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Xclaudin 1 is required for the proper gastrulation in *Xenopus laevis*

Da-Jung Chang [a], Yoo-Seok Hwang [b], Sang-Wook Cha [c], Jeong-Pil Chae [a], Sung-Hun Hwang [a], Jang-Hee Hahn [d], Yong Chul Bae [e], Hyun-Shik Lee [f], Mae Ja Park [a,*]

[a] Department of Anatomy, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[b] Laboratory of Molecular Genetics, NICHD, NIH, Bethesda, MD, USA
[c] Division of Developmental Biology, Department of Pediatrics, Cincinnati Children's Research Foundation, Cincinnati, OH 45229, USA
[d] Department of Anatomy and Cell Biology, School of Medicine, Kangwon National University, Chuchon 200-701, Republic of Korea
[e] Department of Oral Anatomy, School of Dentistry, Kyungpook National University, Daegu 700-412, Republic of Korea
[f] School of Life Sciences, College of Natural Sciences, Kyungpook National Univerity, Daegu 702-701, Republic of Korea

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ABSTRACT

Claudin 1 is one of the tight junctional proteins involved in the tight sealing of the cellular sheets and plays a crucial role in the maintenance of cell polarity. Although its structure and physiological function in intercellular adhesion is relatively well understood, we have little information about its possible involvement in early development of vertebrates. We found Xclaudin 1 is expressed maternally in the oocyte of *Xenopus laevis* and the zygotic expression initiates stage 9 in the animal hemisphere but not in the vegetal hemisphere, limited on the ectoderm and mesoderm until the end of gastrulation. We have investigated a potential role for claudin 1 at gastrulation by gain and loss-of-function studies. Overexpression of Xclaudin 1 resulted in gastrulation defect in a dose-dependent manner. Knockdown of Xclaudin 1 by antisense morpholino oligonucleotides (MOs) blocked convergent extension, whereas ectopic expression of Xclaudin 1-myc mRNA rescued these defects. However, altered expression of Xclaudin 1 did not inhibit mesodermal gene expression. Taken together, our results suggest that Xclaudin 1 is required for proper convergent extension movement during *Xenopus* gastrulation.

1. Introduction

In vertebrates, the body plan and the basic three germ layers are established during gastrulation. Embryonic cells are brought to their right location of each three germ layers through the gastrular movement. In *Xenopus*, gastrulation movements are diverse according to location of the embryonic cells. In vegetal region, endodermal cells initiate vegetal rotation movements prior to gastrulation, providing an essential force for gastrulation. Cells located animally to the blastopore lip undergo radial intercalation to make fewer cellular layers that move vegetally in a process called epiboly. Within the marginal zone, mesodermal and endodermal cells involute through the blastopore to internalize. Following involution, head mesoderm migrates animally along the blastocoel roof, while trunk mesoderm undertakes the convergent extension (CE) movement, in which cells polarize and elongate along the mediolateral axis and intercalate toward the midline. The highly orchestrated cell movements in different regions of the embryos require dynamic and integrated regulation of cell–cell and cell–matrix interactions, in which a variety of different molecules and signaling pathways might be involved since multiple signaling pathways have been known to be implicated in various aspects of cell movements during gastrulation [1,2].

Claudin 1 is a member of the claudin family proteins comprised of 24 members [3]. Claudins consist of four transmembrane domains, two extracellular loop regions that interact adhesively, and two intracellular domains [4]. Most tissues express multiple claudins with a tissue specific manner, which can interact in both homotypic and heterotypic fashion to form the tight junction strands [5]. Claudins have been known to play an important role in the maintenance of cell polarity and the epithelial tissue integrity as tight junction proteins [3]. In addition, they seem to play a crucial role in the regulation of cell movement, since their altered expression and phosphorylation led to the loss of adhesion of cancer cells and their progression to metastasis [6,7]. Interestingly, claudin 1 appears to be a cancer metastasis suppressor [8]. Overexpression of claudin 1 suppressed tumor cell migration, whereas knockdown of its expression increased cancer cell motility. Downregulation of claudin 1 underlies epithelial–mesenchymal transition (EMT), which is a key event not only in tumor metastasis, but also in embryonic development [9]. Taken together, these results suggest that claudin 1 may be involved in the EMT during gastrulation. However, possible involvement of claudin 1 in

* Corresponding author. Address: Department of Anatomy, School of Medicine, Kyungpook University, 101 Dongin-dong, Jung-gu, Daegu 700-422, Republic of Korea. Fax: +82 53 426 9085.
E-mail address: mjpark@knu.ac.kr (M.J. Park).

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dynamic movements of early embryonic cells has not been reported yet.

In the present study, we investigated roles of Xclaudin 1 during *Xenopus* gastrulation by injecting wild-type Xclaudin 1 mRNA and Xclaudin 1-specific antisense morpholino oligonucleotides into *Xenopus* blastomeres. This study showed that altered expression of Xclaudin 1 resulted in the inhibition of convergent extension movement during *Xenopus* gastrulation, suggesting that Xclaudin 1 is involved in the embryonic cell movement.

2. Materials and methods

2.1. Xenopus embryos manipulation

Xenopus laevis was purchased from *Xenopus* I and Nasco. Eggs were obtained from *X. laevis* primed with 800 U of human chorionic gonadotropin (LG). *In vitro* fertilization was performed as described previously (Newport and Kirschner, 1982), and developmental stages of the embryos were determined according to Nieuwkoop and Faber (1967). Microinjection was carried out in 0.33× Marc's Modified Ringers (MMR) with gentamycin (50 μg/ml) using a Nanoliter Injector (WPI) until they had reached the appropriate stage. The jelly coat was removed with thioglycolic acid (Sigma).

2.2. RT-PCR

Total RNA was extracted from whole embryos or cultured explants with TRIzol reagents (MRC) following the manufacturer's instructions (http://www.mrcgene.com/tri.htm). Reverse-transcription polymerase chain reaction (RT-PCR) was performed with a Revertaid cDNA synthesis kit (Fermentas). Primers used in this study were as follows: XClaudin 1 (forward; 5′-AAGGATGGCCAACG CAGGCT-3′ and reverse; 5′-CACATAATCTTTTCCAGCAG-3′), ornithine decarboxylase (ODC) (forward; 5′-CAGCTAGCTGTGGTGTGG-3′ and reverse; 5′-CAACATGGAAACTCACAC-3′).

Primers for *Chordin*, *Xbra*, *Goosecoid*, *Wnt 11* and *EF1α* were used as described online by De Robertis (www.hhmi.ucla.edu/derobertis/index.html).

2.3. In situ hybridization

Embryos to be used for in situ hybridization were incubated until the proper stages. The collected embryos were fixed with 1× MEMFA (0.1 M MOPS [pH 7.4], 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde, pH 7.4) and stored in ethanol at −20 °C until use.

Whole-mount in situ hybridization was performed as previously described with some modifications, BM purple AP substrate (Roche, Germany) was used (Sive, 1998). For *in vitro* riboprobe transcription, the Megascript kit (Ambion, Austin) was used following the manufacturer's Instructions (http://www.ambion.com/techlib/prot/fm_1340.pdf). Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled probes as described by Harland (1991). An antisense in situ probe of XClaudin 1 was generated by linearizing the pGEM-T XClaudin 1 construct with ClaI and transcribing with the T7 RNA polymerase. Hybridized RNAs were detected with an alkaline-phosphatase-conjugated anti-DIG-antibody (Roche, Germany) and developed using BM purple alkaline-phosphatase (Roche, Germany). Stained embryos were bleached with bleaching solution (1% H_2O_2, 5% formamide, 0.5× SSC [standard saline citrate]).

2.4. Construction of expression vector and microinjections

The open reading frame of XClaudin 1 was PCR amplified from the cDNA clone MGC:53308 IMAGE:5570517(NCBI) using the primers, forward; 5′-AAGGATGGCCAACGCAGGCT-3′ and reverse; 5′-CACATAATCTTTTCCAGCAG-3′ to produce XClaudin 1 mRNA. XClaudin 1 was cloned into the HindII and BamHI sites of the pCS2 + vector. The PCR primer pairs were as follow: forward; 5′-GACAAGGCCTGCTTCAGAGT-3′, reverse; 5′-CACATAATCTTTTCCAG-CAG-3′ for 5′-UTR MO un-target sequence and forward; 5′-AAG-GATGGCCAACGCAGGCT-3′, reverse; 5′-CACATAATCTTTTCCAGCA G-3′ for MO target sequence, respectively. For specificity assay of XClaudin 1 MO, XClaudin 1 was cloned into the BamHI and ClaI sites of the myc-pCS2 + vector. XClaudin 1-myc-pCS2 + was linearized with XhoI. Capped mRNAs were synthesized from linearized plasmids using the mMessagae mMachine kit (Ambion).

2.5. Morpholino oligonucleotide

Antisense morpholino oligonucleotide (MO) was obtained from Gene Tools. MO sequences were as follows: XClaudin 1 MO 5′-CAT-CCTTTGGAGTGGATAAAGTAAG-3′, Control MO 5′-CCTCTTACCTCAG TTACAATTTATA-3′.

2.6. DMZ elongation assay

Embryos were injected with mRNA into either dorsal marginal zone (DMZ) at the 4-cell stage embryos. DMZ explants were excised at stage 10.5 and were cultured in 1× MR containing 10 μg/ml of bovine serum albumin, 50 μg/ml of gentamycin and 5 μg/ml of streptomycin, until stage 18.

3. Results

3.1. Temporal and spatial expression of Xclaudin 1 during Xenopus development

In order to elucidate the presumptive role of claudin 1 in the developmental processes of amphibians, the spatiotemporal expression pattern of claudin 1 was analyzed during *Xenopus* embryogenesis following isolation of Xclaudin 1 cDNA from *Xenopus laevis* stage 12–24 embryos cDNA library. The temporal expression patterns of Xclaudin 1 gene were characterized by a reverse transcriptase-polymerase chain reaction analysis on whole embryos, using primers that amplify a fragment of the Xclaudin 1 cDNA that encodes a specific portion of the Xclaudin 1 gene. We detected that Xclaudin 1 was expressed maternally and that zygotic expression persists at all stage examined (Fig. 1A). Particularly, the level of Xclaudin 1 gene expression was low until midblastular transition (MBT), and then increased markedly after stage 9. This temporal expression pattern looks similar with that of claudin 4L or 7L during *Xenopus* embryogenesis [10].

Using in situ hybridization on whole embryos, we have analyzed the location of Xclaudin 1 expression (Fig. 1C–I). Comparing to previous data [10], spatial expression of Xclaudin 1 is very much alike with that of claudin 4L or 7L. That is, Xclaudin 1 transcript was localized in the animal hemisphere, but not in the yolk mass during the period from oocyte stage to blastula stage (Fig. 1C–F). At the gastrula stage, the transcripts were detected in the entire surface ectoderm (Fig. 1G). In the neural stage embryos, Xclaudin 1 was expressed along the dorsal midline of the neural plate and in the overall epidermal progenitor cell layer (Fig. 1H).

As the stage proceeded to tail-bud stage, expression of Xclaudin 1 became localized in the otic vesicles, the eye vesicles, the dorsal fin, the mesencephalon, the pronephros, pronephric duct and branchial arch (Fig. 1I). The expression pattern of Xclaudin 1 is similar to that of claudin-4L1, -4L2 and -7L1 during the tail-bud stage. Exceptionally, Xclaudin 1 expresses in the eye vesicle and dorsal fin where the expression of claudin 1-4L1, -4L2 and -7L1 was not