

## ABSTRACT

XIE, JIANZHEN

Analysis of *Xenopus laevis* claudin (*Xcla*) tight junction genes in development

Under the direction of Dr. Brenda Judge Brizuela

Eight *Xenopus laevis* claudin genes, *Xcla1*, *Xcla4B*, *Xcla5*, *Xcla6*, *Xcla12*, *Xcla16*, *Xcla18* and *Xcla19*, were cloned and sequenced. Their normal mRNA expression was determined from cleavage stage to tadpole stage by whole mount *in situ* hybridization. The protein expression of *Xcla5* was detected at the neural stage by whole mount immunostaining. Overexpression of *Xcla5* by injection of synthetic mRNA into embryos caused morphological defects similar to those in embryos exposed to Bisphenol A (BPA). Altered patterns of claudin gene expression in the presence of BPA can be correlated with these developmental defects. The results suggest that claudins may play an important role in neural crest cell migration, epithelial-mesenchymal transition and ultimately organogenesis during embryonic development.

**ANALYSIS OF *Xenopus laevis* CLAUDIN (*Xcla*) TIGHT JUNCTION GENES IN  
DEVELOPMENT**

by

**JIANZHEN XIE**

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**APPROVED BY:**

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Dr. Brenda J Brizuela  
(Chair of Advisory Committee)

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Dr. Patricia A Estes

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Dr. Robert M Grossfeld

## BIOGRAPHY

I was born in February of 1975 in Fujian, China. My younger brother was born in April of 1979. Time passed, I grew up, did many things, went many places, etc. Along the way, I developed an interest in several subjects but at some point chose to focus on life science. I continued to do so while attending the Peking Union Medical College and Fujian Medical University. In 2002, as a graduate student's wife, I moved to Houghton, Michigan. I enjoyed the friends, the snow, the beaches, and the Lake Superior in Houghton. At 4: 15am on May 6<sup>th</sup> of 2003, my son Marcel Nuo Zhang was born in WakeMed hospital, Raleigh, USA. While Marcel was three month old, I decided to continue my studies by pursuing a master degree in Zoology. For that reason, I joined the lab of Dr. Brizuela to study the mechanism of morphogenesis.

*“Nothing in Biology makes sense, except in the light of evolution”*

*T.H. Dobzhansky*

(Learned from Dr. Atchley's class. GN703 spring of 2005)

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## LIST OF ABBREVIATIONS

BBB	Blood Brain Barrier
BLASTN	Basic Local Alignment Search Tool for Nucleotides
MBT	midblastula transition
BMP	bone morphogenetic protein
BPA	Bisphenol A
TBLASTX	Basic Local Alignment Search Tool for Transcription
bp	base pair
CATCH-22	<u>c</u> ardiac defects, <u>a</u> bnormal faces, <u>t</u> hymic hypoplasia, <u>c</u> left palate, <u>h</u> ypocalcemia, associated with chromosome <u>22</u> microdeletion
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
Dig	digoxigenin
EDTA	ethylenediamine tetraacetic acid
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
EST	expression sequence tag
hCG	human chorionic gonadotropin
kb	kilobase
kDa	kilodalton
MDCK	Madin-Darby canine kidney

MGC	Mammalian Gene Collection
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
PBS	phosphate buffered saline
PBT	Triton in PBS
PCR	polymerase chain reaction
SSC	sodium saline citrate
TER	transepithelial electrical resistances
TBE	Tris-Borate-EDTA
TJ/TJs	tight junction/tight junctions
TMVCF	transmembrane protein deleted in velo-cardio-facial syndrome
VCF	velo-cardio-facial syndrome
<i>Xcla</i>	<i>Xenopus laevis</i> claudin
ZO	zonula occludin

# CHAPTER 1

## INTRODUCTION

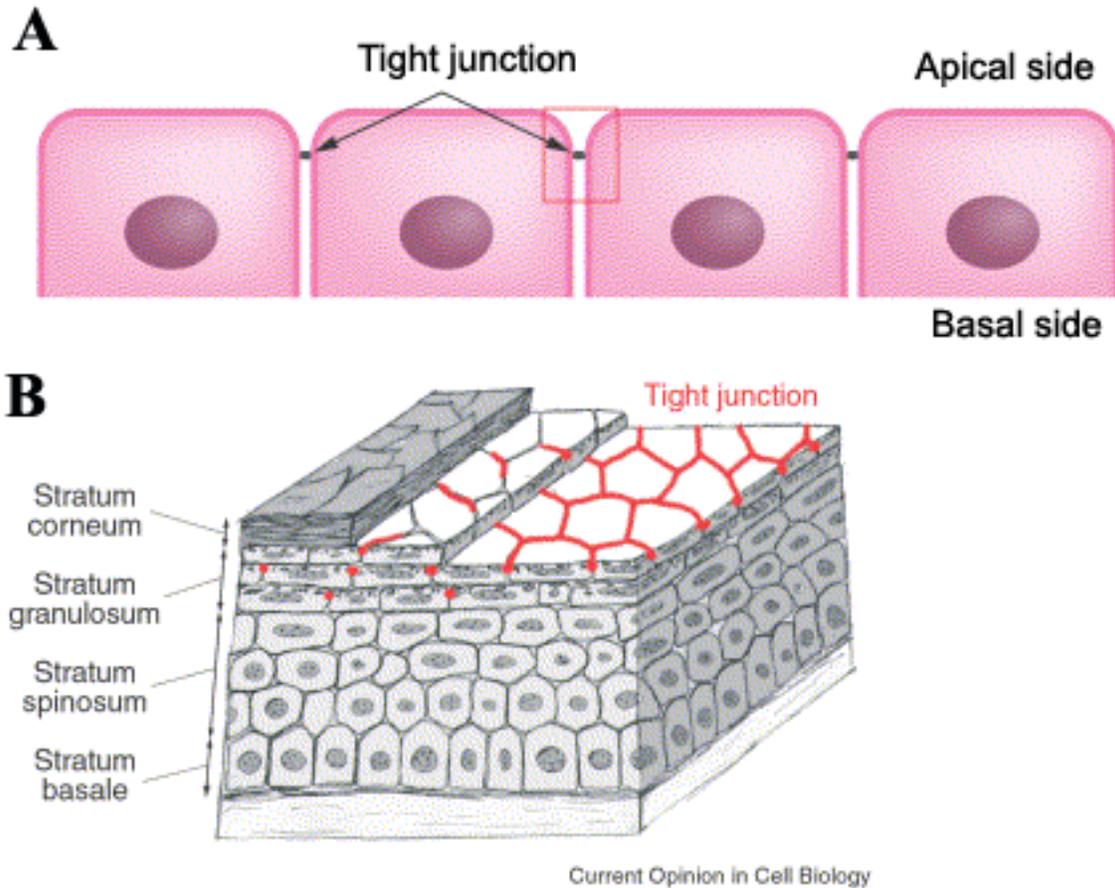
### **What are tight junctions?**

Epithelial tissues are the fundamental building blocks of all animals. Tight junctions (TJs) and septate junctions in epithelial tissue are two important intercellular seals for compartmentation in vertebrates and invertebrates, respectively. These intercellular seals create selective barriers, which allow tissues to regulate paracellular movements of solutes down their electro-osmotic gradients. These intercellular seals also generate cellular polarity, which allows the apical and basolateral membrane surfaces to recognize signals directionally or to transport material across the epithelial sheet. Septate junctions are in general found in non-chordates whereas tight junctions are mainly found in chordates (Green et al., 1983). However, septate junctions have also been found in the node of Ranvier in the vertebrate nervous systems (Genova and Fehon, 2003) and tight junctions also occur in adult arthropods (Baumgartner et al., 1996).

There are three major findings of tight junctions over the past years. First, in mammals, TJs are located at the most apical region of simple epithelia (Figure 1-1A). But recently, tight junctions were also found at the stratum granulosum of stratified epithelia (Figure 1-1B), where they regulate water balance as well (Tsukita and Furuse, 2002; Furuse et al., 2002). Second, in mammals, TJs connect two matching strands of intra-membrane particles in the opposed cell membranes. In freeze fracture studies, the intercellular space of TJs is obliterated, which is different from mammalian adherens junctions and gap junctions and also different from non-chordate septate junctions. It has now been

accepted that pores (or paracellular channels) exist in the paired TJ strands and that these pores have ion- and size- selective properties (Gow et al., 2004; Colegio et al., 2003) . Third, not only do claudins act as an important structural protein (maintaining cell polarity and regulating paracellular permeability), but also claudin tight junction proteins may act as important adhesion molecules, which regulate epithelial-mesenchymal transition and organogenesis in development. The supporting evidence came from gain of function studies, which demonstrated that claudin causes left and right randomization in the development of *Xenopus laevis* (Brizuela et al., 2001).

Tight junction related proteins such as occludin, claudin, zonula occludin (ZO), and junctional adhesion molecules (JAM) have been identified. Indeed, over the past seven years, scientists have gained important insights into the TJ's molecular complexity (D'Atri and Citi, 2002), the physiological paracellular transport mechanism (Van Itallie and Anderson, 2004a; Anderson et al., 2004) and phylogenetic conservation (Wu et al., 2004).



**Figure 1 –1** Tight junctions are in simple and stratified epithelia. (A) TJs are located at the most apical region of lateral membrane in a typical intestine simple epithelium. Claudins are the main components of the tight junction strands. (B) Tight junctions are found in stratified epithelia. The epidermis consists of a single cell layer of stratum basale and several cell layers each of strata spinosum and granulosum, which are covered with stratum corneum. Continuous tight junctions (red) were found in the second layer of the stratum granulosum in newborn mice. Tight junctions have also occasionally been detected in the first and third layers of stratum granulosum (Tsukita and Furuse, 2002).

## The discovery of claudins

In 1993, the report on occludin from the Tsukita group started a new era of molecular dissection of the tight junction complex (Furuse et al., 1993). Occludin can induce the formation of tight junction strands on L-fibroblast cells lacking endogenous tight junctions. Therefore, occludin had been considered the integral membrane protein of tight junctions. However, in occludin knockout mice, tight junction strands can still be formed (Saitou et al., 2000), which suggested the existence of other unidentified integral membrane proteins in tight junctions.

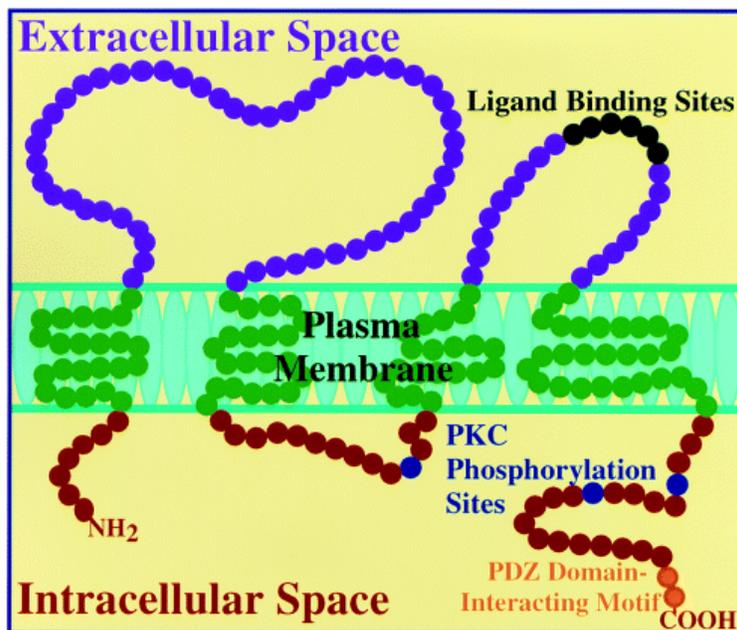
Using microsome fractionation, a developmentally regulated transmembrane protein was isolated, named MI1 (Brizuela et al., 1998). BLAST analysis showed that MI was related to a transmembrane protein deleted in velo-cardio-facial syndrome (VCFS) (Morita et al., 1999b) and to a rat ventral prostate gene (RVP-1) (Brizuela et al., 1999; Briehl and Miesfeld, 1991; Peacock et al., 1997). During the time the Tsukita lab re-examined the isolated junction fraction and isolated two full-length cDNAs encoding 22-KD proteins consisting of 211 and 230 amino acids, respectively, which were different from occludin. Hydrophilic analysis was consistent with the topology predicted by Brizuela et al., who showed that *Xenopus* claudin (*Xcla*) has four predicted transmembrane domains, with two extracellular loops and a cytoplasmic carboxy terminus containing a PDZ binding domain (Figure 1-2) (Brizuela B., 1998). Further, in the Tsukita lab, immunofluorescence and electron microscopy showed that these proteins targeted to TJ strands. Thus they were termed claudin-1 and claudin-2 (Furuse et al., 1998; Furuse et al., 1998). Also in the Tsukita lab, the cDNA of occludin, claudin-1 and – 2 were transfected into mouse L fibroblasts lacking TJs. It was found that claudin-1 and –

2 could induce the formation of well-developed continuous TJ strands, while occludin only induces a small number of short TJ strands (Saitou et al., 1998). This reinforced the result from Brizuela et al. This evidence strongly demonstrated that claudins are mainly responsible for the formation of TJ strands while occludin is an accessory protein in TJ strands (Tsukita and Furuse, 1999).

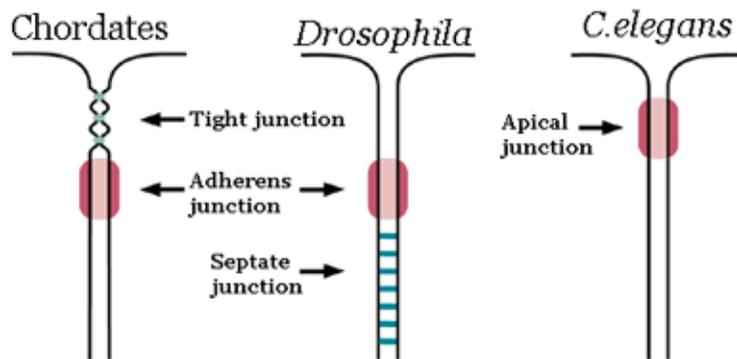
*Xcla* can induce the formation of TJ strands after *Xcla* cDNA is transfected into mouse L fibroblasts lacking TJs. In addition, overexpression of *Xcla* caused defects in cell adhesion (Brizuela B., 1999). Whole mount *in situ* hybridization using *Xcla* antisense mRNA showed the expression pattern of *Xcla* in developing embryos. Overexpression of *Xcla* by microinjection of synthetic mRNA at the 4-cell stage causes randomization of left-right body axis (Brizuela et al., 2001). This study strongly suggested that claudins play an important role in embryogenesis. It was also shown that the PDZ binding site of *Xcla* is critical for claudin's localization on the membrane. Recently, Anderson et al also argued that the cytoplasmic carboxyl PDZ-binding motif of claudin influences the stability of claudin composition in TJ and subsequent functions (Van Itallie et al., 2004).

Sequence analysis in *Drosophila* and *C. elegans* has identified non-chordate claudin orthologs in their septate junctions. In *Drosophila*, there are two claudin orthologs identified, Mega (Behr et al., 2003) and Sinuous (Wu et al., 2004). Mega is essential in septate junctions for epithelial barrier function. Sinuous is required for septate junction organization and epithelial tube size control. In *C. elegans*, there are four claudin orthologs identified (Asano et al., 2003). By immunofluorescence analysis and permeability studies, two of them have been characterized for their distribution and barrier function. VAB-9 of *C. elegans* is the claudin-like protein also located in septate

junctions (Simske et al., 2003). It is very interesting to note from this paper that VAB-9 makes an essential contribution to intercellular adhesion through its positive modulatory role in the cadherin-catenin complex. Interestingly, both VAB-9 and claudins belong to the tetraspan superfamily. But the relationship between claudin and tetraspan protein is still being investigated. The functional study of non-chordate claudins and chordate claudins provided evidence that the common molecular basis for the barrier in Nematoda, Insecta and Vertebrata are claudin proteins (Figure 1-2).



**Figure 1- 2** The predicted structure of claudin 4 protein. Claudin 4, a typical claudin superfamily protein with a molecular weight of ~21kDa, contains four highly hydrophobic transmembrane segments (green). Three putative PKC phosphorylation sites (blue) and one PDZ domain-interacting motif (red) are present in its cytoplasmic portions. The second extracellular loop contains binding sites (black) for ligands, such as enterotoxins (Heiskala et al., 2001)

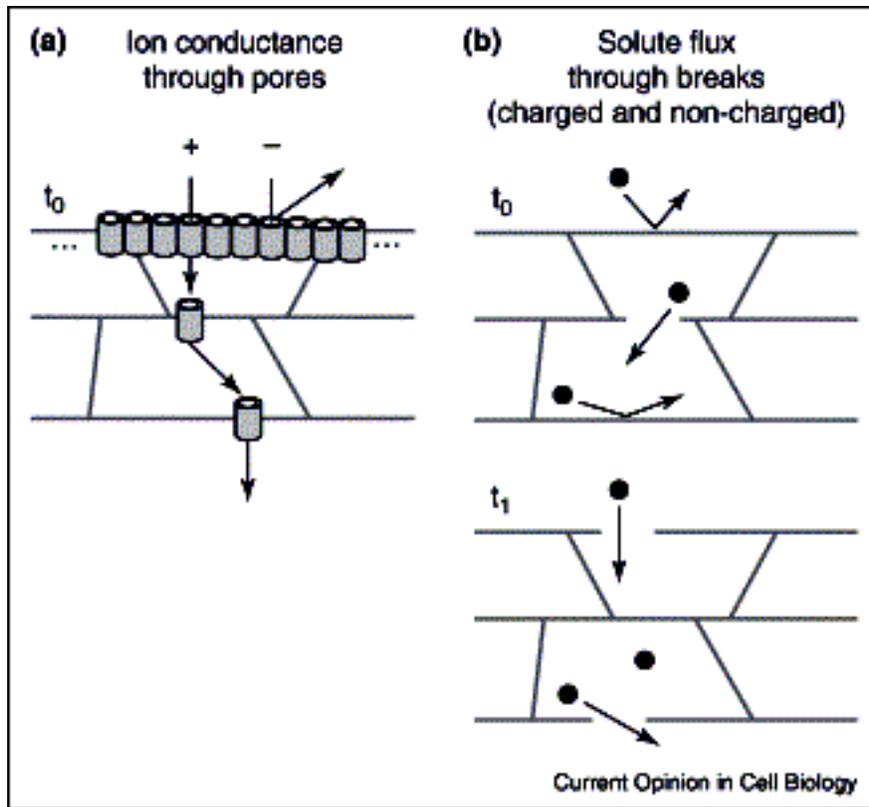


**Figure 1-3** Apical junctional complex in chordates, *Drosophila* and *C.elegans*. Tight junctions and adherens junctions are found in chordates (desmosomes which form spots, rather than circumferential bands, are not shown). Adherens junctions and septate junctions combine in a typical junctional complex of non-chordate animals, such as *Drosophila*, *C.elegans* displays only a single element in its apical junctional complex, which is often simply referred to as the apical junction (Tepass, 2003).

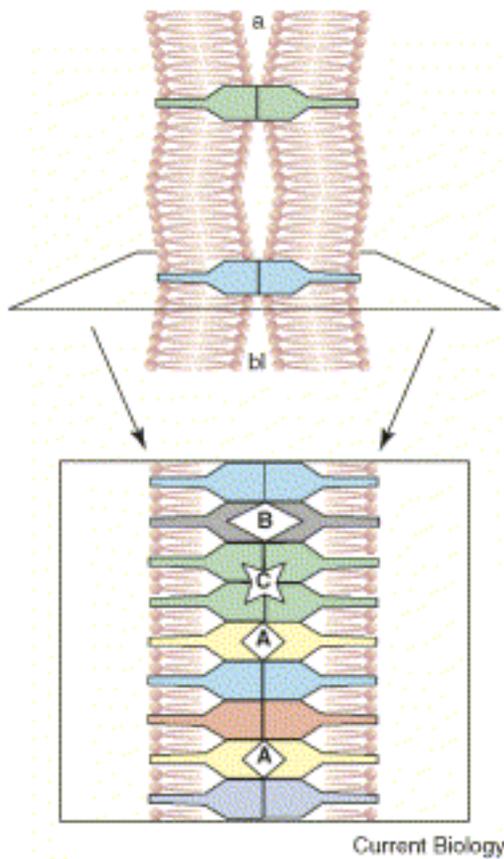
### **Claudins in modern electrophysiology**

Today, scientists are debating whether tight junctions are conventional ion channels or merely aqueous pores. The consensus is that claudin-based barriers have different charge selectivity (mainly in terms of the ratio of  $P_{Na^+}/P_{Cl^-}$ ) and size selectivity (4-40Å) among different tissues (Anderson et al., 2004; Van Itallie and Anderson, 2004b; Yu et al., 2003). The hypothesis from Anderson et al illustrated in Figure1-4, is that claudin create aqueous pores to regulate paracellular transport. The hypothesis from Peter and Goodenough illustrated in Figure 1-5 is that the claudins create paracellular ion channels (Peter and Goodenough, 2004).

The methods used in this field combine molecular biology and traditional electrophysiology (Van Itallie et al., 2001). First, different claudin constructs (mutated or normal) are transfected into epithelial cell lines such as Madin-Darby canine kidney (MDCK) II cells. Second, immunoblots or immunofluorescence microscopy is used to confirm the existence of newly made claudin protein. Finally, by using the voltage-current clamping technique, transepithelial electrical resistances (TER) are measured, which is the main index for the measure of *tightness* at the tight junctions. Meanwhile, in addition to TER measured in voltage-current clamping, ion compositions are substituted (equimolar amounts of NaCl, KCl, LiCl, RbCl, and CsCl) to measure the charge selective properties. Using this experimental approach, Anderson's group has shown that (a) claudins create charge-selective channels in the paracellular pathway between epithelial cells (Colegio et al., 2002); (b) reversal of charge selectivity for cations or anions was created in MDCK cell lines by expressing different claudins (Nitta et al., 2003); and (c) claudin extracellular domains (claudin 2 and 4) can determine the paracellular charge selectivity and resistance but not tight junction fibril architecture (Colegio et al., 2003). However, the experiment from Goodenough et al proposed that discrete ion channels are present in tight junctions. Their evidence was that: tight junctions shared biophysical properties with conventional ion channels, including size (4.9-7 Å) and charge selectivity, dependency of permeability on ion concentration, competition between permeant molecules, anomalous mole-fraction effects, and sensitivity to pH (Tang and Goodenough, 2003). They argued that it was the absolute number of channels that accounts for the variation in TER.



**Figure 1-4** Anderson's pore model to explain why the barriers for ions and solutes behave differently. The barrier strands are formed by rows of charge-selective claudin pores. (a) Shows strands formed by cation-selective claudins, which permit instantaneous transjunctional passage when measured at  $t_0$ . Anions experience relatively lower permeability. (b) By contrast, non-charged solutes that cannot pass through pores as readily as the ions must wait for breaks in the strands to pass. Their step-wise progression takes much longer. One break pattern is shown at  $t_0$  and another at  $t_1$  (Anderson et al., 2004).



**Figure 1-5** Goodenough's tight junction paracellular ion model. The upper part of the figure shows an apical/basal section of two interacting plasma membranes at a tight junction. The interacting membrane proteins (claudins) separate the apical (a) from the basolateral (bl) extracellular spaces. A section perpendicular to the drawing at one of the cell-cell interactions reveals that the claudins (and occludin) form a continuous linear polymer (lower part of the figure), interrupting the external leaflet of the lipid bilayer. The claudins may interact between cells to form a variety of ion-selective channels (A, B or C) joining the two extracellular spaces (Peter and Goodenough, 2004).

### Expression profiles of claudins

Different epithelia have different permeability and different claudin distribution, although some claudin species are ubiquitous. For example, claudin 5 is specifically located in endothelial cells such as blood vessels of blood brain barrier, lung, and heart (Xie and Brizuela, unpublished). It is possible that the tightness of TJ in different tissues is controlled by different combinations of claudin species. But it is not known how different claudin isoforms are assembled to function. The study from Anderson's lab suggested that the carboxyl terminal domain might contribute to it by stabilizing protein-protein interactions within a claudin complex (Van Itallie et al., 2004)

Three major methods are widely used in studying claudin expression: RT-PCR, *in situ* hybridization, and immunostaining. The major claudin expression profiles are listed in table1-1.

**Table 1-1** Claudin expression in different tissues

Claudin species	Tissue or cell lines	Main reference
Claudin-1	Skin, liver (hepatocytes), ear, pancreas, colon, placenta, choroid plexus, prostate MDCK	(Morita et al., 2004a), (Furuse et al., 1998)
Claudin-2	MDCK-C7*, MDCK-C11*, ear, intestine, lung, choroid plexus	(Furuse et al., 1998; Lipschutz et al., 2005)
Claudin-3	Ear, intestine, liver, kidney, testis, lung, colon, prostate, uterus	(Fujita et al., 2000)
Claudin-4	Intestine, lung, kidney, colon, prostate, breast, lung, uterus	(Michl et al., 2001; Rangel et al., 2003)
Claudin-5	In the endothelial cells of all tissues except in kidney (only in arteries), alveolar epithelial cells of lung	(Ishizaki et al., 2003; Kojima et al., 2002; Li et al., 2004; Liebner et al., 2000; Li et al., 2004; Nitta et al., 2003; Wang et al., 2003)

Claudin-6	Fetal tissues	(Turksen and Troy, 2002a; Turksen and Troy, 2001)
Claudin-7	Lung, kidney, ovary, prostate, colon	(Blackman et al., 2005; Li et al., 2004)
Claudin-8	Ear, lung, kidney, colon, breast	(Yu et al., 2003; Li et al., 2004)
Claudin-9	Ear, brain, tumors	(Heiskala et al., 2001)
Claudin-10	Ear, brain, tumors	(Cheung et al., 2005)
Claudin-11	Ear, brain, testis, spinal cord	(Gow et al., 2004; Hellani et al., 2000; Kitajiri et al., 2004)
Claudin-12	Ear, prostate, colon, uterus	(Heiskala et al., 2001)
Claudin-13	Embryonic tissue	(Heiskala et al., 2001)
Claudin-14	Ear, heart	(Ben-Yosef et al., 2003; Wilcox et al., 2001)
Claudin-15	Small intestine, MDCK	(Van Itallie et al., 2003; Colegio et al., 2003)
Claudin-16	Kidney, tumors, female genitalia, intestine (Goblet cells)	Ozken et al., 2004; (Hirayama et al., 2004; Weber et al., 2001)
Claudin-17	Skin	(Heiskala et al., 2001)
Claudin-18	Ear, lung, stomach	(Niimi et al., 2001)

Claudin-19	NA	(Luk et al., 2004)
Claudin-20	Small intestine	(Heiskala et al., 2001)

\*Madin-Darby Canine kidney cells

### **Genetic studies of claudins**

Claudin knockout mice have been generated as shown in (Table 1-2). Claudin-1 deficient mice died within one day of birth, and showed severe defects in the permeability barrier of the epidermis. Genetic studies provide a new insight into the barrier mechanism of stratified epithelia, especially the skin (Figure.1-1B) (Furuse et al., 2002). Claudin-5 deficient mice died within 10 days of birth and showed size-selective loosening of the blood brain barrier (BBB) (Nitta et al., 2003). In these two studies, the lethality is probably due to the defects in permeability resulting in dehydration. In claudin-11 deficient mice, the gross morphology is normal but CNS myelin and Sertoli cell tight junctions are absent and the mice also have hind leg paralysis (Morita et al., 1999a). These mutant mice suffer from deafness and show defects in the basal cell barrier (Gow et al., 2004). Claudin-14 knockout mice are indistinguishable from wild type mice but have profound hearing loss with normal vestibular function (Ben-Yosef et al., 2003). Overexpressing claudin-6 transgenic mice have also been generated (Turksen and Troy, 2002a). These transgenic mice showed permeability barrier dysfunction and died from dehydration. From the above genetic gain or loss of function studies, it is obvious that claudins play an important role in maintaining homeostasis. Interestingly, overexpression of *Xenopus laevis* claudin caused left-right asymmetry defect (Brizuela et al., 2001).

Gross morphology in mutant mice may be normal because multiple claudin species might be functionally redundant. The supporting evidence is that claudin-4 is intact in

stratified epithelia in claudin-1 deficient mice, and claudin-12 is intact in the BBB. The structural reciprocity and functional redundancy increase the difficulty of studying function for one specific claudin.

**Table 1-2** Genetic study of claudins

Claudin species	Lethality stage	Major phenotypic changes	Reference
Claudin-1 knockout mice	1 day	Epidemic defect	(Furuse et al., 2002)
Claudin-5 knockout mice	10 days	Size loosening of BBB	(Nitta et al., 2003)
Claudin-6 transgenic mice (overexpression)	1-2 days	Small size, skin defect	(Turksen and Troy, 2002b)
Claudin-11 knockout mice	NO	CNS myelin defect, Sertoli cell defect in testis, deafness	(Gow et al., 2004)
Claudin-14 knockout mice	NO	Hearing loss with normal vestibular function	(Ben-Yosef et al., 2003)
<i>Xcla</i> ( <i>Xenopus</i> claudin <i>Xcla4A</i> ) overexpression	NO	Left-right asymmetry defect	(Brizuela et al., 2001)

### Claudins and human diseases

Claudin-related diseases have been studied intensively (Table 1-3). Mutations in claudin genes can be used to create disease models based on this information. Null mutation of PCLN-1/Claudin-16 results in chronic interstitial nephritis (Hirano et al.,

2000), while a mutation of claudin-14 causes autosomal recessive deafness DFNB29 (Wilcox et al., 2001). Claudins 2, 3, 4, and 5 are reported to be involved in tumorigenesis (breast cancer) (Soini, 2004), ovarian cancer (Heinzelmann-Schwarz et al., 2004) and prostate cancer (Long et al., 2001) .

**Table 1-3** Claudins are associated with human diseases

Claudin species	Disease name	Claudin associated etiology	Reference
Claudin1/4	Squamous cell carcinoma, Bowen's disease	Ectopic expression	(Morita et al., 2004b)
Claudin3/4	William-Beuren syndrome	Haploinsufficiency	(Paperna et al., 1998)
Claudin-5	Velocardiofacial syndrome and DiGeorge's syndrome	Deletion	(Sirotkin et al., 1997)
Claudin-7	Breast cancer	Cell dissociation abnormality	(Martin et al., 2004b; Martin et al., 2004a)
Claudin-11	Sclerosis Sterility paralysis	Autoantigen	(Bronstein et al., 2000b)
Claudin-14	Autosomal recessive deafness <i>DFNB29</i>	Cation permeability defect	(Ben-Yosef et al., 2003)

Claudin-16	Chronic interstitial nephritis	Mg <sup>2+</sup> /Ca <sup>2+</sup> channel dysfunction	(Konrad et al., 2004; Simon et al., 1999)
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### Claudins and development

Cell differentiation, migration, cell-cell communication and epithelial-mesenchymal transition (EMT) are basic events during embryogenesis. Claudin 6 affects epidermal cell differentiation (Turksen and Troy, 2002a) and claudin 6 expression is regulated in mouse embryogenesis (Turksen and Troy, 2001). Claudin-11 deficiency reduces oligodendrocyte migration. Also the migration of oligodendrocytes was inhibited significantly in the presence of anti-claudin-11 antibody (Tiwari-Woodruff et al., 2001; Bronstein et al., 2000a; Bronstein et al., 2000b). *Snail* is a transcriptional repressor that plays a central role in EMT, by which epithelial cells lose their polarity. Claudins are integral membrane proteins localized at tight junctions, which are responsible for establishing and maintaining epithelial cell polarity. Claudin 3, 4, and 7 were down regulated by *Snail* during EMT (Ikenouchi et al., 2003). Also protein mobility shift assays showed that *Snail* can directly bind to the E-box on claudin promoter region (Cano et al., 2000). In addition, Brizuela et al has shown by animal cap assay that *Xcla* affected cell-cell interaction in *Xenopus laevis* embryogenesis (Brizuela et al., 2001).

In this thesis study, the author cloned and sequenced eight *Xenopus laevis* claudins: *Xcla1*, *Xcla4B*, *Xcla5*, *Xcla6*, *Xcla12*, *Xcla16*, *Xcla18*, and *Xcla19*. The RNA expression patterns of *Xcla4A*, *Xcla4B*, *Xcla5*, and *Xcla7* from cleavage stage to tadpole stage were identified. The ectopic expression patterns of *Xcla4A*, *Xcla4B*, *Xcla5*, *Xcla7*, and BMP4

after exposure to xenoestrogen Bisphenol A (BPA) were identified. Some preliminary data from overexpression of *Xcla5* are reported.

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## CHAPTER 2

### EXPERIMENTAL PROCEDURES

#### Molecular Cloning

The human and mouse claudin nucleotide sequences were retrieved from the National Center for Biotechnology Information (NCBI). They were searched against the *Xenopus* expression sequence tag (EST) database using the Basic Local Alignment Search Tool for Nucleotides (BLASTN) program to search sequence databases in order to identify *Xenopus* claudin homologs. The identified *Xenopus* claudin ESTs or Mammalian Gene Collection (MGC) sequences were then searched against the human, mouse, and zebrafish protein database using TBLASTX to confirm that they, in fact, code for claudins and identify their putative human orthologs.

As described in detail in the Results, eight *Xenopus* ESTs and MGCs clones with sequence homology to human claudins were found. They are shown in Table 2-1. The cloning vectors were transformed into TOP10 Chemically Competent *E. coli* cells using one-step heat shock protocol (Invitrogen). *E. coli* were grown in LB media with Ampicillin resistance for 16-18 hours at 37°C with rocking. The cDNAs were prepared with a QIAGEN mini-prep kit. DNA concentration and quality were measured by NanoDrop spectrophotometer (DN-1000). The inserts were linearized by the relevant restriction enzymes and run on 1% agarose gel to roughly map their identity.

EST clones, to obtain full-length cDNA, were sequenced with Applied Biosystems 3730XL through the University of Chicago Cancer Research Center (CRC). MGC clones were also sequenced by CRC to ensure that no sequence errors had occurred. Standard sequencing primers (M13, T7, T3 or Sp6) used for each clone sequencing can be found in

the relevant cloning vector, *pBluescript* and *pCMV-SPORT6* as shown in Table 2-2.

Sequence analysis software was Chromos.2.2 and Macintosh Vector7.0

**Table 2-1** Claudin GeneBank accession numbers and other resources

<i>Xenopus laevis</i> claudin species	EST or MGC number	Human orthologs Accession number	Mouse orthologs Accession number	Chick orthologs Accession number	Lab or vendor
<i>Xcla 1</i>	BC042293	AY358652			Biosystems
<i>Xcla4A</i>	AF224712 AF359435	BT006989			Dr. Brizuela
<i>Xcla4B</i>	AW159511 AW158148 *AY919668	BT006989			Cold Spring Harbor Labs
<i>Xcla 5</i>	BC060483	BC002404	NM-013805	AF334678	Biosystems
<i>Xcla 6</i>	BX846721	NM_021195			RZPD, Germany
<i>Xcla12</i>	CA983044	BC068532			Biosystems
<i>Xcla16</i>	CF546671	BC069759			Biosystems
<i>Xcla18</i>	BC060425	NM_016369			Biosystems
<i>Xcla 19</i>	BX850217	NM_148960			RZPD, Germany
*Claudin 7L1	AB072910	**	**	**	Dr. Taira

Note: \*:GeneBank Accession number of full length cDNA

\*\**Xenopus* claudin 7 was used in this study.

**Table 2-2** Claudin vector and promoter information

<i>Xenopus</i> claudin species	Vector	Sequencing primers	mRNA Expression patterns	Promoters and enzyme to make anti- sense mRNA
<i>Xcla1</i>	<i>pCMV-SPORT6</i>	N/A	Available	T7, <i>Sal1</i>
<i>Xcla4A</i>	<i>pCS2<sup>+</sup></i>	N/A	Available	T7, <i>EcoR1</i>
<i>Xcla4B</i>	* <i>pBluescript</i> and <i>pCS2<sup>+</sup></i>	T7/T3 in <i>pBluescript</i>	Available	T3, <i>EcoR1</i>
<i>Xcla5</i>	* <i>pCMV-SPORT6</i> and <i>pCS2<sup>+</sup></i>	T7/M13R	Available	T7, <i>Sal1</i>
<i>Xcla6</i>	<i>pCMV-SPORT6</i>	M13F/M13R	N/A	N/A
<i>Xcla7</i>	<i>pBluescript SK-</i>	N/A	Available	T7, <i>EcoR1</i>
<i>Xcla12</i>	<i>pBluescript SK-</i>	M13F/M13R internal primer walking	N/A	N/A
<i>Xcla16</i>	<i>pCMV-SPORT6</i>	Sp6/M13R	Available	T7, <i>Sal1</i>
<i>Xcla18</i>	<i>pCMV-SPORT6</i>	N/A	Available	T7, <i>Sal1</i>
<i>Xcla19</i>	<i>pCMV-SPORT6</i>	M13F/M13R internal primers walking	N/A	N/A

Note\* There are two versions of these plasmids, *pBluescript* vector is for *in situ* hybridization, *pCS2<sup>+</sup>* vector is for making mRNA for microinjection.

N/A Full length sequence comes come GeneBank; expression patterns have not been finished

## **Plasmid Constructs**

Sense and anti-sense RNA were made on the cloning vector *pBluescript* or *pCMV-SPORT6*. mRNA for injection was made on the *Xenopus* expression vector *pCS2<sup>+</sup>*. *Xcla4B* and *Xcla5* were subcloned into *pCS2<sup>+</sup>* from cloning vector *pBluescript* and *pCMV-SPORT6*, respectively. *Xcla4B* was digested with *XbaI* and ligated into *pCS2<sup>+</sup>* *XbaI* sites. *pCS2<sup>+</sup>* vector was treated with Calf Intestinal Alkaline Phosphatase (CIAP) to dephosphorylate the ends to avoid self-ligation. Inserts were cleaned with a gel purification kit (Promega). The ligation reaction was carried out overnight at 16°C in a PCR machine. The recombinant plasmids were transformed into TOP10 *E.coli* competent cells (Invitrogen) and selected by Ampicillin resistance. For *Xcla4B* construct, the promoter orientation was confirmed by restriction enzyme mapping. The appropriate constructs were selected according to the criterion that sense RNA can be synthesized by Sp6 RNA polymerase. The new constructs were sequenced by CRC to ensure that no mutation had occurred in the subcloning procedure. *Xcla5* was cloned into *pCS2<sup>+</sup>* as above but using *XhoI* restriction enzyme. *NcoI* and *EcoRI* mapping confirmed the orientation of Sp6 promoter. The construct of GFP tagged with *c-myc* was kindly provided by Dr. Klymkowsky.

## ***Xenopus* Whole Mount *in situ* Hybridization**

### **Synthesis of mRNA probes**

Synthetic sense and anti-sense mRNA were made based on the protocol from the DeRobertis lab

[http://www.hhmi.ucla.edu/derobertis/protocol\\_page/XenopusWholeMountInSitu.pdf](http://www.hhmi.ucla.edu/derobertis/protocol_page/XenopusWholeMountInSitu.pdf)

Approximately 10 ug claudin 5 cDNA were linearized with 5' overhang restriction enzyme *Sal* I and *Not* I, respectively at 37°C overnight. Electrophoresis on 1% TBE agrose gel ensured the completeness of digestion. The template was cleaned by extracting two times in phenol/Chloroform followed by one time with chloroform. The template was then precipitated by 100% ethanol for 15 minutes at -80°C and washed with 70% ethanol. NanoDrop (DN-1000) measured the template concentration and quality. Template with an OD number 1.8 was used for the transcription reaction. In a total volume of 20 ul transcription reaction the template was more than 0.6 ug but less than 1.2 ug.

The transcription reaction was RNase free and based on the protocol provided by the manufacturer (Roche 1277073). RNA guard was used to protect against RNase. T7 RNA polymerase and Sp6 RNA polymerase were used for *Sal* I and *Not* I digested template respectively. Digoxigenin-labeled UTG was incorporated into synthetic mRNA (Roche 881767). The reaction was carried out at 37°C in a water bath for 2 hours and terminated by 0.2 M EDTA. A native 1% agrose gel was run to ensure the RNA products. The DNA template was not removed from the products. The unincorporated NTP was removed by ethanol with LiCl precipitation at -80°C for 15 minutes. RNA was washed with 70% ethanol and quantified by NanoDrop (DN-1000). Sense and anti-sense mRNA were aliquoted and stored at -80°C until needed for hybridization.

### **Preparation of *Xenopus* embryos**

*In vitro* fertilization was carried out based on the protocol from the DeRobertis lab [http://www.hhmi.ucla.edu/derobertis/protocol\\_page/FertilizationOfFrogEggs.pdf](http://www.hhmi.ucla.edu/derobertis/protocol_page/FertilizationOfFrogEggs.pdf)

Female albino *Xenopus* frogs were primed one week ahead with 30-40 units human chorionic gonadotropin (hCG) and induced for ovulation with 400 units hCG. Two percent cysteine pH7.8 was used to take off the jelly coat. Embryos were staged according to the Nieuwkoop normal table (Nieuwkoop and Faber J, 1994). Different stage embryos were fixed at room temperature (R/T) with MEMFA (0.1M MOPS, pH 7.4, 1 mM EGTA, 2 mM MgSO<sub>4</sub>, and 3.7% formaldehyde) for 45 minutes to 90 minutes. The embryos were dehydrated at R/T by sequential washings in 25% methanol/PBSW, 50% methanol/PBSW, 75% methanol/PBSW for 5 minutes, and finally 100% methanol two times, 5 minutes each time. Then the embryos were stored at -20°C for up to 6 months ready to be used for *in situ* hybridization.

### **Hybridization procedures**

The embryos were rehydrated at room temperature by sequential washings in 75% methanol/ PBSW, 50% methanol/PBSW, 25% methanol-75% PBSW and finally PBST, each for 5 minutes. In order to facilitate RNA penetration of the cell membrane, embryos were digested with proteinase K for around 8 minutes and refixed with 4% paraformaldehyde/0.2% glutaraldehyde at R/T for 15 minutes. Embryos were prehybridized in hybridization solution (1%Boehringer block, 50%formamide, 25%20xSSC, 10%Torula RNA (10 mg/ml in water; filtered), 0.2% Heparin (50 mg/ml in1xSSC), 5% Tween-20 (20% Tween-20), 1% CHAPS (10% CHAPS), and 1% EDTA (0.5M)) for 3 hours at 65°C with rocking. Then embryos were hybridized with sense and anti-sense RNA probes overnight at 70°C with rocking. The endogenous alkaline phosphatase was inhibited by MAB solution (100 mM Maleic acid, 150 ml NaCl, pH 7.5). Anti-Digoxigenin antibody conjugated with alkaline phosphatase (Roche) was

diluted 10,000 fold from a stock of 150 units per 200 ul and preblocked with antibody buffer (10% heat inactivated goat serum, 1% Boehringer block, and 0.1% Tween-20 in PBS, vortexed and filtered with 0.45um membrane). Embryos were incubated with anti-Dig-fragment in antibody buffer overnight at 4°C with rocking. BM purple (Roche) staining indicates the mRNA location. The embryos were dehydrated by methanol series as above and stored at -20°C.

### **Bleaching of pigmented embryos**

For pigmented embryos, the pigment was bleached with bleach solution (6% H<sub>2</sub>O<sub>2</sub>, 5% formamide, 0.5xSSC) for 2 hours under the light with rocking.

### **Wax embedding after *in situ* hybridization**

This protocol is based on the protocol from the Moss lab:

<http://www.chuq.gc.ca/labomoss/Protocols/Protocols%20X%20ooc%20emb.html>

After whole mount hybridization, the embryos were dehydrated stepwise for 10 min each through: 50% methanol / 50% saline (0.85% NaCl), 70% methanol in water, 85% methanol in water, 95% methanol in water, 100% methanol twice. Before embryos were transferred into paraffin wax, they were processed through the following transition for 30 minutes each time: 100% dry methanol; 50% dry ethanol / toluene; 100% Toluene. Then embryos were immersed in paraplast I for one hour. After that embryos were switched into paraplast II and immersed overnight. Paraplast I and II were melted at 60 °C to 62 °C overnight in the ratio of paraplast: tissueprep 2: 1 (both Fisher Brand). Finally the embryos were removed into the metal moulds and were rapidly oriented under a dissecting microscope while the wax slowly solidified on a heated platinum wire.

### ***Xenopus* Whole Mount Immunostaining**

Embryos were fixed with Dents fix (4 volumes methanol and 1 volume DMSO) for 1 to 2 hours at room temperature with gentle rocking (Dent et al., 1989). The pigmented embryos were bleached with 10% H<sub>2</sub>O<sub>2</sub> under the lamp light for 2 days until the embryos became completely white. Embryos were switched into 100% methanol (changed two times) and stored at -20°C until immunostaining.

Embryos were washed with PBS (phosphate -buffered saline) 2 times, 10 minutes each time. Then the non-specific staining was blocked with 20% Fetal Bovine Serum in PBT (0.1% Triton in PBS) for 1hour at room temperature with rocking. In this experiment, Claudin 5 rabbit anti-mouse polyclonal antibody (Cat.No.34-1600, Zymed laboratories Inc) was used. The secondary antibody was FITC conjugated affinity Pure Donkey anti-rabbit IgG (Code number: 771-095-152 Jackson Laboratory Inc.) The primary and secondary antibodies were diluted in PBT serum appropriately and collected after use for the future use. The embryos were incubated in the primary antibody at 4°C overnight with rocking. Embryos were washed in PBT for 5 hours at room temperature with rocking. Embryos were incubated in Cy3 or FITC-conjugated secondary antibody at 4°C overnight. The embryos were washed with PBT and then were photographed.

### **mRNA Microinjection System**

#### **Capped mRNA synthesis**

For mRNA microinjection, first, inserts were ligated into an expression construct under the Sp6 promoter orientation. Sequencing ensured no reading frame shifts. Capped mRNAs were synthesized using the Ambion Message Machine kit (Ambion.) The

procedure provided by Ambion was strictly followed. DNase I removed the DNA template. The quantity and purity of mRNA were measured on a NanoDrop spectrophotometer. After Ambion's column cleaning procedure, the mRNA with OD value 1.9 was used for injection. The mRNA was quantified, aliquoted and stored in DEPC treated water at  $-80^{\circ}\text{C}$  until use.

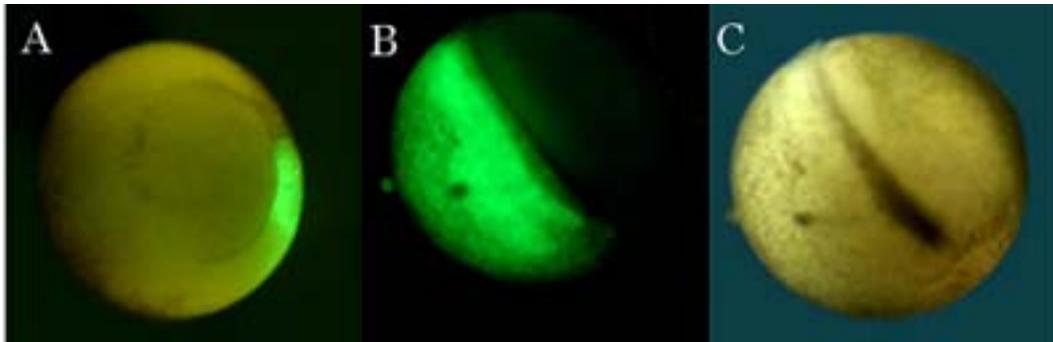
### **mRNA microinjection**

All procedures were carried out in a nominally RNase-free environment. RNA was injected into blastomeres by pneumatic pressure (PL1-100, picoinjector; Harvard Apparatus, Cambridge, MA) using Narishige manipulators (Medical Systems Corp., Great Neck, NY). Glass micropipettes were pulled with a micropipette puller (Model P-97 micropipette puller, Sutter instrument, Novato USA).

The original RNA concentrations of *Xcla5* and GFP were 159ng/nl and 170ng/nl, respectively. They were diluted 1:1 for injection. Eight nanoliter mixed RNA was injected into different stage blastomeres in 1 x Barth solution (88 mM NaCl, 1 mM KCl, 0.7 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , 5 mM HEPES, pH 7.8, 2.5 mM  $\text{NaHCO}_3$ ). Embryos were kept to stay for a while in 1x Barth for healing. Then embryos were transferred into 0.1xBarth (8.8 mM NaCl, 0.1 mM KCl, 0.07 mM  $\text{CaCl}_2$ , 0.1 mM  $\text{MgSO}_4$ , 0.5 mM HEPES, pH 7.8, 0.25 mM  $\text{NaHCO}_3$ ) and incubated at room temperature. The un-injected side was used as an internal control. Injection of GFP alone under the same concentration and volume was used as a negative control. Un-injected embryos from the same mother and equivalent fertilization time were used as a control as well. Different *Xcla5* RNA concentrations were tried by different mixtures with GFP RNA (the ratio of GFP and *Xcla5* was 2:1 or 3:1)

### **GFP mRNA used as a lineage tracer**

The lineage tracer, synthetic GFP (green fluorescent protein) mRNA is clear enough and not toxic to embryos within the injection range, i.e. the injection volume should be less than 10nl and the mRNA amount should be less than 5ng (Sive et al., 2000). As shown in Figure 2-1, GFP protein was synthesized by embryos and emitted green fluorescence. The un-injected side was used as an internal control showing no GFP fluorescence.



**Figure 2-1** GFP mRNA was used as a lineage tracer. Image A was taken under both GFP filter and normal light. Images of B and C are from the same embryo under GFP filter and normal light, respectively.

### **Microscopy and Photography**

A Leica-M-Series MZFLIII Stereo microscope was used for imaging. The pictures were taken with a Nikon Digital Camera DXM 1200. The software was ACT-1 version2.6. The pictures were taken at 3200x2560 resolution and saved as TIF files. Adobe Photoshop 7.0 was used to make figures.

#### Reference List

1. Dent, J.A., Polson, A.G., and Klymkowsky, M.W. (1989). A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. *Development* *105*, 61-74.
2. Nieuwkoop, P.D. and Faber J (1994). Normal Table of *Xenopus laevis* (DAUDIN)  
. A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis (Abstract)
3. Sive, HL., Grainger, RM., and Harland, R. (2000). Early development of *Xenopus laevis*. A laboratory manual. Cold Spring Harbor Laboratory Press (Abstract)

## CHAPTER 3

### RESULTS AND DISCUSSION

#### **The sequences of *Xenopus laevis* claudins**

*Xenopus laevis* claudin cDNAs were cloned and sequenced. Their relationship to claudins of other species is shown in the phylogenetic tree of Figure 3-1. Claudin proteins are present evolutionarily in phylum vertebrate from class Amphibia to Mammalia. *Xenopus* claudins are homologous to zebrafish, mouse, and human claudins in amino acid sequence. But it is not known whether their functions are similar.

The predicted multiple amino acid sequence alignment of *Xenopus* claudins is shown in Figure 3-2. The amino acid sequence is conserved in the first and fourth transmembrane domains as well as in the first and second extracellular loops but diversified in the second and third transmembrane domains. C-termini of most of the claudins end with YV, with the exception of *Xcla16* and *Xcla18*, in which the amino acid sequences in the cytoplasm are diversified (data not shown).

Using the computer programs (<http://www.sbc.su.se/%7Eerikw/toppred2/> and <http://us.expasy.org/prosite/>), *Xcla5* topology and schematic functional sites were predicted and shown in Figure 3-3 and Figure 3-4, respectively. The carboxyl termini and amino termini are in the cytoplasm, with 7 amino acids in amino termini and 25 amino acids in the carboxyl termini. The first extracellular loop has 53 amino acids with a claudin family signature (amino acid site 49-64); the second extracellular loop has 28 amino acids with one protein kinase C phosphorylation site (amino acid site 155-157); the intracellular loop has 12 amino acids with one protein kinase C phosphorylation site

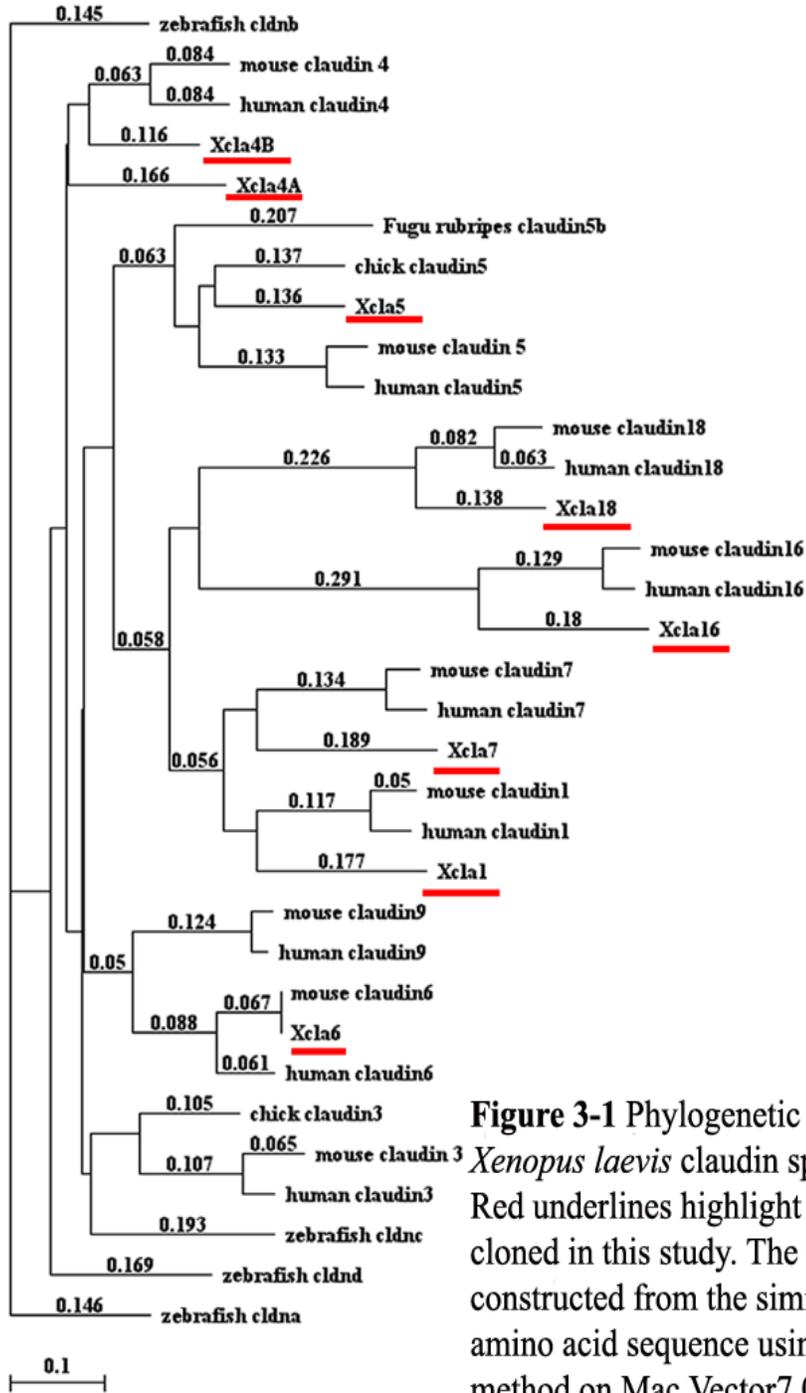
(amino acid site 112-114); the program also predicts seven N-myristoylation sites which may help *Xcla5* localize on the lipid bilayer membranes (amino acid sites not shown).

#### **Nomenclature of *Xenopus laevis* claudin 4**

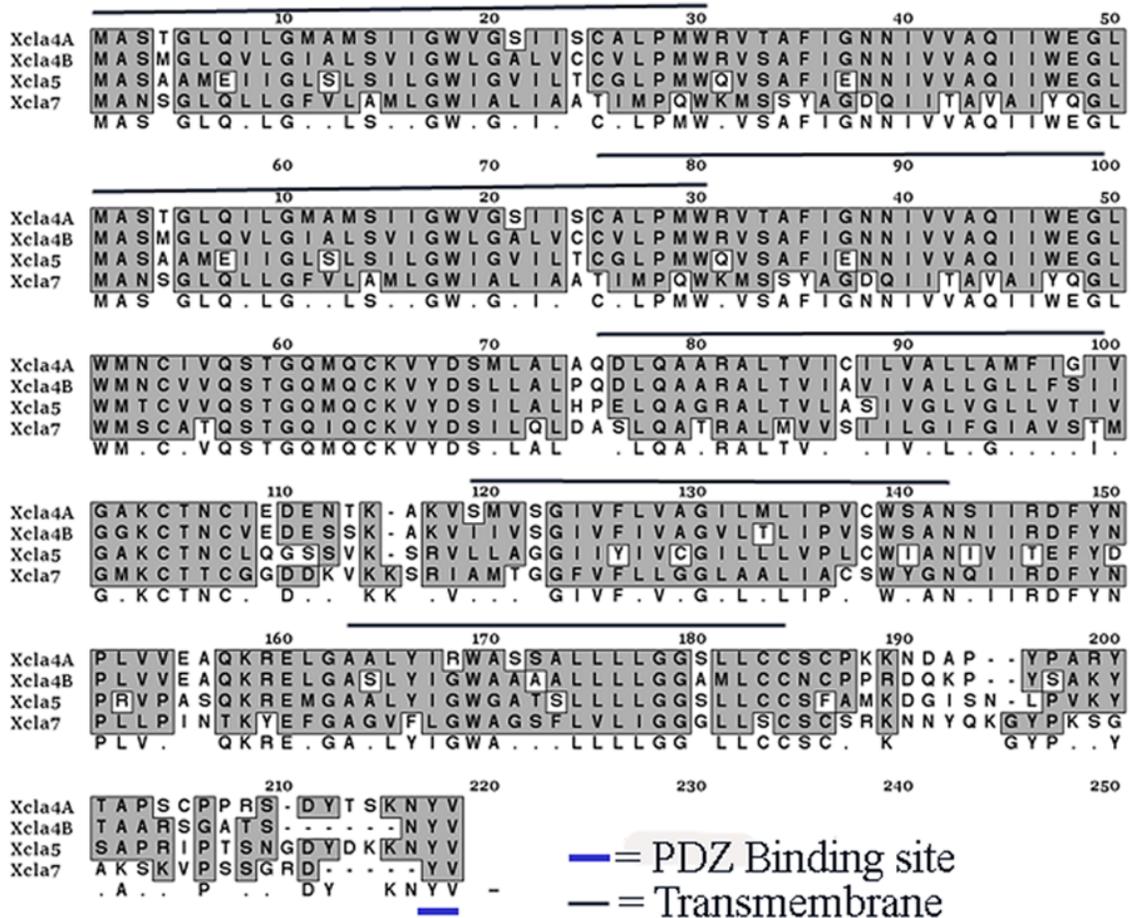
*Xcla 4A* was originally reported as *Xcla* (Brizuela et al., 2001). *Xcla* has 92.5% amino acid and 88.5% DNA identity to *Xenopus claudin 4L1* (Furuse et al., 1998), has 77% amino acid similarity to *zebrafish cldnb*, and 74% similarity to *zebrafish cldna*. Although Hudspeth AJ *et al* (Kollmar et al., 2001) referred to *Xcla* as *cldna*, it is actually not the same as the *Xcla A* gene. Masanori et al suggested that *claudin-4L1* and *Xcla* may correspond to A and B genes of *Xenopus* pseudotetraploid.

A new clone called C11 was pulled out in this study. Based on tblastx searching in NCBI (using nucleotide sequence search against translated database), C11 has 65% (136/209) similarity and belongs to the human claudin 4 homologs (Clostridium perfringens enterotoxin receptor 1, CPE-R1, Williams-Beuren syndrome chromosome region 8 proteins). C11 is also similar to mammalian claudin 3, 4, 6, 9 and *zebrafish cldnd*. C11 has 75% similarity with human claudin 3 (Clostridium perfringens enterotoxin receptor 2, CPE-R2, ventral prostate 1 protein, RVP1, located in chromosome 7. Pair-wise alignment (amino acid sequence comparison in software Vector Mac 7.0) shows that C11 and *Xcla* share 76% similarity. Since C11, *claudin4L1*, *claudin4L2*, and *Xcla* are very similar in sequence, it is possible that these genes are 4 similar copies of claudin 4 in *Xenopus* pseudotetraploid. Therefore, the nomenclature here will refer to *Xcla* as *Xcla4A* and C11 as *Xcla4B*. All other claudins isolated from *Xenopus laevis* will follow this nomenclature: *Xenopus* claudin (*Xcla*) and the number of the gene, i.e., *Xenopus* claudin 5 will be referred to as *Xcla5*.

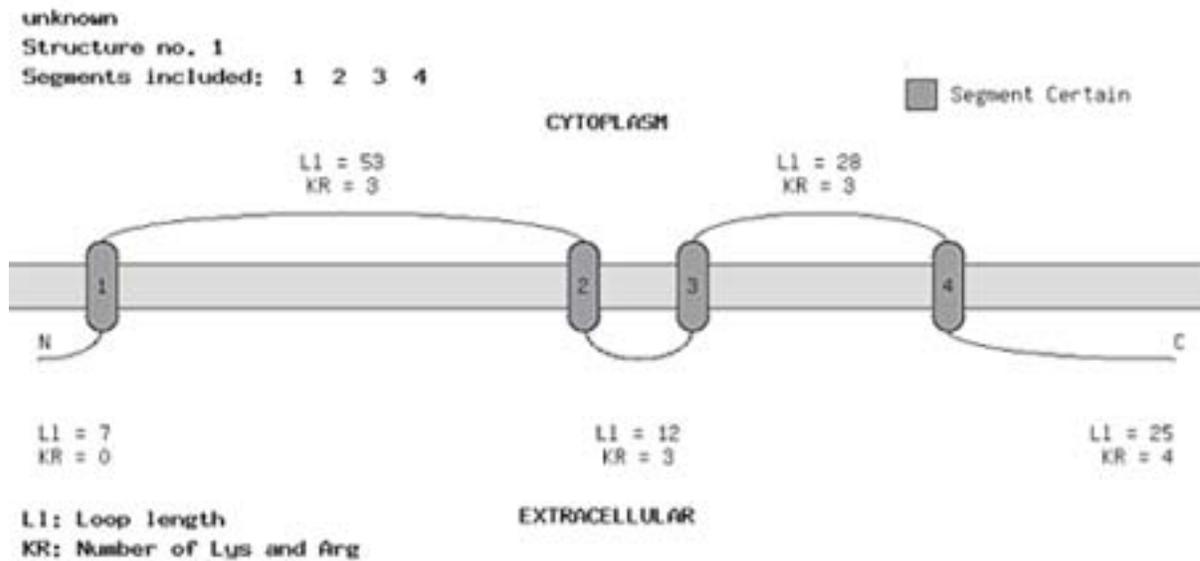
ClustalW (v1.4) Multiple Alignment Parameters:  
 Open Gap Penalty = 10.0; Extend Gap Penalty = 0.1; Delay Divergent = 40%  
 Gap Distance = 8; Similarity Matrix = id



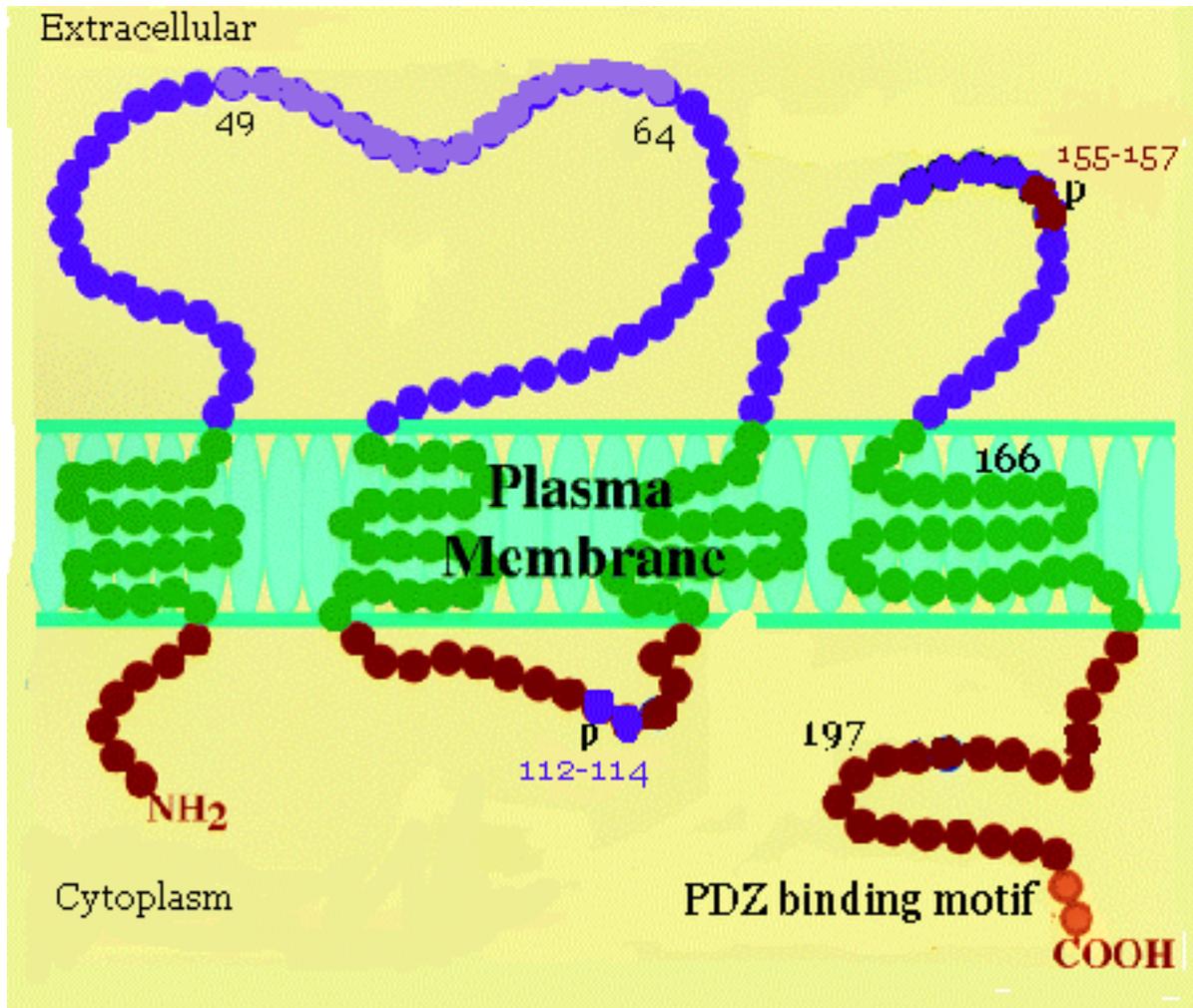
**Figure 3-1** Phylogenetic analysis of *Xenopus laevis* claudin species. Red underlines highlight the cDNAs cloned in this study. The tree was constructed from the similarity of amino acid sequence using the UPGMA method on Mac Vector7.0 software



**Figure 3-2** Amino acid sequence comparison of *Xenopus* claudins *Xcla4A*, *Xcla4B*, *Xcla5*, and *Xcla7*. *Xenopus* claudin proteins have four predicted transmembrane domains, two extracellular loops, one intracellular loop, and a cytoplasmic carboxyl terminus, which ends with YV, a PDZ binding site.



**Figure 3-3** Topology of *Xcla5* predicted by TopPred 2. There are 7 amino acids in the N-terminus and 25 amino acids in the C-terminus. The first extracellular loop has 53 amino acids and the second extracellular loop has 28 amino acids. The intracellular loop has 12 amino acids.



**Figure 3-4** Schematic structures of *Xenopus laevis* claudin 5 protein (Xcla5) predicted by TopPred 2 and proSite. Amino acid residues from 49-64 are the claudin family signatures, and from 166-197 are the protein kinase ATP binding regions, from 112-114 and from 155-157 are the protein kinase C (PKC) phosphorylation sites. Their location in this figure is based upon assumed similarity to the predicted structure for claudin 4 illustrated in Figure 1-2. That puts residues 112-114 on an intracellular loop for both claudins but predicts residues 155-157 to be on an extracellular loop; it may be that that region of the protein actually is associated in claudin 5 with the membrane or C-terminus.

## **mRNA expression of *Xenopus laevis* claudins**

### **Claudin 5**

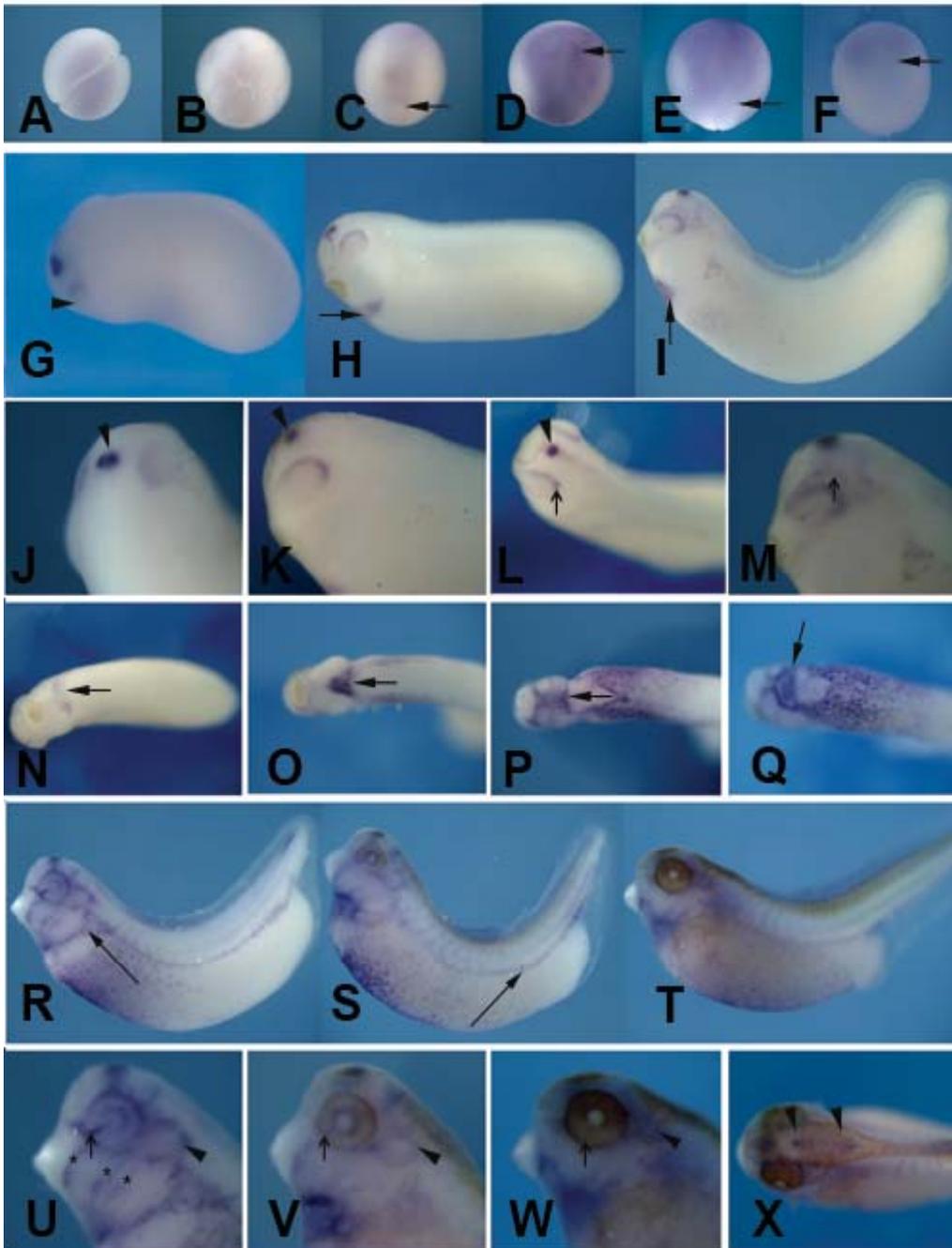
Mouse claudin 5 is ubiquitously expressed in the endothelial cells of all tissues, except in kidney (only in arteries) (Morita et al., 1999b). Further, it was confirmed that, like vascular endothelial-cadherin, claudin 5 is an endothelial specific claudin species (Morita et al., 1999a). In physiology, claudin 5 is important in determining and regulating vascular permeability, especially in regulating the tightness of the blood brain barrier (BBB). In claudin 5 knockout mice, the BBB was size-selectively loosened, but without bleeding and edema, which promotes the idea that claudin 5 can be regarded as a potential target for developing a new drug delivery method for the central nervous system. However, claudin 5<sup>-/-</sup> mutant mice died within 10 hours of birth. The relationship between BBB impairment and death is under investigation (Nitta et al., 2003a).

Claudin 5 is also called a transmembrane protein deleted in velo-cardio-facial syndrome (TMVCF) and maps to human chromosome 22q.11.2 region (Sirotkin et al., 1997). VCF syndrome is a developmental defect that exhibits craniofacial and cardiac defects resulting from abnormal development of the third and fourth neural crest-derived brachial arches and brachial arch arteries (Lammer and Opitz, 1986). VCF syndrome is grouped into CATCH-22 (cardiac defects, abnormal faces, thymic hypoplasia, cleft palate, hypocalcemia, associated with chromosome 22 microdeletion). Numerous studies have been done on the signaling cascade involved in CATCH-22 (Yamagishi et al., 1999; Thomas et al., 1998; Srivastava et al., 1997). Therefore, claudin 5 may play an important

role in regulating the developmental process (Ikenouchi et al., 2003), especially in regulating neural crest cell migration.

### ***Xcla5* expression pattern**

The dynamic expression pattern of *Xcla 5* is shown in Figure 3-5, from the cleavage stage to the tadpole stage. Maternal *Xcla5* mRNA is only weakly expressed at cleavage and blastula stages and appears stronger after gastrulation. Two areas of expression can be seen. One is over the dorsal lip and the second is in the neural plate that is moving more anteriorly and more vegetally as neurulation proceeds. These two areas may demark the future head and heart fields. At the late neural stage *Xcla5* is expressed in the brain and primordium of heart, and vessels. As development proceeds, endothelial cells in the cardiovascular system are strongly stained, especially the aortic arch and common cardinal veins. Expression is seen at a sharply defined region on the midbrain. Expression in the eye begins as a single crescent shape at the late neural stage and progresses to a complex pattern, which may define the blood vessels around the retina by the tailbud stage. Also, there is a line of expression at the ventral fissure as the lens is forming and around the area outside of the otic vesicle region.



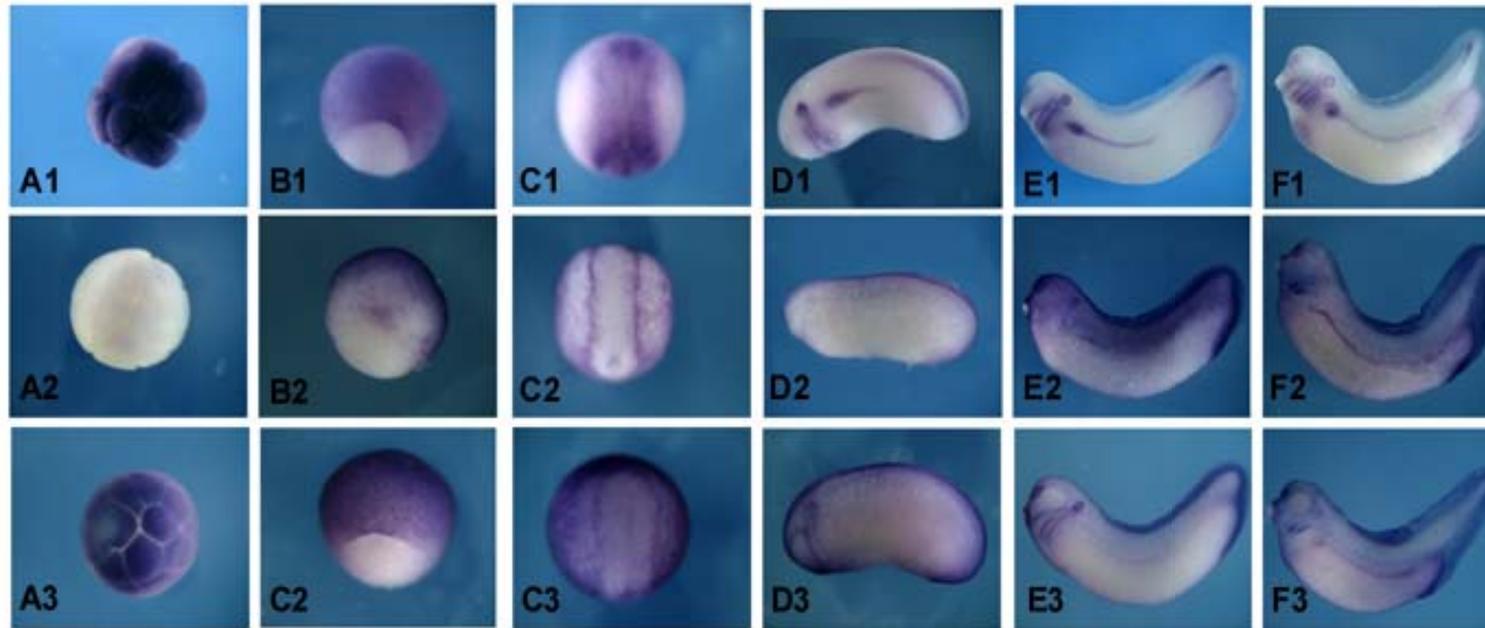
**Figure 3-5** Expression pattern of *Xcla5* in development. Little RNA was detected before blastula stage (A and B). At gastrula stage, RNA was detected at dorsal lip (C). At neural stage (D-F), transcripts were strongly detected at dorsal side including neural plate and ectoderm. At early tailbud stage (G), RNA was detected at the anlage of heart (black arrow head) and brain; At middle tailbud stage (H-M), RNA was detected at the primordium of heart (ventral view, black arrowhead in N-Q) and the brain (J-L, black arrowhead) and eye (L-M, arrowheads). As the embryos develop, the staining in the endothelial cells indicates gradual formation of cardiovascular system (O-T). At tadpole stage (U-X), the endothelial cells were stained at different organs, mainly eye, heart, and different vessels.

### **Comparison of *Xcla4A*, *Xcla4B*, and *Xcla7* expression patterns**

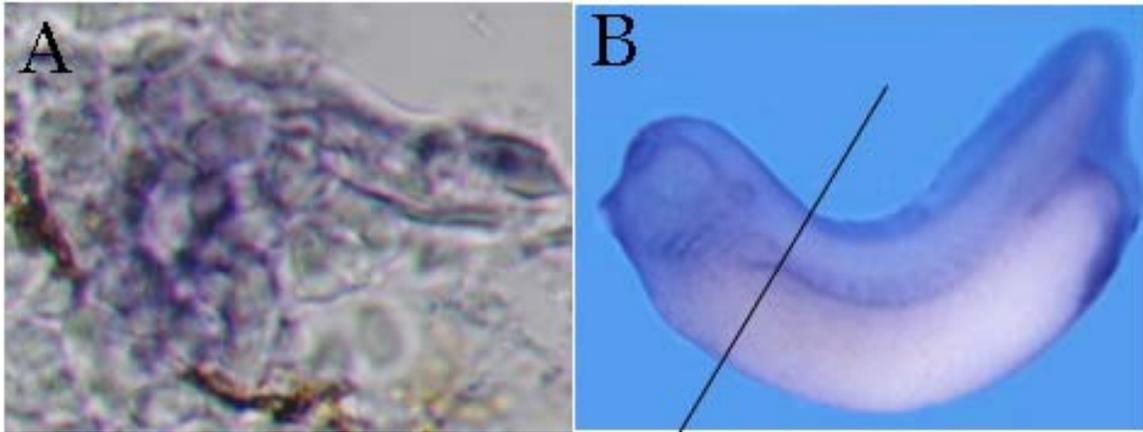
*Xcla4A*, *Xcla4B*, and *Xcla7* have high homology in their sequence (data from pair-wise amino acid sequence comparison are not shown). From the literature, they are expressed in similar organs such as in brachial arches, ear, rectal diverticulum, olfactory placode, and kidney. In this study, comparison of the expression patterns suggested that functional redundancy of different claudin species might result from the overlapping temporal and spatial expression.

As shown in Figure 2-6, *Xcla7* and *Xcla4A* are strongly expressed maternally, whereas *Xcla4B* transcripts seem to be strongly expressed only after midblastula transition (MBT), when zygotic genes are transcribed. The organ expression pattern shows that *Xcla4A* is expressed at the tubules and ducts of pronephros (beginning at the neural stage), whereas *Xcla4B* and *Xcla7* show expression only in the ducts and the rectal diverticulum, which begins later, at the tailbud stage. In addition, *Xcla4B* and *Xcla7* are expressed in the surface of ectoderm. At the neural stage, *Xcla4B* and *Xcla7* are expressed at the epidermis outside of the neural plate, whereas *Xcla4A* is strongly expressed in a complementary pattern in the neural plate. The other interesting difference in expression pattern is in the brachial arches. For *Xcla4A*, as development proceeds, RNA expression is seen as the arches develop, first in two brachial arches, then in three brachial arches, finally four brachial arches, progressively activated from anterior to posterior in the visceral arches. *Xcla7* is expressed first in two brachial arches and then three brachial arches and appears to be in the surface layer, while *Xcla4B* has only ectoderm expression in the gills. *Xcla5* does not have this pattern. However, the protein can be seen by immunostaining and appears to be expressed in the endoderm layer (Figure 3-8).

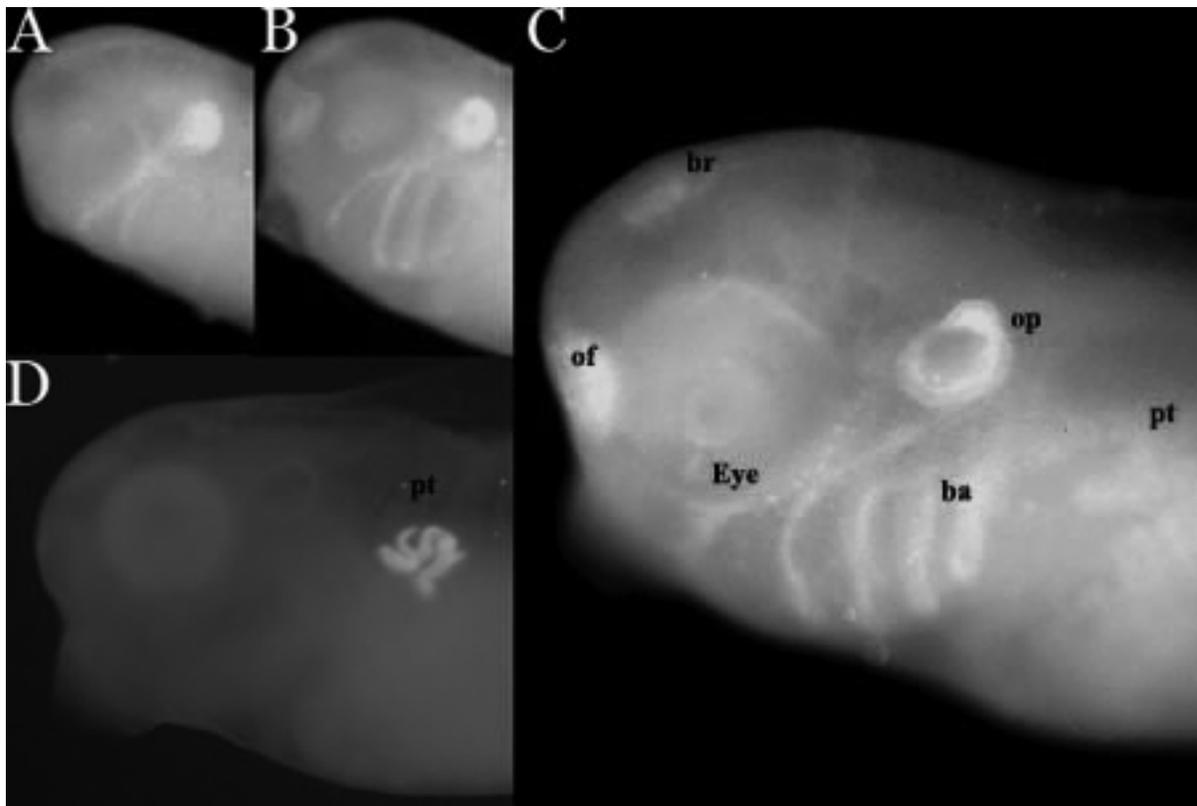
Therefore, these expression results support the hypothesis that different associations of claudin isoforms regulate the tightness of tight junction. But, very little is known about the molecular mechanism of how different claudin species are assembled. For example, is the tightness of the tight junction regulated in response to different environments? Such questions will be left for physiological studies. In this study, we focus on developmental expression of claudins.



**Figure 3-6** Comparison of *Xcla4A*, *Xcla4B* and *Xcla7* mRNA localizations. A1-F1 represent *Xcla4A*; A2-F2 represent *Xcla4B*; A3-F3 represent *Xcla7*. Cleavage stage expression patterns are shown in column A; gastrula stage in column B; neural stage in column C; late neural stage in column D; early and late tailbud stages E-F. *Xcla4A* and *Xcla7* expression are seen from maternal stage (A1 and A3), whereas, *Xcla4B* transcription turns on at midblastula stages (A2-B2). At neural stage, *Xcla4B* and *Xcla7* are expressed outside of the neural plate (C2-C3), whereas *Xcla4A* is expressed in a complementary pattern in the neural plate (C1). *Xcla4A* expression is gradually seen from brachial arch 1 to 4 as they develop and in the otic vesicles, pronephric tubules and ducts from late neural to tailbud stage (D1-F1). *Xcla4B* and *Xcla7* show only late expression in the ducts and more superficial layers of the ectoderm (D2-F2 and D3-F3). *Xcla4B* is seen in the ectoderm of the gills (F2) and *Xcla7* expression is seen from brachial arch 2-3 in the surface layer (E3-F3).



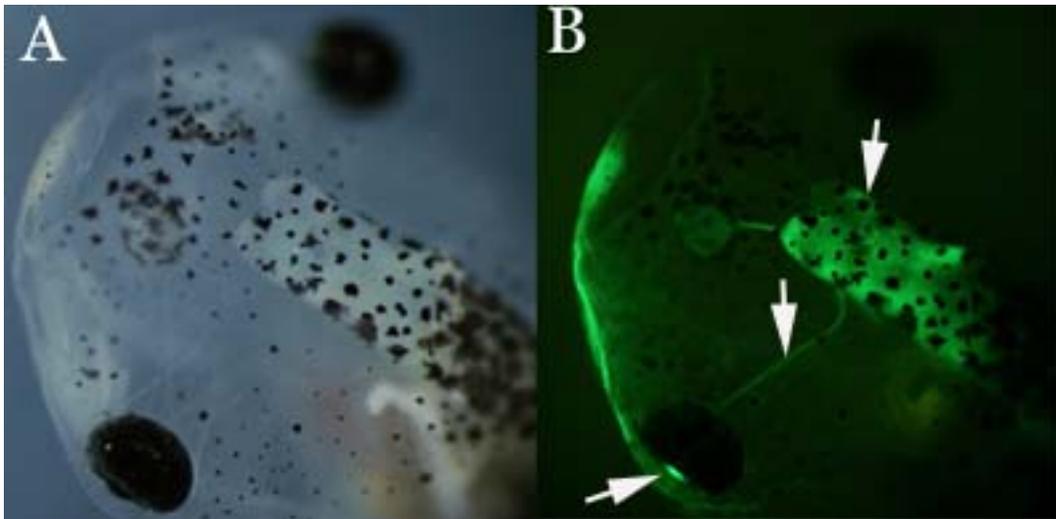
**Figure 3-7** Paraffin section of the embryos stained with anti-sense *Xcla 4B* RNA. (A) Cross section showing the pronephric tubules in the embryos after *in situ* hybridization using *Xcla 4B* probe. The blue color (BM purple from *in situ* hybridization) indicates the RNA location. Hence, *Xcla4B* RNA was detected at pronephric tubules. The yellow color is the debris from sectioning. (B) Where the embryos were sectioned. The black line indicates the location of section.



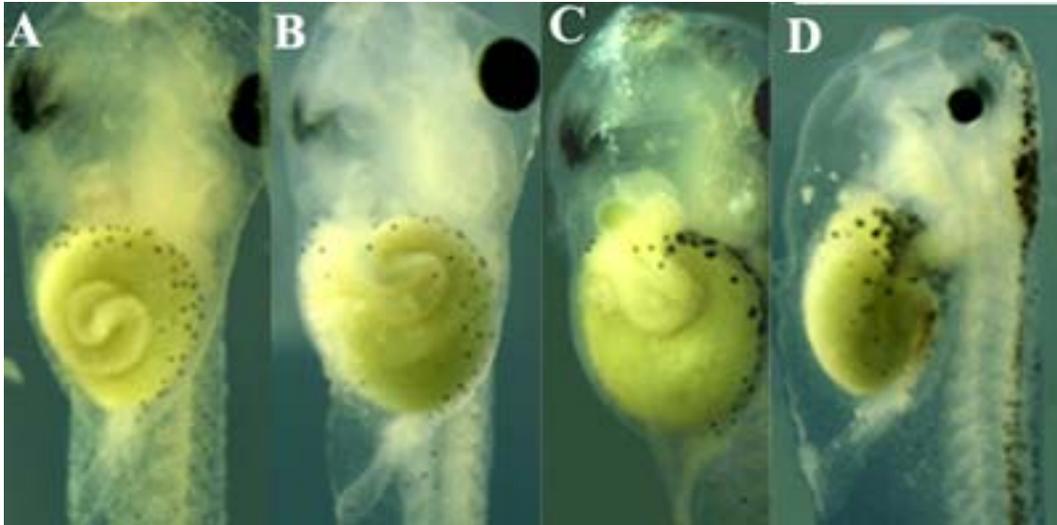
**Figure 3-8** *Xcla5* protein expression. (A) Stage 27-28 embryos. (B) Stage 29-30 embryos. (C) Stage 35-36 embryos. (D) 3G8 positive control showing stained pronephric tubules. br represents brain; of represents olfactory placode; ba represents brachial arches; op represent otic placode; pt represent pronephric tubules.

### Preliminary data from overexpression of *Xcla5* mRNA

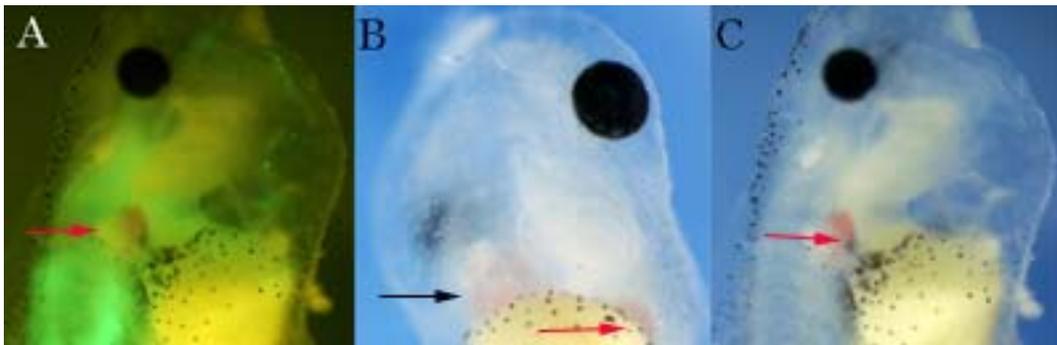
*Xcla5* mRNA injection was titrated to non-lethal concentrations. Until the tadpole stage, embryos showed normal viability. The morphology at the organ level has shown phenotypic change, especially in the group of embryos injected with 340 pg *Xcla5* mRNA. The cellular level has not been tested yet. As shown in Figure 3-9, after injection *Xcla5* protein was seen at eyes, forebrain and cement gland. Compared with control embryos, most of these embryos have shown abnormality in gut looping (Figure 3-10) and retina (data not shown). Interestingly, blood tissue (Figure 3-11) and extra heart (Figure 3-12) were found in these embryos, which may suggest that *Xcla5* is involved in vasculargenesis or angiogenesis.



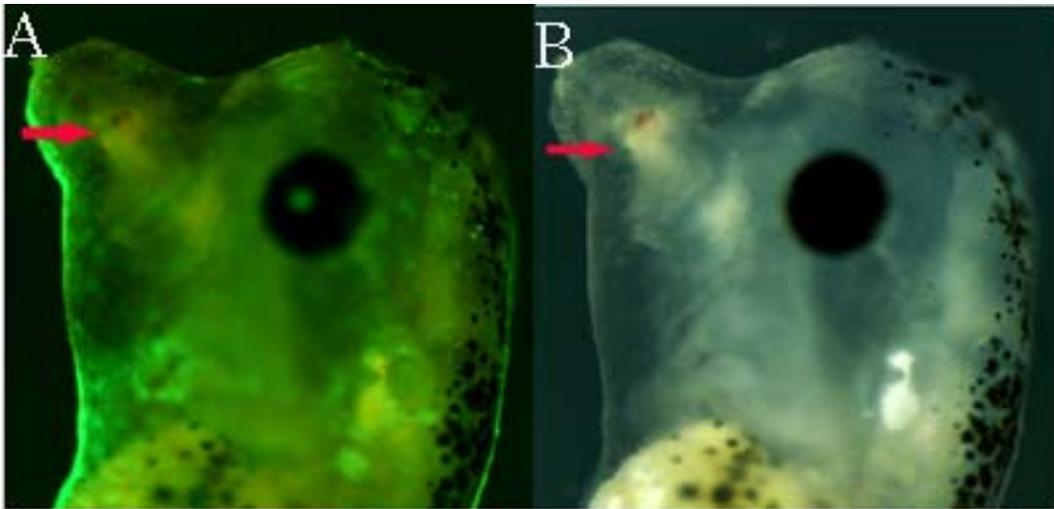
**Figure 3-9** *Xcla5* mRNA was seen at eyes, forebrain and cement gland. (A) The image was taken under the normal light. (B) The image was taken under the GFP filter.



**Figure 3-10** Abnormal gut and heart looping was found in embryos injected with *Xcla5* mRNA. (A) Control tadpole. (B-D) Embryos were injected with *Xcla5* mRNA.



**Figure 3-11** Extra blood tissue was found in the embryos injected with *Xcla5* mRNA. Red arrows indicate extra blood tissues. Black arrow in (B) indicates the heart.



**Figure 3-12** Extra hearts were found on the embryos injected with *Xcla5* mRNA. (A) The image was taken under the GFP filter. (B) The image was taken under the normal light. Red arrows indicate extra hearts.

Currently, the overexpression experiment is still being optimized, especially the injection dosage, the injection time, and the injection spots. So far the evidence suggests that *Xcla5* plays an important role in the developmental process. To ensure the success of the experiments, several preliminary factors were considered. **First**, claudins are in a multiple gene family. The function may be redundant in some family members. In contrast with anti-sense morpholino injection (loss of function), the result from overexpression (gain of function) may give more information due to the difficulty in complete knockdown of all the associated claudin species in order to obtain a null phenotype. **Second**, in claudin 5 knock-out mice, the gross morphology was normal until 10 days of birth. Therefore, claudin 12 may compensate for claudin 5 in maintaining normal function of the BBB (Nitta et al., 2003b). Therefore, double knockout by co-injecting *Xcla5* and *Xcla12* anti-sense morpholinos (chemical form of mRNA which is more stable than nature mRNA) may be tested to produce more severe phenotypes.

Because claudins are important for homeostasis of an organism, double knockout could lead to lethality. Another strategy may be chimeras using an inducible plasmid construct that could allow overexpression or knockdown expression only in selected cells.

This experiment has shown that the mRNA made was non-toxic at the concentrations and conditions used and could be translated into protein. In the future, to ensure that non-GFP protein can be made appropriately, Western Blotting will be needed to confirm the production of protein from injected mRNA. Double *in situ* hybridization or immunostaining of the RNA stained embryos using neural crest cell markers (*Xsnail* or *Xslug*) could provide a way to know the cellular phenotypic change. Also, markers for cardiac tissue will allow determination of the effect of *Xcla5* on ectopic heart development.

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## CHAPTER 4

### BISPHENOL A (BPA) REGULATES CLAUDIN EXPRESSION

#### INTRODUCTION

Bisphenol A (4,4' isopropylidenediphenol or 2,2'-bis(*p*- hydroxyphenyl propane)) is a man-made monomer of polycarbonates that is widely used in industry to make plastic products. The BPA structure is like estrogen and some studies have reported that BPA exhibits hormone-like properties. For this reason, BPA is known as an endocrine disrupter compound (EDC) or more accurately termed a *hormonally active agent*, coined by the U.S National Academy of Science <http://bisphenol-a.org>.

Plastic products provide much convenience for our daily life. It is known that the polycarbonate linkages of BPA are normally resilient but can be hydrolyzed at high temperatures, in which case BPA can be released into the environment (Krishman and Pueppke, 1993). In addition, studies reported that the *hormonally active agent* -BPA is released to the environment from the inner coating of food cans (Brotons et al., 1995), dental composites (Olea et al., 1996), baby-feeding bottles (Mountfort et al., 1997), and plastic waste samples (Yamamoto and Yasuhara, 1999). The potential risk of exposure to BPA for human health has gained great attention in society.

A number of theories have been proposed to explain the potential mechanism of BPA action. One of the theories is that BPA binds to an estrogen receptor (ER) and triggers the ER signaling pathway and function. It is well known that estrogen regulates the permeability of endothelia in different vascular beds (Arnal et al., 2000; Niklaus et al., 2003). (Albrecht and Pepe, 2003; Tatchum-Talom et al., 2003; Tatchum-Talom et al.,

2002)The effect of estrogen on human endothelial permeability is by modulating the paracellular transport via an endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) related mechanism (Cho et al., 1999). Cho *et al* suggested that the estrogen-dependent increase in permeability in endothelium was  $Ca^{2+}$  independent and associated with tight junctions. They reported the effects of estrogen on tight junctional resistance in cultured human umbilical vein endothelial cells (Cho et al., 1998). Indeed, tight junctions are the main regulator of the paracellular permeability in endothelial cells. Claudin and occludin protein are the backbones of the tight junction strands, but more importantly, the assembly of tight junctions via claudin is  $Ca^{+}$  independent as well (Kubota et al., 1999). The influence of  $17\beta$ -estradiol on the expression of tight junction molecules has been investigated and shown to have biphasic effects on human vascular endothelial cells (Ye et al., 2003). The above evidence implies that BPA might function via claudin tight junction molecules.

Amphibians are good vertebrate models to study the effects of environmental chemicals on the development of embryos. It is well known that estrogenic hormones are important in various developmental processes, including sex determination. Like estrogen, BPA has been shown to induce feminization at stage 50-52 in *Xenopus laevis* (Levy et al., 2004). The survival rate of the embryos treated with 5-10  $\mu$ M  $17\beta$ -estradiol after stage 3 decreased markedly by stage 27 and all embryos were dead by stage 42. In addition, these embryos showed malformations after stage 38 (crooked vertebrae, swollen stomach and small head and eyes located close to each other compared with the control). Meanwhile the detection of estrogen receptor in the unfertilized eggs and fertilized embryos suggests that estrogen receptors are involved in the induction of developmental

defects in estrogen-treated *Xenopus* embryos (Nishimura et al., 1997). Interestingly, the malformations of embryos induced by exposure to BPA have been shown to be similar to those induced by 17 $\beta$ -estradiol (Oka et al., 2003). In their study, they also showed the specific apoptosis of central nervous system after exposure to BPA during early development of *Xenopus laevis* embryos.

In this study, early stage embryos were exposed to BPA. Morphological changes observed confirmed the previous reports. *Xcla4A*, *Xcla4B*, *Xcla5*, *Xcla7*, and BMP4 expression patterns were changed after exposure to BPA.

## MATERIALS AND METHODS

### Exposure of embryos to BPA

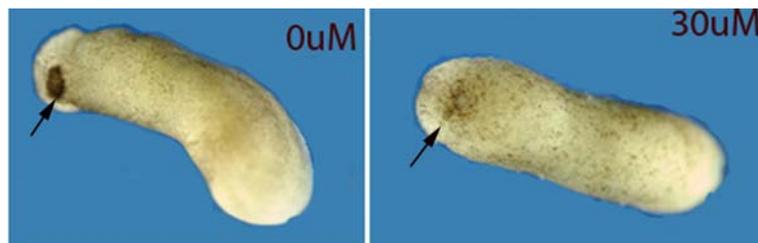
Embryos were obtained as described in chapter 2 and were exposed to BPA from stage 6-10 until the early tadpole stage. Bisphenol A was dissolved in ethanol and diluted in 0.1xBarth (refer to chapter 2). The final concentration of ethanol in the solution was less than 0.2%. Control embryos were developed in solutions containing ethanol alone without BPA. Whole mount *in situ* hybridization was carried out on the selected embryos. The detailed experimental procedures were described in chapter 2.

## RESULTS

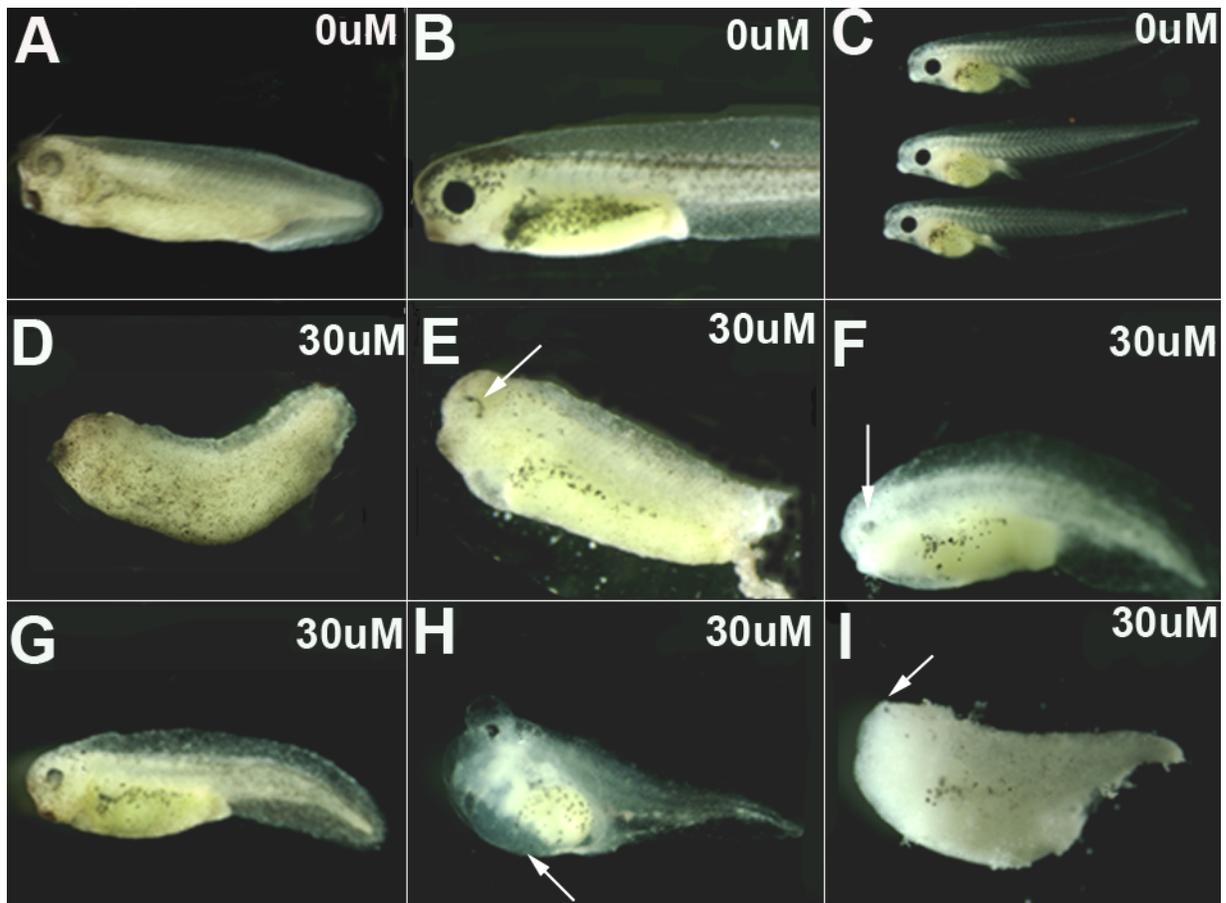
### Effects of BPA on early developmental stages

*Xenopus laevis* embryos were exposed to 0 $\mu$ M, 10 $\mu$ M, 20 $\mu$ M, and 30 $\mu$ M BPA in 0.1xBarth from stage 6-10. Among embryos exposed to BPA, 46% (101 out of 221) died in 30 $\mu$ M BPA. There were few dead embryos (2-3 out of 200) in 20 $\mu$ M, 10 $\mu$ M, and 0 $\mu$ M

BPA. Exposure to BPA concentration above 40 $\mu$ M causes lethality at the gastrula stage (Oka et al., 2003). The developmental abnormalities observed in early –stage embryos occurred when the embryos were exposed to as little as 20 $\mu$ M (data not shown) and the 30 $\mu$ M (Figure 4-1 and Figure 4-2). The embryos in 30 $\mu$ M group showed more severe defects than in the 20 $\mu$ M group. Various abnormalities were observed, as follows: (i) All of the exposed embryos (in 20 $\mu$ M and 30 $\mu$ M) showed growth retardation compared with 0 $\mu$ M controls (Figure 4-1 and Figure 4-2). There was little gross effect in the 10 $\mu$ M group; (ii) the formation of the cement gland (Figure 4-1) and the formation of the eye showed retarded development (Figure 4-2D, 2E); (iii) The skin was rough compared with the smooth skin of the control embryos (Figure 4-1); (iv) the vertebrae were either bent (Figure 4-2D) or crooked (Figure 4-2F); (v) the tails of the tadpole were crooked (Figure 4-2G); (vi) the tissues were dissociating (Figure 4-2E); (vii) the body cavity was swollen; (viii) some of the embryos lost their eyes (Figure 4-2I).



**Figure 4-1** Developmental retardation in the early *Xenopus laevis* embryos. Control embryo is shown on the left. The black arrow in the figure on right indicates the retarded formation of cement glands.



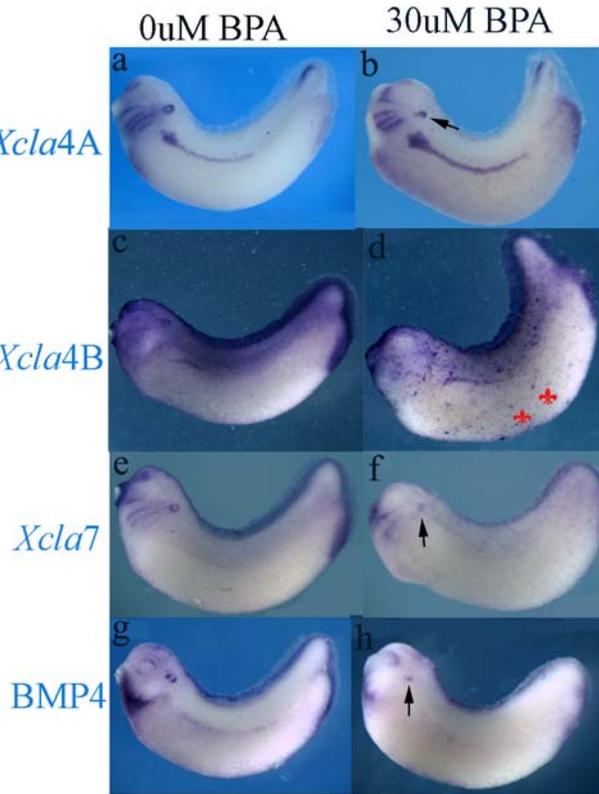
**Figure 4-2** Developmental abnormalities in early *Xenopus laevis* embryos. (A-C) Control embryos. (D-I) Embryos exposed to 30 $\mu$ M BPA. The arrows in (E, F, and I) indicate the abnormality of eyes. The white arrow in (H) indicates the swollen abdomen.

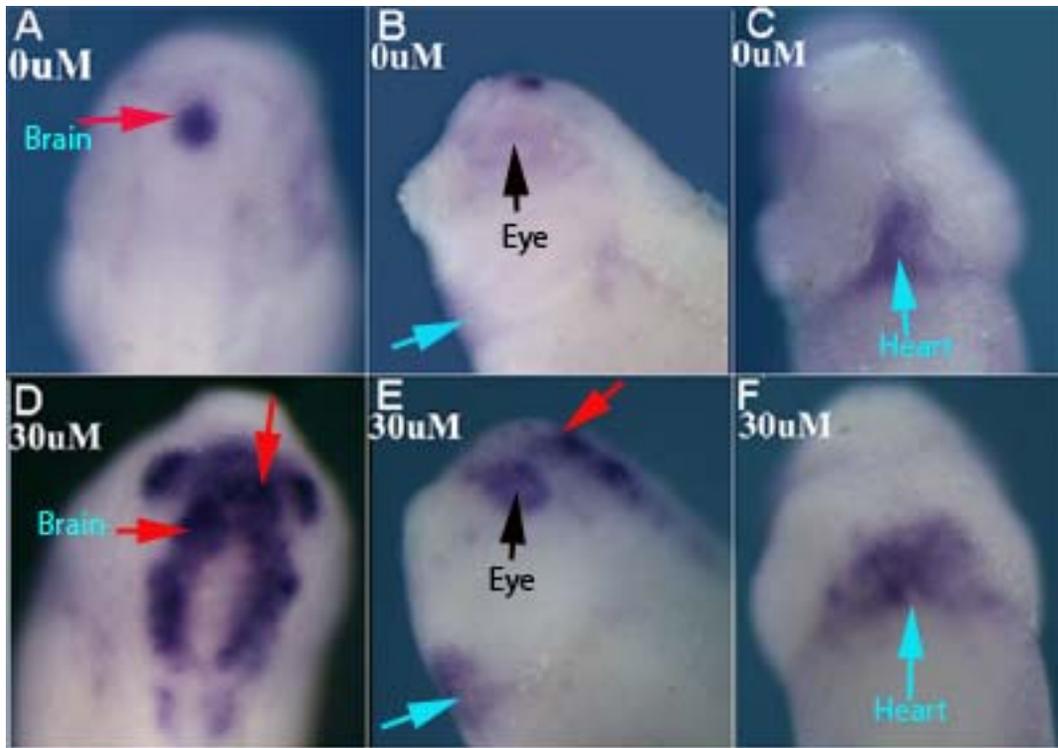
#### **Claudin and BMP4 expression patterns change after exposure to BPA**

Whole mount *in situ* hybridization was carried out on the selected embryos, due to the BPA treatment, the gross morphology is mildly abnormal. The probes used were claudin *Xcla4A*, *Xcla4B*, *Xcla5*, *Xcla7*, and transcription factor BMP4. Various abnormalities at the RNA level were observed, including: (i) ear formation was abnormal (Figure 4-3b, 3f, 3h); (ii) the skin was rough (Figure 4-3d); (iii) the brain was severely malformed (Figure

4-4AD); (iv) the formation of eyes was retarded (Figure 4-3h, Figure 4-4B) and (v) the heart formation was abnormal (Figure 4-4B and 4C).

**Figure 4-3** The ectopic expression of claudins and BMP4 in embryos after exposure of BPA. *Xcla4A*, *Xcla7*, and BMP4 transcripts showed the defects in ear and brachial arches. *Xcla4B* transcripts showed the abnormal skin. Red symbols indicate the rough skin. Black arrows in the right panel indicate the defect in eyes.





**Figure 4-4** *Xcla5* ectopically expressed in embryos after exposure to BPA. (A-C) Control embryos. (D-E) Embryos exposed to 30 $\mu$ M BPA. Red arrows indicate abnormality of brain formation. Blue arrows indicate the heart defects. Black arrows indicate the eye defects.

## DISCUSSION

*Xenopus laevis* has been used as an animal model to study the effects of different chemicals in the environment (Kloas et al., 1999) (Lutz and Kloas, 1999), including the effects of endocrine disruptors on metamorphosis and sex differentiation (Hayes et al., 2002). This study provides further evidence, at the molecular level. Another advantage of using *Xenopus laevis* comes from the fact that intensive research has been done on *Xenopus laevis* embryos and plenty of resources are available.

## **BPA is harmful to amphibian development**

This study showed that BPA could affect *Xenopus* embryonic development at a concentration as low as 20 $\mu$ M. The data collected are in agreement with the previous morphological report (Oka et al., 2003). In addition, it was reported that BPA has strong estrogenic effects on *Xenopus laevis*, teratogenic effect in early development (Iwamuro et al., 2003) and feminization in tadpoles. In a 1997 study, a BPA manufacturing site in the U.S had BPA concentrations from 2 to 8 micrograms/L (i.e.20-80 $\mu$ M) upstream and from 7 to 8 micrograms/L (i.e.70-80 $\mu$ M) downstream (Staples et al., 2000). Even though it is controversial whether BPA is harmful to human beings, there is no doubt that BPA is harmful to early vertebrate development in amphibians.

## **The mechanism of BPA effects**

BPA is known as an estrogen-active agent from its structure. Also, studies have shown its estrogenic effects on embryos (Iwamuro et al., 2003). Therefore, it is possible that BPA may affect development by interrupting the estrogen-signaling cascades. Estradiol can activate extracellular-regulated kinases (ERKs) in the pituitary tumor cell line (Bulayeva and Watson, 2004). Probably BPA has a similar pathway. cDNA microarray assays have pulled out 179 up-regulated and 103 down-regulated genes which are regulated by BPA (Sone et al., 2004). But much work remains. For example, little is known about the target genes. Claudins are probably a target of BPA. Our current knowledge is that claudins are the components of tight junctions in epithelial tissues and that estrogen regulates endothelial permeability through tight junctions. Therefore, it is possible that BPA may trigger some signaling cascades such as ER signaling and BMP4 signaling which may target tight junction protein claudins, causing abnormal phenotypes.

In the future, it will be interesting to test the expression of claudins after direct exposure of embryos to estradiol.

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