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Roles for *Xenopus aquaporin-3b* (*aqp3.L*) during gastrulation: Fibrillar fibronectin and tissue boundary establishment in the dorsal margin

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ABSTRACT

Aquaporins and aquaglyceroporins are a large family of membrane channel proteins that allow rapid movement of water and small, uncharged solutes into and out of cells along concentration gradients. Recently, aquaporins have been gaining recognition for more complex biological roles than the regulation of cellular osmotic homeostasis. We have identified a specific expression pattern for *Xenopus aqp3b* (also called *aqp3.L*) during gastrulation, where it is localized to the sensorial (deep) layer of the blastocoel roof and dorsal margin. Interference with *aqp3b* expression resulted in loss of fibrillar fibronectin matrix in Brachet's cleft at the dorsal marginal zone, but not on the free surface of the blastocoel. Detailed observation showed that the absence of fibronectin matrix correlated with compromised border integrities between involuted mesendoderm and noninvoluted ectoderm in the marginal zone. Knockdown of *aqp3b* also led to delayed closure of the blastopore, suggesting defects in gastrulation movements. Radial intercalation was not affected in *aqp3b* morphants, while the data presented are consistent with impeded convergent extension movements of the dorsal mesoderm in response to loss of *aqp3b*. Our emerging model suggests that *aqp3b* is part of a mechanism that promotes proper interaction between cells and the extracellular matrix, thereby playing a critical role in gastrulation.

1. Introduction

Previous work on the regulatory role of *zic* family genes in early *Xenopus laevis* development identified an aquaporin gene, *aqp3b*, whose expression is directly regulated by the Zic1 transcription factor (Cornish et al., 2009). This suggested a potential role for *aqp3b* (also called *aqp3.L)* in early *Xenopus* development.

Aquaporins (Aqps) are a large family of membrane channel proteins that allow rapid movement of water into and out of cells along osmolality gradients. Aquaporins assemble as homotetramers within the cell membrane, with each pore-forming subunit acting independently (Jung et al., 1994; Hub and de Groot, 2008). Aquaporin 3 is an aquaglyceroporin, since it also allows passage of glycerol and other small solutes. Analysis of *Aqp3* knockout mice and cell culture analysis of *Aqp3*-deficient cells have suggested roles for this protein in cell migration (Levin and Verkman, 2006; Hara-Chikuma and Verkman, 2008; Stroka et al., 2014). Knockout mouse studies and *in vitro* studies have provided substantial evidence that *aquaporin* genes regulate cellular volume (Chen et al., 2005; Karlsson et al., 2013). Thus, it has been speculated that aquaporins may have roles in facilitating cell shape changes, which may affect both migration and adhesion (Zhang et al., 2014). However, little has been suggested with regard to the role of aquaporins during early development.

Fibronectin (FN) is a glycoprotein constituent of extracellular matrices. It is critical for the guidance of cell movements during embryonic development, such as mediolateral intercalation during convergent extension and radial intercalation during epiboly (Davidson et al., 2004, 2006). FN-deficient mice are not viable and display severe defects in mesoderm development (George et al., 1993; Georges-Labouesse et al., 1996).

In *Xenopus* embryos, FN fibrils first appear at the onset of gastrulation on the inner surface of the blastocoel roof (BCR) (Lee et al., 1984). Although soluble FN is present throughout the blastocoel and FN-interacting integrin receptors are expressed on all cells of the embryo, a fibrillar FN matrix forms only on the inside surface of the blastocoel roof (Lee et al., 1984). The reason for this is the higher cell-cell adhesion that exists on this surface, which generates the cell traction forces necessary for FN fibril assembly, thus limiting fibrillar FN matrix formation to the inner BCR surface (Dzamba et al., 2009).

As gastrulation commences, dorsal mesendoderm initiates movement by folding up along the inside surface of the blastocoel roof. Thus,

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the FN matrix comes to lie between the outer ectoderm and the involuting mesendoderm, and as a result, the FN matrix becomes a partition between ectoderm and mesendoderm (Lee et al., 1984). This region is called Brachet's cleft (Gorny and Steinbeisser, 2012) and is flanked by the dorsal marginal zone (DMZ). During gastrulation, mesendoderm cells in the dorsal blastopore lip (which constitutes the posterior tip of the DMZ) involute. The FN matrix acts as a guide for mesendoderm to migrate from the dorsal blastopore lip in an anterior direction along the inside surface of the blastocoel roof. In this process, the FN matrix provides directional cues towards the animal pole in *Xenopus* embryos and interfering with fibrillar FN assembly causes mesoderm cells to move in random directions (Winklbauer and Nagel, 1991).

Two morphogenetic processes, radial intercalation and convergent extension, help drive the advancement of the mesendoderm (Keller and Shook, 2008) and both are FN-dependent (Marsden and DeSimone, 2001, 2003; Davidson et al., 2006). During epiboly, when the blastocoel roof thins from 5 to 6 cell layers to only 2 cell layers (Keller, 1980), radial intercalation of the ectoderm requires the presence of fibrillar FN (Marsden and DeSimone, 2001; Rozario et al., 2009). Injecting embryos with peptides or antibodies that interfere with fibrillar FN assembly results in defects in blastopore closure, radial intercalation, and convergent extension (Marsden and DeSimone, 2001; Davidson et al., 2002, 2006; Rozario et al., 2009). Thus, fibrillar FN assembly plays a central role in the control of gastrulation movements.

In addition to FN binding to α5β1 integrins and cell tension exerted by cadherins (Wennerberg et al., 1996; Dzamba et al., 2009), FN fibril assembly requires Wnt/PCP signaling and focal adhesion kinase (FAK) (Zhong et al., 1998; Yoneda et al., 2007; Ilić et al., 2004; Dzamba et al., 2009). In vivo, assembly of thick FN matrices often coincides with periods of morphogenetic changes during development. However, it is not well understood, how assembly of this matrix is regulated. In this study, we have identified an essential role for *Xenopus* Aqp3b in the assembly of the fibrillar FN matrix and thus proper gastrulation. This effect was specific to areas in which the FN matrix was sandwiched between cell layers during gastrulation (Brachet's cleft). Further, radial intercalation of the ectoderm was unaffected in *aqp3b* morphants, while our data are consistent with a requirement of *aqp3b* for convergence of the axial mesoderm. Potential mechanisms through which Aqp3b may affect FN matrix assembly are discussed.

2. Materials and methods

2.1. Embryos and immunofluorescence

Embryos were obtained and cultured using standard methods and staged according to (Nieuwkoop and Faber, 1994). After fixation in MEMFA, embryos were hemisected through the blastopore lip using a thin razor blade. Bisected embryos were immunostained with combinations of mouse anti-FN mAb at 1:2000 (clone 4H2, generous gift from Dr. Douglas DeSimone, University of Virginia), chicken anti-EGFP polyclonal antibody at 1:500 (Abcam #ab13970), rabbit anti-β-catenin polyclonal antibody at 1:200 (Abcam #ab16051), and mouse anti C-cadherin and mouse anti-β1-integrin mAbs (Developmental Studies Hybridoma Bank, clones 6B6 and 8C8-c, respectively), both at 1:200. The secondary antibodies goat anti-mouse-Alexa-568 (Life Technologies, Carlsbad, CA), goat anti-chicken IgG-Alexa-488 (Life Technologies, Carlsbad, CA), and goat anti-rabbit-DyLight-633 (Thermo Scientific/Pierce Biotechnology) were used at 1:500. Phalloidin-Alexa-488 (Life Technologies) was used at 10 units/ml. Antibody incubations were performed in 1x PBS/0.1% Saponin/1% BSA. After staining, bisected embryos used for confocal microscopy were dehydrated in methanol (or isopropanol for phalloidin staining) and cleared overnight in Murray's Clear (Dodt et al., 2007).

2.2. Plasmid and sense RNA synthesis

The pCMVSPORT-*aqp3b* plasmid (Open Biosystems/GE Dharmacon, Lafayette, CO) was linearized with Sca1 and sense RNA transcribed with SP6 polymerase. *aqp3bMM* was constructed to contain only the coding region and five third position codon variations to render it unrecognizable by either *aqp3b* MO. The PCR primers used to construct *aqp3bMM* were (mismatched residues in lower case): FWD: 5'-CGGAATTC GGaCGtCAaAAaGAcTTcGTtAACAAATGTAACCAGATGCT-3' and REV: 5'-GGGCTCGAGTCAGATTCTGTCTTTGGGTTTG-3'. The PCR product was digested with *Eco*RI and *Xho*I and subcloned into *pCS2+ATG*, which contains a Kozak consensus sequence between the *Bam*HI and *Eco*RI sites of *pCS2+* (Merzdorf and Sive, 2006). The resulting *aqp3bMM* coding region starts at the Kozak sequence methionine and includes an additional glutamate and phenylalanine prior to the *aqp3b* coding region. The *mem-EGFP* plasmid was a gift from Dr. Jennifer Gutzman (University of Wisconsin-Milwaukee). The *mem-EGFP* and *aqp3bMM* plasmids were linearized with *Not*I and sense RNA was transcribed with SP6 polymerase. *lacZ* RNA was synthesized as previously described (Gammill and Sive, 1997).

2.3. Morpholino oligonucleotides and validation of their specificity

Two translation-blocking morpholino oligonucleotides were designed to *Xenopus aqp3b*: one spanning the translation start site (*aqp3b* MO1): 5'-CAAAATCCTTCTGGCGACCCATGTT-3' (start codon underlined) and the other in the 5'UTR (*aqp3b* MO2): 5'-TTGGCTGGATCTCAGTTTGTCCTTA-3' (Gene Tools, Philomath, OR). Specific control MOs containing 5 mismatched bases were designed for each *aqp3b* MO.

Since *aqp3b* is maternally expressed, we were unable to use splice-blocking MOs or CRISPR/Cas9 as alternative methods due to the lingering presence of maternal mRNA in early gastrula embryos (Blum et al., 2015). Thus, in order to demonstrate specificity, two different morpholinos were employed, both were rescued (Fig. 2), and synergism was demonstrated (Figs. 4 and 7). For these experiments, the amounts of MO were determined by titration. While 4 ng of each individual MO did not yield a phenotype, co-injecting 4 ng of each *aqp3b* MO resulted in the same phenotype as 16 ng of each MO alone. The ability of *aqp3b* MO1 to block translation of *aqp3b* sense RNA was tested in co-injection experiments, where antibody staining showed that translation of *aqp3b* RNA with intact MO recognition sequence (*aqp3b-HA*) was inhibited by *aqp3b* MO1, while translation of *aqp3bMM* was not inhibited by *aqp3b* MO1 (Fig. S1).

2.4. Microinjections

To target the dorsal marginal zone, one dorsal blastomere of 4-cell embryos was co-injected with 16 ng MO plus cell tracer [200 pg *mem-EGFP* RNA or 16.5 ng rhodamine-dextran (Life Technologies, Carlsbad, CA)]. Tailbud embryos were injected in both dorsal blastomeres of 4-cell embryos. For synergism experiments, both *aqp3b* MOs or control MOs were each used at ¼ of the normal concentration (4 ng of each MO for a total of 8 ng MO), along with tracer. For rescue experiments, the embryos were pre-injected at the 1-cell stage with 1 ng of *aqp3bMM* or *lacZ* (injection control) RNA, before injection at the 4-cell stage as described.

2.5. Whole mount in situ hybridization

Fixed albino embryos or bleached wild type embryos were analyzed by *in situ* hybridization (Harland, 1991) with digoxygenin-labeled *aqp3b* and fluorescein-labeled *otx2* antisense RNA probes or with digoxygenin-labeled *chordin* antisense probe or digoxygenin-labeled *aqp3b* sense probe. Probe synthesis was as described: *aqp3b* (Cornish *et al.*, 2006); *otx2* (Gammill and Sive, 1997), *chordin* (Sasai et al.,