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

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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

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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

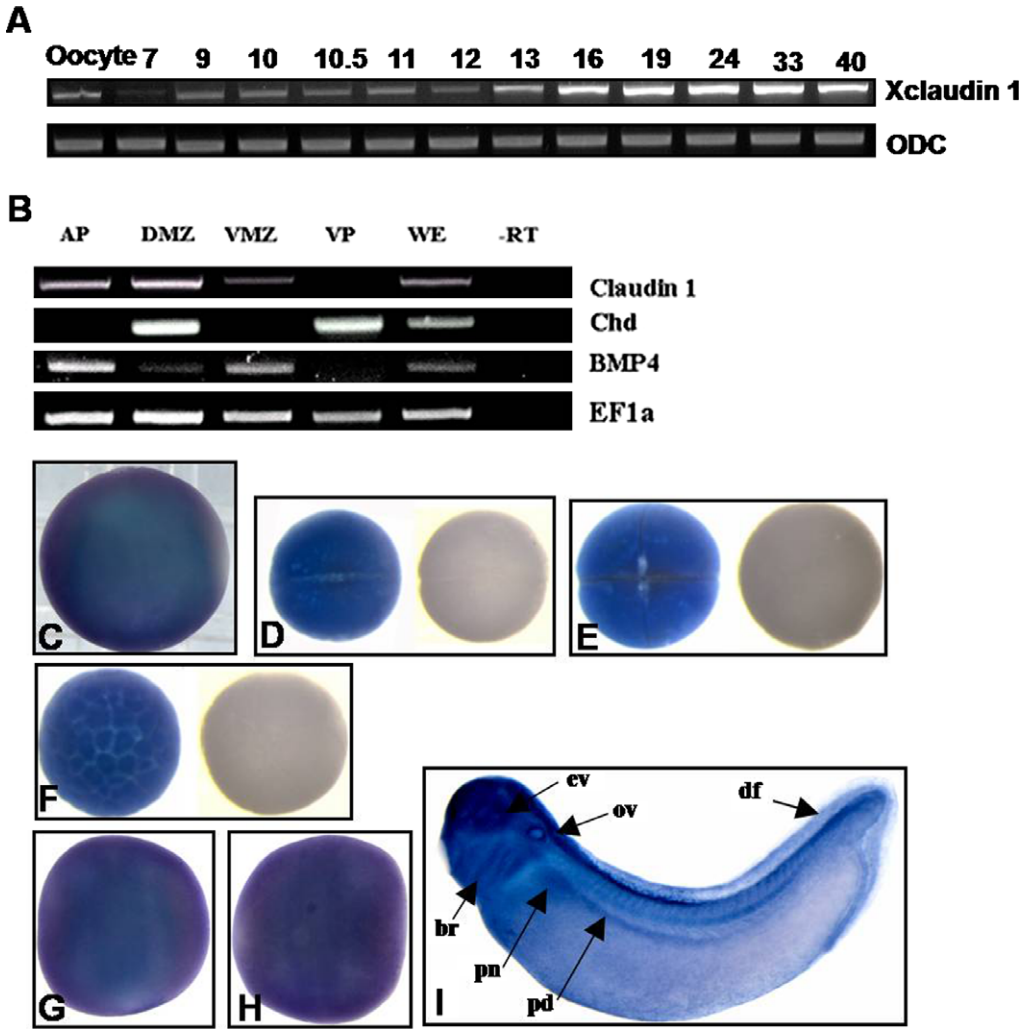

Fig. 1. Temporal and spatial expression pattern of Xclaudin 1 during *Xenopus* development. (A) Temporal expression pattern of Xclaudin 1 was detected by RT-PCR. Ornithine decarboxylase (ODC) primers were used to normalize the amount of cDNA template. (B) RT-PCR of gastrula stage embryos (st.10.5). Xclaudin 1 mRNA is enriched in animal pole region and dorsal marginal zone in embryos. Chd (chordin) and BMP 4 were used as each gene control of explanted tissue and EF1α also used as a loading control. (C–J) In situ hybridization of spatial expression of Xclaudin 1 during early *Xenopus* development. Whole-mount in situ hybridization was carried out using a digoxigenin-labeled riboprobe. (C) Oocyte, animal view. (D) 2-cell stage, animal view and vegetal view. (E) 4-cell stage, animal view and vegetal view. (F) One hundred and twenty-eight cell stage, animal view and vegetal view. (G) Gastrular stage, animal view. (H) Neurula stage, animal view. (I) stage 29/30 lateral view. pd, pronephric duct; pn, pronephrus; ov, optic vesicle; df, dorsal fin; ev, eye vesicle; br, branchial arch.

detected. These data indicate that Xclaudin 1 has a strong expression pattern in the ectoderm including the dorsal marginal zone (DMZ) that undergoes dynamic convergent extension movements during gastrulation.

3.2. Over-expression or loss of expression of Xclaudin 1 results in gastrulation defects

We investigated the function of Xclaudin 1 in early embryo development through assessments of the effects of gene over-expression or knockdown on the *Xenopus* gastrulation. Xclaudin 1 mRNA injection into dorsal or animal blastomeres resulted in severe gastrulation defects (Fig. 2A and B). Interestingly, the phenotypes of Xclaudin 1 over-expression were locus-specific and, at the same time, dose dependent. Injection of Xclaudin 1 mRNA into dorsal or animal blastomeres led to more severe gastrulation defects than into ventral or vegetal blastomeres (Fig. 2B). When Xclaudin 1 mRNA (500 pg) was injected into dorsal or animal blastomeres of 8-cell embryos, embryos showed no obvious delay in blastopore formation (stage 11, data not shown). However, this led to a failure in complete blastopore closure at stage 13 and showed severe gastrulation defects, of which subsequent embryos showed phenotypic spectrum of shortened axis to bent axis at tail-bud stage (Fig. 2A and B). Additionally, as the injection dose increases, the severity of phenotype also increased (Fig. 2C).

To confirm the involvement of Xclaudin 1 during early *Xenopus* development, we performed loss-of-function studies by using Xclaudin 1 antisense morpholino oligonucleotides (MO) as efficient blockers of specific mRNA translation. An antisense morpholino oligonucleotide was designed to be complementary to the translation initiation sequence of Xclaudin 1 mRNA. Translation of Xclaudin 1 mRNA containing 5′-UTR was effectively blocked by its morpholino oligonucleotide (Fig. 3A). Control MO (CTL MO) had no effect on translation of the Xclaudin 1 mRNA. Interestingly, Xclaudin 1 MO-injected embryos resulted in gastrulation defects, which included delay in blastopore closure, failure of neural tube closure and abnormal anterior/posterior axis formation (Fig. 3B and C). Additionally, morphology of Xclaudin 1 knockdown embryos was similar to that of the Xclaudin 1 over-expression ones showing gastrulation defects (Fig. 3D).

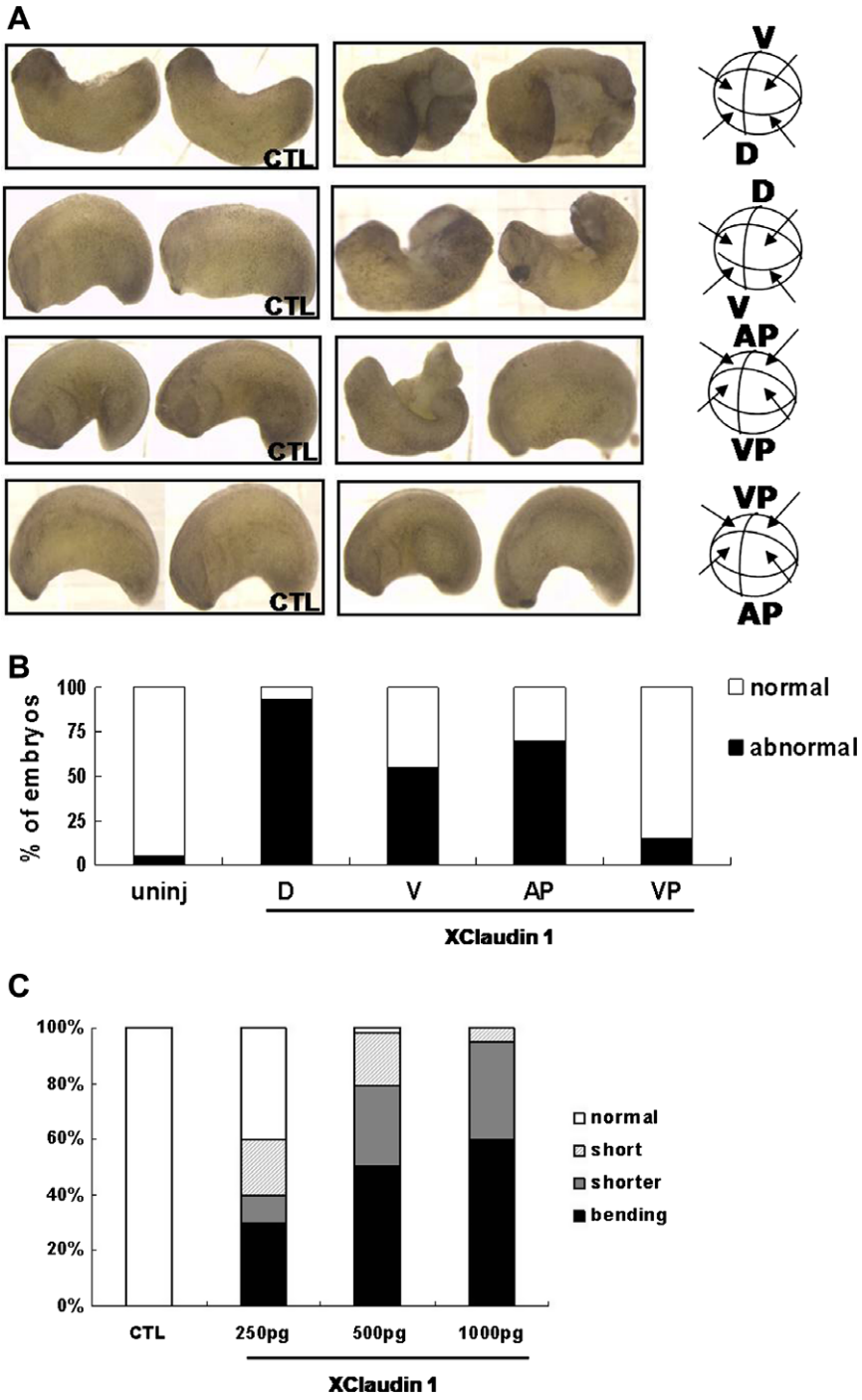

Fig. 2. Gastrulation defects from over-expression of Xclaudin 1. (A) Injection of Xclaudin 1 mRNA 500 pg/embryo ($n = 63$) into designated blastomeres at 8-cell stage resulted in embryos with dorsally bent trunk and shorter or short anteroposterior axis in a dose-dependent manner. (B) Injection of Xclaudin 1 mRNA (500 pg/embryo) at four blastomeres of dorsal ($n = 70$), ventral ($n = 65$), animal ($n = 68$) or vegetal side ($n = 65$) of 8-cell stage embryos. The embryos were observed and analyzed at stage 23. (C) Morphology of embryos injected with various doses of Xclaudin 1 mRNA (250 pg/embryo ($n = 58$), 500 pg/embryo ($n = 63$) or 1 ng/embryo ($n = 60$)) and un-injected embryos ($n = 67$) was analyzed according to category of phenotypes. Numbers of embryos represents the percentage of embryos with normal morphology (open bar), a short (slanting line bar), shorter (gray solid bar), anteroposterior and dorsally curved trunk axis (black solid bar) out of total embryos examined.

3.3. Xclaudin 1 is required for convergent extension during gastrulation

In *Xenopus* embryo, mesodermal convergent extension movement is a primary driving force of gastrulation [11]. To understand the role of Xclaudin 1 for CE (convergent-extension) movements at the cellular level during gastrulation, we next examined elongation of DMZ tissues. DMZ explants exogenously over-expressing Xclaudin 1 showed retarded elongation. Gene knockdown by claudin 1 MO also inhibited elongation of DMZ explants (Fig. 4A and B). This failure of convergent extension either by over-expression or knockdown was partially, but significantly recovered when both treatments were combined in co-injection, addressing this morphant explants' phenotype is the result of specific gene knockdown, also by comparing to the case of sole over-expression, suggesting a optimum level of this protein is required for proper CE movement.

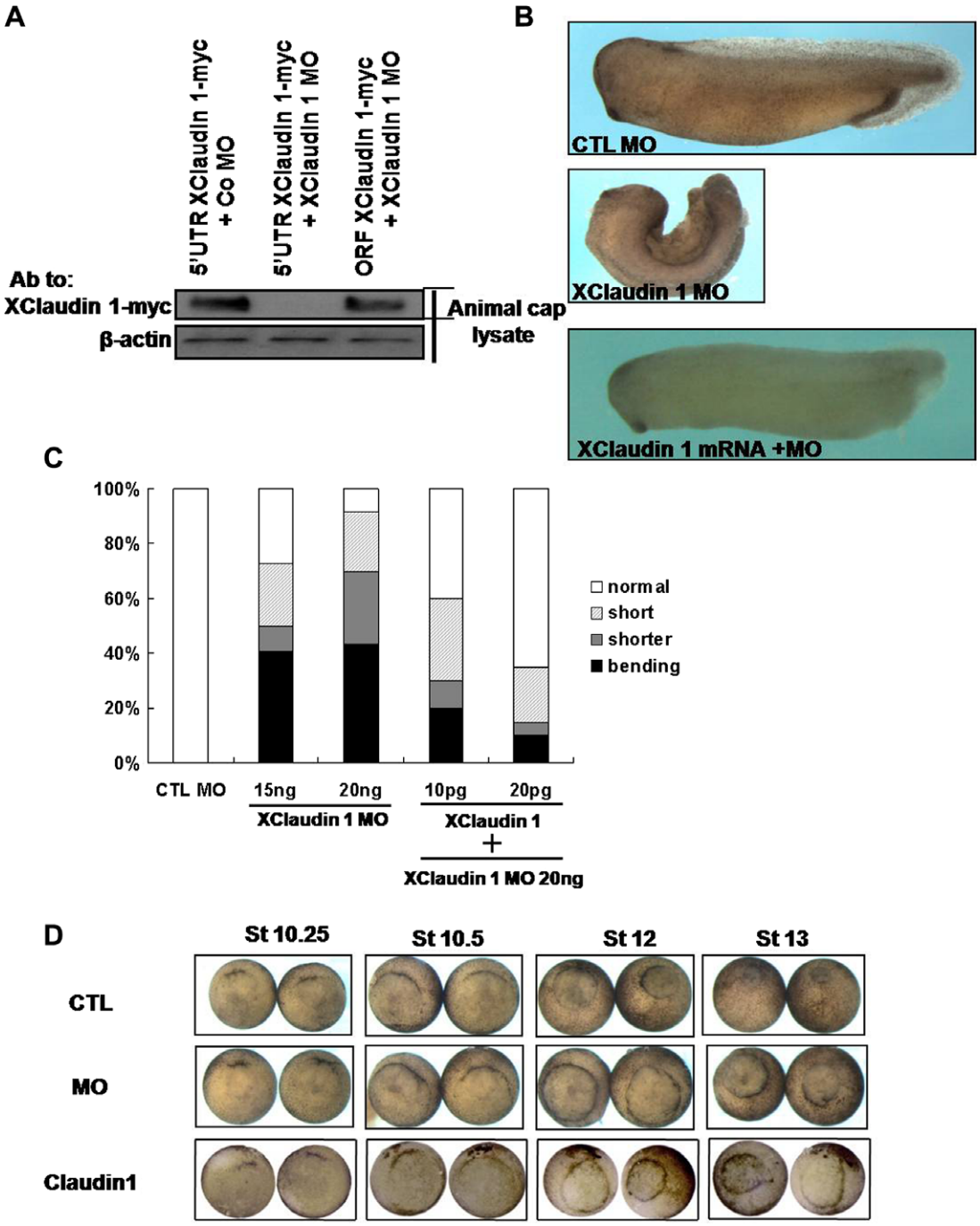

Fig. 3. Gastrulation defects from inhibition of Xclaudin 1. (A) Verification of Xclaudin 1 MO in translation blocking efficiency by using Western blotting co-injection of Xclaudin 1 MO (20 ng) completely blocked the translation of co-injected Xclaudin 1 mRNA (500 pg/embryo) that bears MO-targeted 5′-UTR sequence, but not the one that misses MO-targeted sequence (500 pg/embryo) but bears only the whole coding region. Animal cap explant lysates from early gastrula stages following 2-cell stage injection at animal pole region were used for immunoblot (IB) analysis with anti-myc antibodies. β-Actin served as a loading control. (B) Four blastomeres at 8-cell stage embryos were injected at the dorso-animal blastomeres with Xclaudin 1 MO (20 ng). Unlike control embryos injected with control MO embryos ($n = 70$), embryos injected with Xclaudin 1 MO ($n = 65$) displayed slightly opened neural folds, dorsally bending trunk and shortened axis. These phenotypes were rescued by co-injection of Xclaudin 1 mRNA (ORF Xclaudin 1-myc) and MO ($n = 67$). (C) Gastrulation defects caused by Xclaudin 1 MO were rescued distinctively by the co-injection of Xclaudin 1 mRNA. The number of rescued embryos was increased when 20 pg of Xclaudin mRNA ($n = 58$) was injected from that with 10 pg ($n = 54$). Graph of numbers of embryos, which showed normal embryo (open bar), a short (slanting line bar), shorter (gray solid bar) anteroposterior and dorsally bending trunk axis (black solid bar) in injection of Xclaudin 1 mRNAs. (D) Xclaudin 1 knockdown retarded blastopore closure ($n = 72$). Frames from a representative time-lapse sequence showed that blastopore closure was delayed in either Xclaudin 1 MO (20 ng)- or mRNA-injected embryos until sibling control embryos reach equivalent midgastrula stage 13.

Dorsoventral patterning of the mesoderm could affect CE movements indirectly by changing cell fates in DMZ [12]. To examine whether the phenotypes of Xclaudin 1 over-expression or knockdown were caused by its possible effect on either a dorsal mesodermal differentiation or on cell movement, we performed RT-PCR analysis and whole-mount in situ hybridization using several mesodermal marker genes (Fig. 4C and D). Xclaudin 1 mRNA-injected embryos exhibited normal endogeneous expression of dorsal mesodermal markers, *chordin* (*Chd*) and *goosecoid* (*Gsc*), and pan-mesodermal marker, brachyury (Xbra). These results

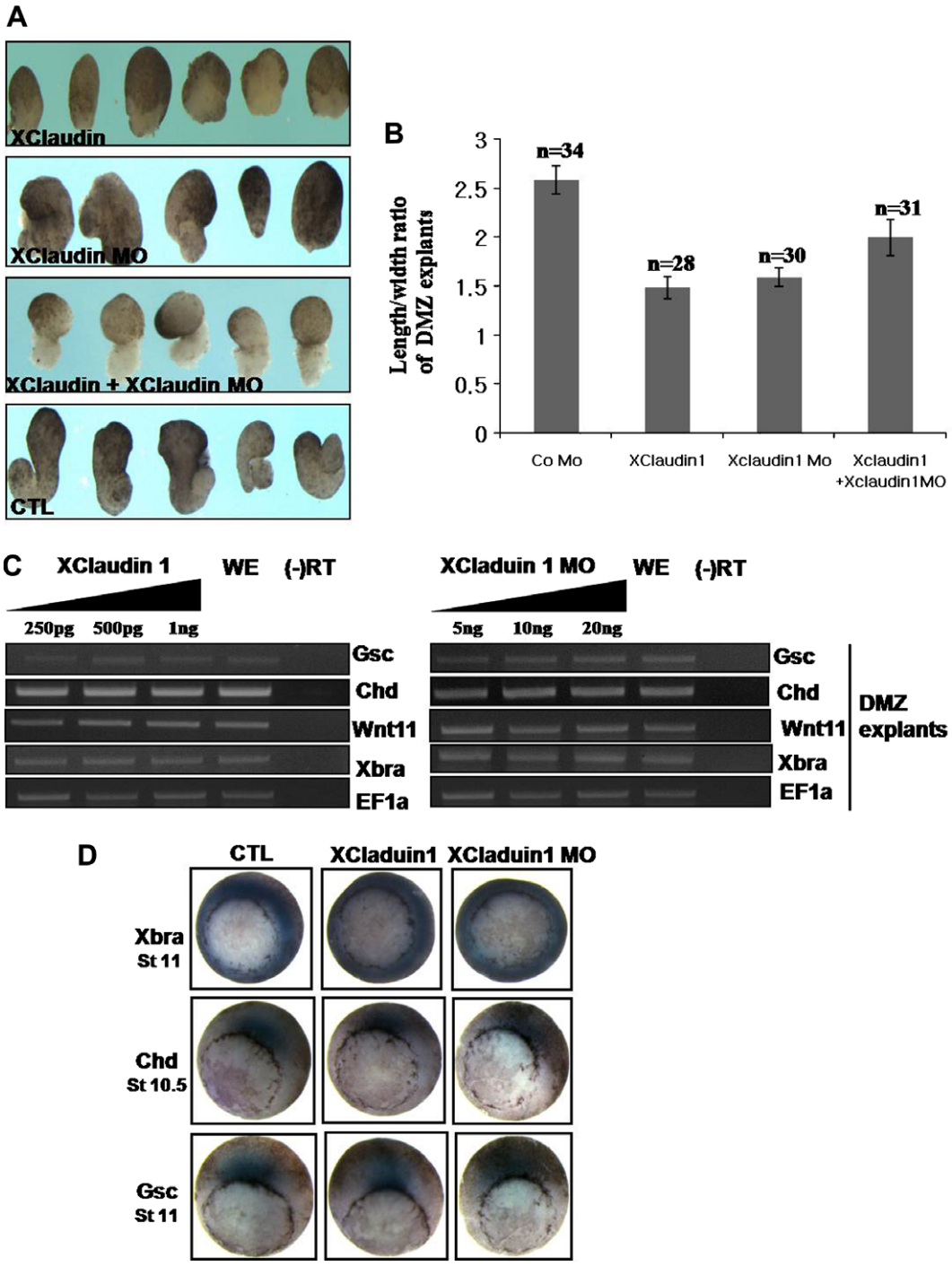

Fig. 4. Injection of embryos with either Xclaudin 1 MO or mRNA inhibits convergent extension movements. (A) Two dorsal blastomeres of 4-cell stage embryos, onto their equatorial region were injected with the Xclaudin 1 mRNA (500 pg; $n = 28$) or the Xclaudin 1 MO (20 ng; $n = 30$) separately, or together (mRNA, 250 pg, MO, 20 ng; $n = 31$). Dorsal marginal zone was dissected at early gastrula (st.10.5) and then cultured until stage 18. The elongation of explants was inhibited by injection with either Xclaudin 1 mRNA or Xclaudin 1 MO. The inhibition was partially rescued by co-injection of Xclaudin 1 mRNA. (B) We measured length/width ratio of injected DMZ explants in DMZ elongation assay. *n*, total number of explants. Error bars indicate means ± SD. (C) RT-PCR analysis showed that injection with either Xclaudin 1 mRNA (250 pg, 500 pg and 1 ng), or Xclaudin 1 MO (5, 10 and 20 ng) do not affect organizer cell fate specification from DMZ explants tissues. The expression of Chd, Gsc, Wnt11 and Xbra was analyzed using primers specific for them. EF1α is a loading control. (−) RT, minus reverse transcription; controls sample. (D) Whole-mount in situ hybridization analysis was performed after injection of either Xclaudin 1 mRNA (1 ng/embryo) or MO (40 ng/embryo) into equatorial regions of two blastomeres at 2-cell stage. Mesodermal marker Xbra and dorsal mesodermal genes Chd and Gsc were used in WISH. Neither Xclaudin 1 mRNA nor Xclaudin 1 MO changed the expression of the mesodermal fate markers.

suggest that the gastrulation defects by Xclaudin 1 over-expression were affected not by the defective cell differentiation but by abnormal cell movements. These data support the idea that Xclaudin 1 is required for proper convergent extension movements without affecting cell fate specification during early *Xenopus* development.

4. Discussion

In this study we have investigated what role claudin 1 plays in early vertebrate embryogenesis. At the gastrula stage, it was strongly expressed in the entire ectoderm including the dorsal

marginal zone that undergoes dynamic convergent extension movements. Altered expression of Xclaudin 1 interfered with convergent extension movement during gastrulation without disruption of dorsal mesodermal fate specification, suggesting that Xclaudin 1 is required for embryonic cell movements in *Xenopus*.

Our in situ hybridization and RT-PCR results showed that Xclaudin 1 is strongly expressed in the dorsal marginal zone, suggesting its possible involvement in convergent extension movements. This suggestion is supported by gain and loss of function experiments to investigate the role of Xclaudin 1 in gastrulation. We showed that over-expression of Xclaudin 1 inhibited convergent extension during gastrulation. Likewise, previous study reported that over-expression of *Xenopus* claudin (Xcla) causes significant loss of tissue integrity and randomization of the left–right body axis [4]. In the early stage of EMT, tight junction disruption allows attached epithelial cells to separate from each other and migrate through the extracellular matrix. Snail plays a role in the dynamic regulation of tight junction through downregulation of claudin 3, 4 and 7 by binding E-box on claudin promoter region during the EMT [13,14]. Runx3 has a role in suppressing gastric carcinogenesis via its binding to the RUNX consensus sites and up-regulates caludin1 to promote cell–cell contact [18]. The tight junction is constantly remodeled for maintenance of the dynamic tissue integrity, and its turnover is affected by endogenous factors, pathogens and cytokines. For example, some toxins from *Escherichia coli, Helicobacter pylori* and *Clostridium perfringens* have been known to enhance dissociation of claudins and inhibit reformation of them [19]. Cytokines such as interferon-γ, tumor-necrosis factor (TNF)-α and interleukin-13 induces claudin breakdown or downregulation of its synthesis and it leads to disruption of tight junction [15,16]. Through this process, epithelial cells lose their integrity and acquire cellular motility. These studies show that alteration of claudin synthesis or unbalanced turnover of claudin causes disruption of tissue integration and leads to functional disturbances. In fact, downregulation of Xclaudin 1 has been shown to enhance metastatic movement of cancer cells [17]. On the other hand, our experiments demonstrated that downregulation or over-expression of Xclaudin 1 result in abnormal gastrulation as well. It suggests that alteration of Xclaudin 1 expression causes abnormal embryonic cell movements by blocking the dissociation of tight junction between mesodermal cells. Taken together, Xclaudin is important for the well-regulated tight junction remodeling for the directed embryonic cell movements during *Xenopus* gastrulation.

We demonstrate here that Xclaudin 1, a tight junctional protein, is required for proper gastrulation movement of the *Xenopus* embryos.

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