of the present study was to determine how loss of Prickle1a function and disruption of non-canonical Wnt signaling impact fibronectin assembly and to identify underlying mechanisms. Similar to Vangl2, we demonstrate that Prickle1a is required for fibronectin assembly during zebrafish gastrulation. We report that Prickle1a directly binds and positively regulates the expression of Vangl2. In contrast to Vangl2 and Prickle1a, we show that the non-canonical Wnt/Glypican4/Frizzled7 signaling branch of the PCP pathway inhibits fibronectin assembly through negative regulation of cadherin-mediated cell adhesion. Our results provide the first evidence that Vangl2/Prickle1a and non-canonical Wnt signaling function by distinct mechanisms to regulate ECM organization in the zebrafish embryo.

Materials and methods

Zebrafish husbandry and embryo manipulation

Adult zebrafish were maintained under standard conditions, and embryos were collected after natural spawnings. Embryos were raised in egg water (ultra pure water, 60 mg/L Instant Ocean) and staged according to morphological criteria (Kimmel et al., 1995). Strains utilized in this study: wild type (AB* and TL), *vangl2*/*trilobite*^{m209} (Solnica-Krezel et al., 1996; Jessen et al., 2002), *glypican4*/*knypek*^{m119} (Solnica-Krezel et al., 1996; Topczewski et al., 2001), and maternal-zygotic (MZ) *frizzled7a/7b* (Quesada-Hernandez et al., 2010).

Morpholinos, mRNA, and embryo microinjection

The *vangl2* (Williams et al., 2012a), *prickle1a* (Carreira-Barbosa et al., 2003), *mmp14a/b* (Coyle et al., 2008), and *N-cadherin*/*cdh2* (Lele et al., 2002) morpholinos (MOs) were obtained from Gene Tools, LLC and have been described previously. The *vangl2* and *mmp14a/b* MOs were validated by our lab and shown to cause both PCP and convergence and extension phenotypes in gastrula stage embryos (Coyle et al., 2008; Williams et al., 2012a). The *cdh2* and *prickle1a* MOs were validated by other groups and re-validated by our lab. In our hands, both of these MOs consistently produced loss of function phenotypes identical to the published phenotypes (Lele et al., 2002; Carreira-Barbosa et al., 2003; Veeman et al., 2003; Harrington et al., 2007; Warga and Kane, 2007). Typical MO doses were 5 ng (*vangl2*), 8 ng (*prickle1a*), 5 ng each (*mmp14a* and *mmp14b*), and 6 ng (*cdh2*) per embryo. Synthetic mRNA encoding full-length zebrafish Cdh2 and Prickle1a were generated from pCS2+ vector clones using Ambion's Sp6 mMessage mMachine kit. Single cell stage embryos were injected with mRNA and MO using standard procedures (Gilmour et al., 2002) and grown in egg water until they reached the appropriate stage.

Transmission electron and confocal microscopy

Tailbud stage wild type, *vangl2*, and *glypican4* mutant embryos were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dechorionated, and processed for TEM following standard procedures. Thick cut cross-sections (500 nm) were used to verify