ABSTRACT

ZHANG, BO. The Role of Claudin-5 on Xenopus Heart Development. (Under the direction of Dr. Brenda Judge Grubb.)

Claudin-5 is an important member of the claudin gene family. The expression of claudin-5 in the heart of *Xenopus laevis* was determined by whole mount *in situ* hybridization. RNA over expression and knock down experiments demonstrated that claudin-5 is critical for heart development. Meanwhile, claudin-5 down regulated bone morphogenetic protein 4 (BMP4) expression in early stage through upregulating *chordin* (*chd*). In addition, other pathways such as estrogen hormone and transforming growth factor-β (TGF-β) may also affect claudin-5 activity. The results show that claudin-5 plays an important role in heart development and is involved in a complex pathway of gene regulation. The mRNA expression of claudin-12, another member of claudin protein family was also determined from cleavage stage to tadpole stage by whole mount *in situ* hybridization.

The Role of Claudin-5 on Xenopus Heart Development

by
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BIOGRAPHY

I was born July 25, 1982 in Jinan of China. I was very lucky in a happy family. My parents love me very much and I love them. I want to be a famous person no matter in science or in society. So my parents will be proud of me. I began to learn about biology when I was very young. Biology is very interesting and there are many puzzles in it. I decided to join the wonderful field for my bachelor's degree. I came to United State to join the laboratory of Dr. Grubb in 2005. I have learned so much in research and life from Dr. Grubb and I will never forget my experience in these three years. 2008 has been a very special year for me. I got married with my wife Hongfang Chen. She is beautiful and nice. I love her so much. I really appreciate that she came with me to the USA for my study. After completing my master's degree, I will start my PhD project. My Chinese name means "Doctor" in China given to me by my mother. So I will pursue a PhD in the USA for my name, my wife, my parents and me. After that, I go come back to China. I will live with my happy family and enjoy my life.

"*If a thing is worth doing it is worth doing well.*"

ACKNOWLEDGEMENTS

Here, I want to say thank you to my advisor, Dr. Brenda Grubb for all of her help in my research and thesis. She gave me really useful suggestions and support. Also, I would like to thank my committee members, Dr. Betty Black and Dr. Jane Lubischer. They provided me with great advice and guidance in my master project. Meanwhile, I want to thank the entire great job done by previous students including Jianzhen Xie, Ozkan Ozden and Carmel Martin-Fairey. They did preliminary work critical to the beginning of this project. All the people in the department of Zoology are nice to me and I was very lucky to be instructed by them. I will remember them forever. Thank you!

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AJs	adherens junctions
AP-1	activator protein-1
ALK	activin receptor like kinase
BBB	Brain Blood Barrier
BD	Bowen's disease
BMP	bone morphogenetic protein
bp	base pair
cAMP	cyclic adenosine monophosphate/protein
chd	*chordin*
CPE	Clostridium perfringens enterotoxin
CRs	cysteine-rich domains
Dig	digoxigenin
E.coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
GFP	green fluorescent protein
hCG	human chorionic gonadotropin
IFs	intermediate filaments

kb	kilobase
MAGUK	Membrane-Associated Guanylate Kinase
MO	Morpholino oligos
mRNA	messenger RNA
PBS	phosphate buffered saline
PKA	protein kinase A
SSC	sodium saline citrate
TBE	Tris-Borate-EDTA
TER	transepithelial electrical resistance
TGF	transforming growth factor
TJ	tight junction
TMVCF	transmembrane protein deleted in velo-cardio-facial syndrome
Tsg	Twisted gastrulation
Xld	Xolloid
xMsr	Xenopus Msr
xNkx	Xenopus Nkx
xTbx	Xenopus Tbx
xTro	Xenopus troponon
ZO	zonula occludin

CHAPTER 1 Literature review

1.1 What is the cell-cell junction?

Xenopus development is initiated with fertilization of the single cell oocyte by the sperm. A cascade of signaling events triggers rapid cell divisions, without growth, called cleavage. A hollow ball of cells called the blastula results from these divisions. During the mid-blastula transition, zygotic gene expression begins, the cells become compacted and junctions form between cells (Figure 1.1-1). The presence of cell-cell junction makes sure molecular signals move quickly in the lateral cells; meanwhile, cell-cell junction can prevent signals from other tissues in order to keep the specific communication. At gastrulation, cell movement allows placement of the germ layers and induction to occur creating differentiation of the ectoderm, mesoderm and endoderm, which result in development of distinct tissues and organs. This is known as Morphogenesis. One example for cell-cell junction in development is from the life cycle of *Dictypstelium* (Devreotes, 1989). In the process of amoabac forming the multicellular structure, cAMP (cyclic adenosine monophosphate/protein) binds with receptor on the cell surface and stimulates cAMP synthesis signal in the cell. This signal transfers through cell-cell junction to the adjacent cells, which generates gradient of cAMP from cAMP aggregation center. The concentration of cAMP oscillates and peak moves for some time. This movement cycle results in the multicellular structure. Even within the structure, movement of signal can work as a

development timer and produce the final organs (Devreotes, 1989). Another example can be seen from frog cardiac muscle. The cell-cell junction such as adherens junction together with tight junction mediate intercellular metabolism by controlling Ca2+-dependent, homophilic trans-interactions (Kooistra, 2007).Functionally, cell-cell junction is a chemical and physical barrier between adjacent cells. The cell-cell junction is used to control cellular molecular movement such as Na+ and K+ (Wright, 1968) and also used to maintain the composition stability between the adjacent cells, involve the stability of fluxes such as ions, water or nonelectrolytes (Luis, 1992). The cell-cell junction function is different in various cell types and organs. Cell-cell junction composition is formed in the mucosal epithelia of the stomach, intestine, gall bladder, uterus, and oviduct; in the glandular epithelia of the liver, pancreas, stomach, and thyroid; in the epithelia of pancreatic, hepatic, and salivary ducts; and finally, between the epithelial cells of the nephron , proximal, distal convolution and collecting ducts (Lipschutz, 2005). The cell-cell junction is a critical factor both in heart development and in maintaining heart function. For example, calcium-dependent adhesion-- N-cadherin is important in forming the distinct cardiac compartment (Linask, 1997). In addition to the cardiac compartment separation, N-cadherin also plays an important role in stabilizing commitment of cardiomyocytes and enables phenotypic differentiation (Linask, 1997). On the other hand, cell-cell interaction is important for bone marrow stromal cells to differentiate into cardiomyocytes. In addition to

the heart development, cell-cell junctions also maintain electrical and mechanical structure of cardiac vessels (Peters, 1994).

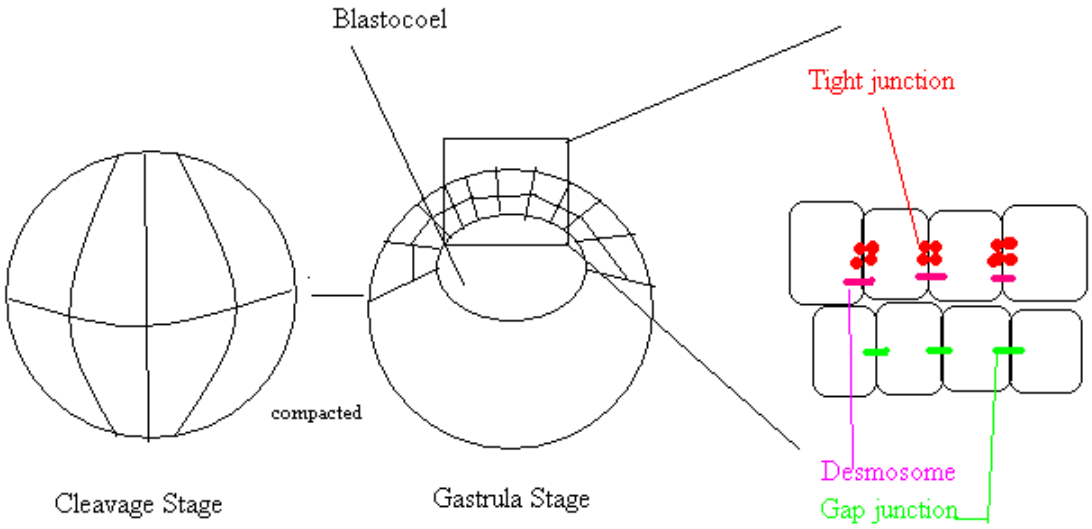

Figure 1.1-1 Cells compact and junctions form.

1.2 Overview of cell-cell junction

The cell-cell junctions include gap junction, tight junction (TJ), adherens junctions (AJs) and desmosome(Figure 1.1-2), with varying function and protein compositions.

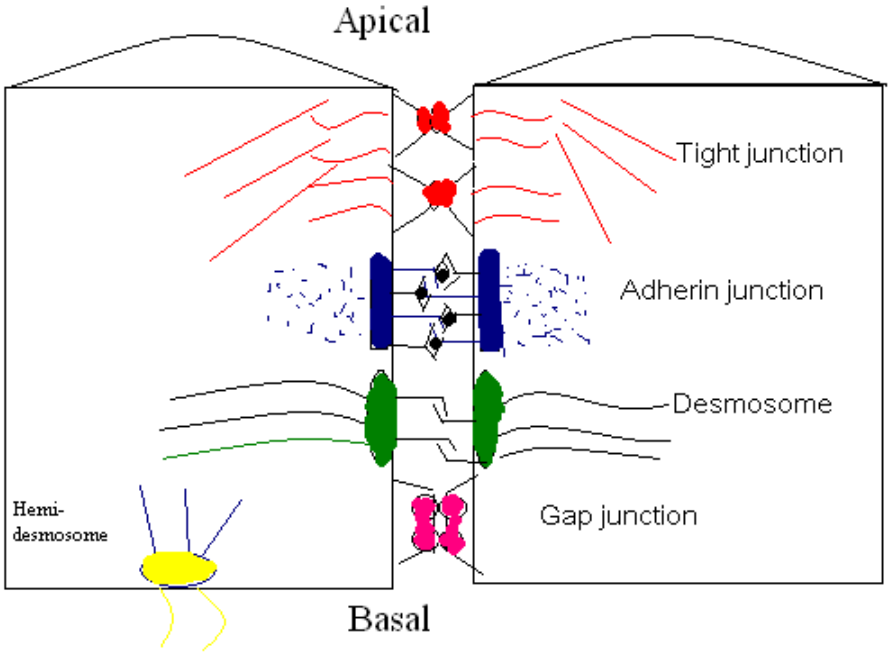

Figure 1.2-1 Main cell-cell junction in the epithelia cell or endothelia cell.

The gap junction is composed of a protein family named the connexons. The connexons form transmembrane hydrophilic channels, which can allow direct exchange of ions and small molecules (Sáeza, 2005). The gap junction works in coordinating endothelial cell migration and replication during repair of injury after mechanical denudation and during angiogenesis (Pepper, 1989). Adherens cell junction is made of the cadherins protein family. The cadherins are transmembrane glycoproteins, which form a very complex intracellular undercoat network with cytoplasmic proteins and actin microfilaments (Geiger, 1992; Kemler, 1993).The cadherins have two conserved regions. One is a highly conserved

cytophasmic region; another one is an extracellular region containing Ca+2 binding motifs. So the adherens cell junction involves the Ca+2 dependent cell-to-cell recognition (Takeichi, 1991; Kemler, 1993). Desmosomes are specialized junctions called “anchoring junctions” (Yin, 2004). The desmosome junction uses outer and inner plaque to connect cytoskeletal elements to the plasma membrane at cell–cell or cell–substrate adhesions (Yin, 2004). The desmosome consists of at least three different gene families: cadherins, armadillo proteins, and plakins (Huber, 2003;Getsios, 2004). Different from other junctions, the desmosome can bind with some stress-bearing intermediate filaments (IFs) at strong intercellular adhesion points (Yin, 2004). This is a very useful protection from mechanical damage. This protecting mechanism is in the epidermis and the heart. The tight junction is a special cell-cell junction located in the apical of epithelia and endothelia tissue. Most tight junctions are located in the apical side of epithelia and endothelia cells, which can separate apical space from the lateral plasma (Anderson, 1995; Tsukita, 1996).Recently, more research has been focused on the tight junction, the main protein studied in this project.

1.3 What are tight junctions?

The tight junctions are most located in the apical side of cells in the epithelial and endothelia tissues. It is seen as a continuous, anostomosing network of intramembranous particle strands (Staehelin, 1974) using the freeze fracture technique. Many proteins are

included in the tight junction complex: occludin, claudins, MAGUKs, cingulin (Citi, 1988), 7H6 (Zhong, 1993), symplekin (Keon, 1996), ZA-1TJ (Kapprell, 1990) and others to be discovered later. The barrier and fence functions are most important functions for tight junction. For the barrier function, the tight junction acts to seal the "sheet" of cells and prevent the tissue permeability. Another function of tight junction is called fence function (Schneeberger, 1992; Gumbiner, 1987;1993; Anderson, 1995). It is used to helps keep the cell polarity. At this time, there are a lot of popular topics relating to tight junction. The blood-brain barrier (BBB) and cancer research are two of them.

The BBB was recognized and demonstrated by German microbiologist Paul Ehrlich in the late-19th century. The function of the BBB is to separate the brain from blood circulation. This protects the brain from hydrophilic and toxic substances (Jiang, 2000). Adherens junctions and tight junction are existing cell junction in the BBB (Figure 1.2-1).The tight junction has a primary effect in limiting transcellular flux of BBB, due to the barrier and fence function. In the BBB, the cell junction not only separates the toxic substances, but also provides nutrition in the form of amino acids to the brain. Both of these functions require some active transport. Some of them are receptor mediated. Well known receptors include transferrin receptor (Pardridge, 1997) which has a strong ability to cross the BBB and other receptors with lower ability (Mclay, 1997). These receptors co-localize with the

tight junction and some of the receptors contain some proteins from tight junction protein families.

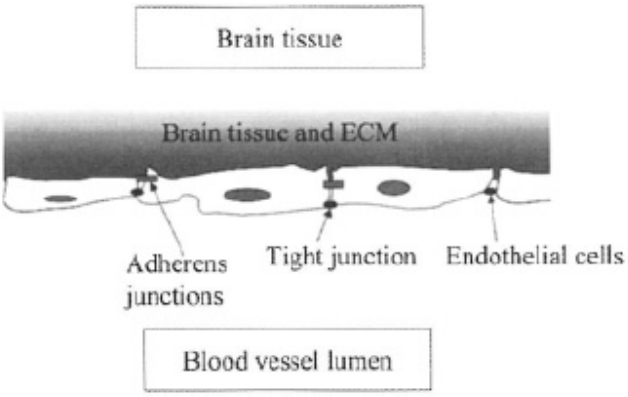

Figure 1.3-1 Cell junctions in the blood- brain barrier.
TJ is thought to play an important role in the BBB (Jiang, 2000).

Cancer research is a very important area of public health in biology. Also, it is a new topic for cell-cell junction study. It is known that tumor cells require long distance movement to the target organ, referring to the formation of metastasis (Hart, 1989; Jiang , 1994). Tumor cells can cross cell layers and enter blood vessels, following in the blood circulation to the target tissues or organs. In the target tissues, tumor cells penetrate endothelia and basal membrane, where tumor cells meet the tight junction. This tumor cell--tight junction interaction is slow but strong and long-term. Also, tumor cells may experience interaction mediated by carbohydrate-carbohydrate reactions (locking), which is faster but weak

compared with tight junction interaction (Jiang, 2000). From this, we may hypothesize if treatment for the tight junction in the target tissue and organ can be good to control the tumor cell diffusion. This will be a significant advance in the area of cancer research.

1.4 The anatomy of the tight junction

Much research has been done on techniques to measure the barrier ability of tight junction and several models have been proposed to explain the tight junction. The central technique is measuring transepithelial electrical resistance (TER) (Gitter, 1997). This method is a measurement of the junction barrier to small ions such as Na+ and Cl- in an electrical media. This measures the barrier ability of the tight junction. Another method uses radiolabeled inulin or mannitol (Fanning, 1999). By tracking these radiolabeled materials, cell junction characteristics can be located. A model is needed to explain the fact in the tight junction: why the TER varies by a wide range in the different tissues. For example, the paracellular barriers of collecting ducts are approximately 300ΩXcm2. The very "tight" epithelium of the bladder is about 6,000 to 300,000 ΩX cm2 (Fanning, 1999). At the same time, the size cutoff seems to keep in the same level, 5 Å to 20 Å (Fanning, 1999). The most acceptable model appears to be from Claude and Goodenough. (Figure 1.3-1, Claude, 1973). In figure 1.3-1 the occludin and claudin proteins compose several layers barrier in the cell including the closed channels (Black beads) and open channels (white beads). The molecule (X) can cross the junction only through the open channels (left one). In other cases, the

molecules cannot cross the junction. This model has three advantages in constructing the tight junction model: First, the model is in consistent with the tight junction network proposed by some research. The ratio of occludin and claudin in TJ is random, which explains the diversity of the tight junction network. Second, the model is the one used to explain the tight junction figure under the electron microscope. The micrograph of the tight junction shows that the occludin and claudin proteins from adjacent cells can form the spot-like contact or "kiss"contact (Cereijido, 1992; Farquhar, 1963). The spot-like characteristic is unique to the tight junction, makes the junction dense. This is why we call it "tight junction". Claude's model is identical to the figure. Third, the model explains the logarithmic, rather the linear relationship between the number of barrier and the net paracellular permeability (Fanning, 1999). Claude said the barrier in the model is not fixed but flicked open and closed (Claude, 1978). From Claude's model, the two important proteins in the tight junction-occludin and claudin are recognized.

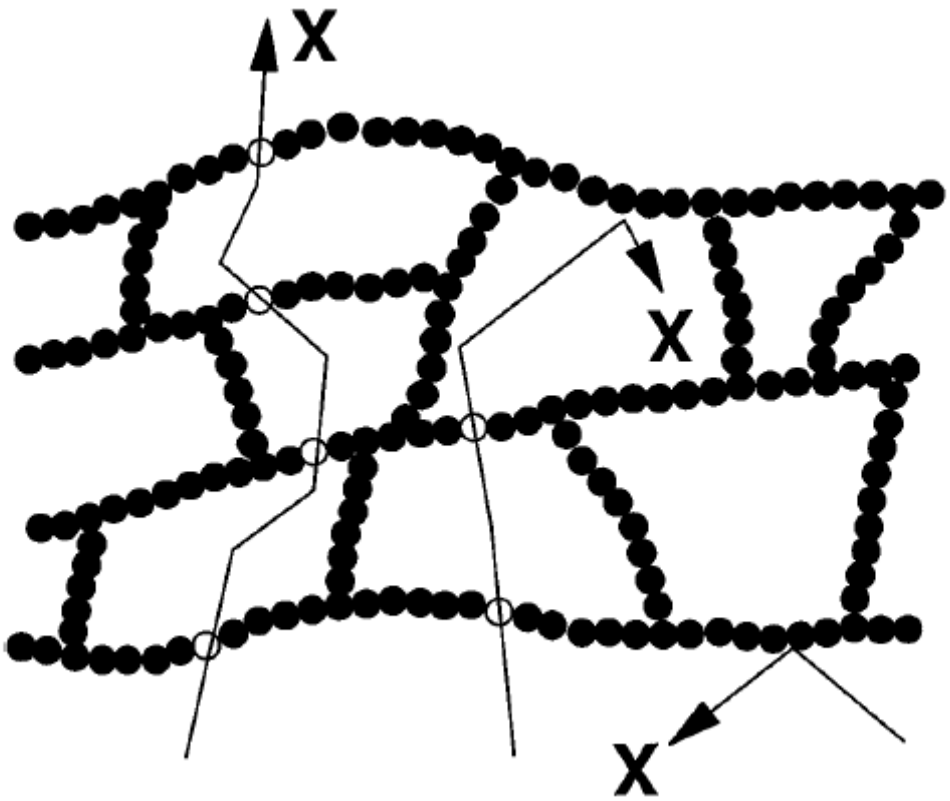

Figure 1.4-1 TJ is composed of many barriers (claudin and occludin).
Some of them can form open channels (white bead), some of them form closed channels (black bead) at a time. Solute molecules(X) can cross membrane only through open channels and be stopped by closed channels. This process is regulated by channel precisely. Such a model explains the logarithmic, rather than linear relationship between the number of tight junction and net paracellular permeability. The molecule on the left passes through membrane successfully (Fanning, 1999).

Since the discovery of the electron microscope in the early 1960s, the different kinds of junctions have been revealed (Mitic, 1998). Biochemical functions and protein compositions of adherens junction and desmosome have been gradually discovered. Meanwhile, research on the tight junction has never stopped. The dynamic network of the tight junction complex has at least three proteins. They are MAGUK protein family,

occludin, and claudin protein family (Figure 1.4-2).

Membrane-Associated Guanylate Kinase (MAGUK) is a large protein family. This family shares common domains of SH3, a region homologous to guanylate kinase (GUK), and one or more PDZ domains (Jiang, 2000). ZO-1 and ZO-2 are the first two proteins found in the tight junction for this family. ZO-1 is 220-kDa (Fanning, 1996; Stevenson, 1986; Willott, 1993). Different from claudin and occludin, ZO protein is peripheral protein not transmembrane protein. Some experiments show that ZO-1 binds directly to a 150-amino acid domain at the cytoplasmic tail of occludin (Furuse, 1994) and may bind to the actin-binding protein spectrin (Itoh, 1993). ZO-2 is the second protein found in the family (Gumbiner, 1993) and is a phosphoprotein of 160kDa and coprecipitate with ZO-1. There is high homology between ZO-1 and ZO-2. ZO-2 contains three PDZ domains, a SH3 domain and a GUK domain. ZO-3 is a new found protein in the family and 130 kDa in size and shares high sequence homology with both ZO-1 and ZO-2 (Stevenson, 1998). ZO-3 can bind with ZO-1 and occludin in the cytoplasm. Of the three proteins, ZO-1 is thought to be the most important. Actually, it is the bridge connecting the tight junction protein and the action cytoskeleton. ZO-1 can interact with AF-6, ZAK, actin catennin and occludin, as well as ZO-2 and ZO-3 in the same family. Interestingly, ZO protein can be found in some tissue without tight-junction, in association with adherens junction.

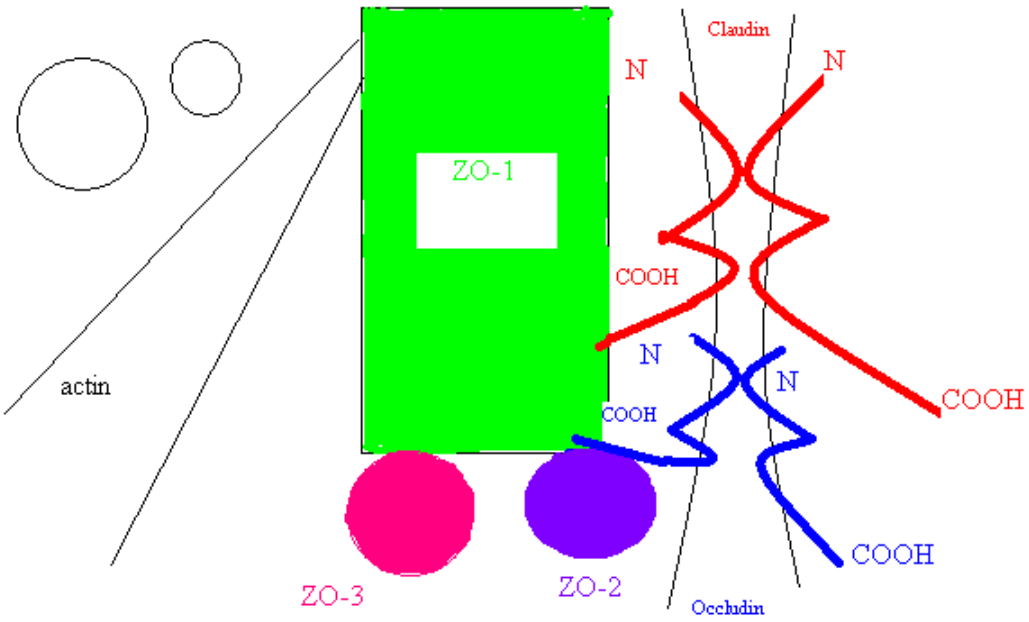

Figure 1.4-2 Network of the tight junction.
Claudin and occludin proteins are transmembrane proteins, while ZO locates intracellularly. Occludin interacts with ZO-1, and 2. ZO-1 is proved to connect actin with occludin and claudin.

Occludin was the first transmembrane protein found in the tight junction (Furuse, 1993). It has a weight of 55.9 kDa. Occludin is similar to claudin in structure. Occludin has four membrane-spanning segments, and two extracellular loops as regions II and I. This is the same as the claudin protein family (Figure 1.4-1). In the two extracellular loops, the first one includes a high content of alternating glycine and tyrosine residues, 36% and 22% (Mitic, 1998). The first loop seems likely to be involved in the tight junction's barrier function and in cell adhesion. High glycine content can form a highly flexible structure; the tyrosine will

stick together (Mitic, 1998). The structures from adjacent cells may interact with each other and create a zipper-like seal. Compared with the claudin protein, the occludin protein has a longer C-terminal in the cytoplasm. The ZO protein family seems important for localization of occludin. Occludin may bind with ZO-1, 2 and 3. Occludin binds mostly with ZO-1 (Balda, 1996; Chen, 1997; Furuse, 1994; Matter, 1998). This may explain why the occludin cannot exist in tissues without the tight junction especially ZO protein. Occludin is widely expressed in epithelial and endothelial cells, with a very high level of occludin in brain endothelial cells, but not in fibroblasts (Saitou, 1997).

Besides the big protein families in the tight junction, there are small proteins, such as cingulin and 7H6. Cingulin was first found by Citi et al (Citi, 1988). It may interact with actin in the junction plaque. 7H6 is another new protein found in the tight junction.7H6 may be involved in the mechanism cells use in response to different environments. Zhong Y 1994 paper states that 7H6 could connect and disconnect with the tight junction complex according to different conditions of ATP level and its expression reduces continuously when cells change from normal to dysplastic to carcinomatous states (Zhong, 1994).

1.5 What are claudin proteins?

Claudin proteins are the main transmembrane proteins in the tight junction. Compared with occludin, claudin proteins also have four tansmembrane domains, and a short C-terminal

(Figure 1.4-1). Claudin proteins share little similarity with each one in the amino acid sequence; different members of the family have different sequences. Claudin has a big family of at least 24 proteins found in mice or in humans (Morita, 1999; Tsukita , 1996). Claudin-1 and –2 are the first two claudin proteins in the family discovered by Furuse from a chick liver. They are 23-kDa integral membrane proteins that are structurally related 38% identical at the amino acid sequence level (Furuse, 1998). It is still not clear how many proteins are in the multigene family. There are 24 claudin or claudin-like proteins discovered. Due to a large family, claudin proteins are co-expressed in the different tissues, which make it hard to investigate the function for a unique protein. Claudin protein can reconstruct TJ strands in the fibroblasts without TJs, while occludin can only constitute a small amount of the network (Furuse, 1998; Brizuela, 2001). This means claudin proteins are important and major components in the TJ network. Claudin proteins interact with other claudin proteins in adjacent cells to form a pair structure. Until now, no evidence shows that claudin proteins can link with occludin or other actin in the TJ. It is hypothesized that claudin may act more as a structural component than a functional component in the TJ, although claudin proteins clearly have multiple functions in the cell.

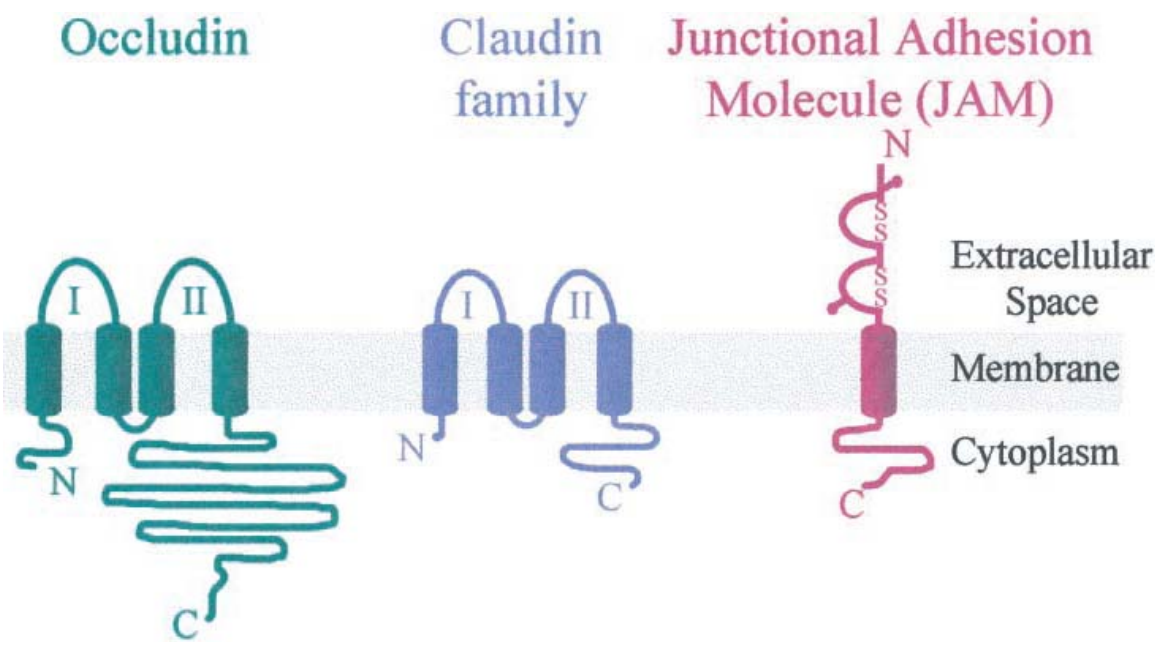

Figure 1.5-1 Occludin, claudin and JAM are three kinds of transmembrane proteins in TJ. Both of occludin and claudin have four spanning regions and two extracellular regions. Occludin has a longer c-terminal than claudin. JAM only has one single spanning protein. It is one of the Ig superfamily (Fanning, 1999).

1.6 Why is claudin protein so important?

The TJ is a complex network and the distribution of TJ is broad and it lies in most epithelia and endothelia tissues. Meanwhile, composition and function are different in the different tissues. All of these can be ascribed to the diversity of the claudin proteins family. From table 1.1, we can see claudin proteins disperse all over the body. For example, claudin-1 is in the skin and liver, claudin-3 in the ear and intestine, claudin-5 in the heart and eyes etc. The claudin proteins can express from skin to heart, almost in every tissue. Claudin genes have become the critical markers for the TJ in molecular research. People rarely use occludin as the TJ marker because occludin does not always go with TJ. On the other hand, claudin exists

in all the TJ. As we discussed before, why TJ is so important is due to their close relation with cancer research. From table 1.3, we can see claudin protein has been involved in many kinds of tumors. For example, claudin-1 and –4 relate to skin cancer, claudin-2, 3, 4, and 5, 7, 22 in breast cancer, claudin-3 and 4 in prostate and ovarian tumors. Claudin-5, 20, 22 are recently found in brain cancer. Meanwhile, the function of claudin protein in cancer seems to be more complex than previous. Some claudin genes such as 1,2,3,4,5,7,11,12 and 15 are expressed in a lot of tissues and many cases of tumors. While, others such as 14,16,17,20, and 22 have more limited expression region (Hasegawa, 2002). Claudin-14 for example is only found in the Cochlea Vestibule and Heart. Claudin-17 is expresseed in low level in normal kidneys. Claudin-20 only exists in the small intestine in very low level.

Same claudin gene may even act as different or reverse function in the different cancers. The extreme case is claudin-17. Claudin-17 has been found downregulated in breast and head and neck cancer, but upregulated in stomach cancer (Johnson, 2005; Kominsky, 2003). Overall, it is very clear the correlation between cancer and TJ is due to correlation with the claudin genes. In addition to the cancer, claudin genes have been implicated in a lot of syndromes in human beings (Table 1.2), such as claudin-2, 3, 4, 5 with Paget's disease (Ylermi, 2004), DiGeorge's syndrome with claudin-5, Williams-Beuren syndrome with claudin-3 (Paperna, 1998).

More importantly, claudins are the proteins located in the cell surface so that they can be good targets for many medical treatments. There is special interest in the Clostridium perfringens enterotoxin (CPE) as a novel chemotherapeutic compound. CPE is a natural ligand for claudin-3 and -4 proteins; it can bind toxins to these claudins, leading to a rapid cytolysis of the cells (Katahira, 1997). So this will let us think about treating cancer or other disease through the claudin protein pathway.

Table 1.6-1 Claudin proteins in different tissues.

Claudin protein	Tissue or cell line	Reference list
Claudin-1	Liver kidney,skin	(Furuse, 1998)
Claudin-2	Kidney liver Madin-Darby canine kidney (MDCK) strain I and II cells Intestinal epithelial cell	(Furuse, 1998) (Lipschutz, 2005) (Enck, 2001) (Fujita, 2008)
Claudin-3	Madin-Darby canine kidney (MDCK) strain I and II cells	(Lipschutz, 2005) (Rangel, 2003)
Claudin-4	Madin-Darby canine kidney (MDCK) strain I and II cells	(Lipschutz, 2005) (Ylermi, 2004) (Rangel, 2003)
Claudin-5	Heart, brain,eye and tail	(Ishizaki, 2003) (Turksen, 2002)
Claudin-6	Liver, kidney, stomach, lung and calvarias embryonic stem cells	(Turksen, 2001) (Hasegawa, 2002)
Claudin-7	Skin Lung, kidney, ovary, colon	(Ylermi, 2004) (Morita, 1999)

Table 1.6-1 continued

Claudin-8	Skin，Cochlea Vestibule Lung, kidney, colon, breast	(Ylermi, 2004) (Kitajiri, 2004) (Morita, 1999) (Heiskala, 2001)
Claudin-9	Cochlea，Vestibule Brain, tumors.	(Kitajiri, 2004) (Heiskala, 2001)
Claudin-10	Skin，Cochlea Brain, tumors Ear	(Ylermi, 2004) (Kitajiri,2004) (Heiskala, 2001) (Cheung, 2005)
Claudin-11	Testis, skin Brain, spinal cord	(Ylermi, 2004) (Bronstein, 1996) (Morita, 1999) (Gow, 1999) (Bronstein, 2000)
Claudin-12	Cochlea Vestibule Brain, prostate, colon, uterus Brain Intestinal epithelial cell	(Heiskala., 2001) (Belanger, 2007) (Fujita, 2008)
Claudin-13	Embryonic tissues	(Heiskala, 2001)
Claudin-14	Cochlea Vestibule Heart	(Kitajiri,2004) (Heiskala, 2001)
Claudin-15	Small intestine	(Heiskala, 2001)
Claudin-16	Kidney,tumors, Genitalia	(Heiskala, 2001)
Claudin-17	Skin Low level normal kidney	(Ylermi, 2004) (Hasegawa,2002) (Heiskala, 2001)
Claudin-18	Stomach and the lungs Cochlea，Vestibule	(Hasegawa, 2002)
Claudin-19	PNS,kidney	(Miyamoto, 2005)
Claudin-20	Small intestine	(Heiskala, 2001)
Claudin-21	Human intestine	(Katoh, 2003) (Krause, 2008)
Claudin-22	Human trachea	(Katoh, 2003) (Miyamoto, 2005)

Table 1.6-1 continued

Claudin-23	Colon, stomach, placenta, human skin, rat duodenum, mouse taste receptor cells	(Katoh, 2003) (Krause, 2008)
Claudin-24	Human intestine	(Katoh, 2003) (Krause, 2008)

Table 1.6-2 claudin study in some model animals.

Claudin protein	Model animal	Symptom	Reference list
Claudin-1	Mice	Epidermis abnormal thinkness with hyperproliferation, Die within one day of birth	(Furuse, 2002) (Buhr,2007)
Claudin-3	Xenopus	Left-right asymmetry defect	(Brizuela, 2001)
Claudin-4	Xenopus	Left-right asymmetry defect	(Brizuela, 2001)
Claudin-5	Mice and Xenopus	Defect in BBB and die in 10 days	(Nitta, 2003) (Xie & Brizuela, 2005)
Claudin-6	Mice	Transgenic newborn mice have smaller size , red, shiny skin	(Turksen., 2001)
Claudin-7	Xenopus		(Fujita,2006)
Claudin-11	Mice	Claudin-11 deficiency mice show CNS defect and sertoli cell defect in testis	(Gow, 2004)
Claudin-12	Xenopus		(This work)
Claudin-14	Mice	Hearing loss	(Yosef, 2003)
Claudin-19	Mice	Claudin-19 deficiency mice suffered from a kind of peripheral neuropathy.	(Miyamoto, 2005)

Table 1.6-3 claudin protein interaction with human being.

Claudin protein	Symptom or disease	Reference list
Claudin-1	Cell carcinoma (SCC) of the skin and Bowen's disease (BD) genotype and weight loss	(Morita, 2004)
Claudin-2	Paget's disease, Breast Carcinoma	(Ylermi, 2004)
Claudin-3	Paget's disease, Breast Carcinoma Prostate and ovarian carcinomas. Williams-Beuren syndrome	(Ylermi, 2004) (Long, 2001) (Hough, 2000) (Paperna, 1998)
Claudin-4	Cell carcinoma(SSC) of skin and Bowen's disease(BD) Paget's disease, Breast Carcinoma	(Morita, 2004) (Ylermi, 2004) (Michl, 2003) (Terris, 2002) (Long, 2001)
Claudin-5	Paget's disease, Breast Carcinoma Velocardiofacial syndrome and DiGeorge's syndrome	(Ylermi, 2004)
Claudin-7	Breast, head and neck cancer and stomach cancer	(Kominsky, 2003) (Johnson, 2005)
Claudin-8	Crohn's disease	(Zeissig, 2007)
Claudin-10	Hepatocellular carcinoma	(Cheung , 2005)
Claudin-11	Sclerosis,sterility paralysis	(Yosef, 2003)
Claudin-14	Human hereditary deafness	(Yosef, 2003)
Claudin-16	Hypomagnesaemia	(Simons, 1998)
Claudin-17	Head and neck cancer, and stomach cancer	(Johnson, 2005)
Claudin-18	Gastric cancer	(Sanada , 2006)
Claudin-19	Familial hypomagnesaemia with hypercalciuria and nephrocalcinosis, visual impairments; hypomagnesaemia mutations in TMD1, 2.	(Sanada , 2006)

Table 1.6-3 continued

Claudin-20	Chondrosarcoma, a brain cancer, and a liver tumor	(Hasegawa, 2002)
Claudin-22	Breast cancer libraries and one brain astrocytoma	(Hasegawa, 2002)
Claudin-23	Downregulation: intestinal-type gastric cancer	(Hasegawa, 2002)

1.7 What is claudin-5 ?

Claudin-5 is a gene found early in the claudin gene family. The claudin-5 gene expresses ubiquitously, in endothelia and epithelia tissue, although most in the endothelia tissues. Work previously done in the Grubb has demonstrated the structure and topology. Claudin-5 protein has four transmembrane regions and two extracellular loops, as found in classical members of the claudin-gene family. There are 7 amino acids in the amino termini and 25 amino acids in the carboxyl termini. Since claudin-5 distributes widely, it is a key protein of tight junction in controlling cell permeability and molecular diffusion. The function of claudin-5 can be reduced to three ways. Claudin-5 is also called the transmembrane protein deleted in velo-cardio-facial syndrome (TMVCF) in human being. TMVCF has many different symptoms such as conotruncal cardiac abnormalities, cleft palate, learning disabilities, and a typical facial dysmorphology (Sirotkin, 1997). Meanwhile, TMVCF is a variable syndrome which has been found in more than 40 phenotypes in clinic study (Goldberg, 1993). Although Digeorge syndrome shares some phenotypes with TMVCF, it is more severe than TMVCF. Some patients with Digeorge appear to also have immune abnormalities and hypocalcaemia.

In the brain, claudin-5 is the primary factor in protection and molecular permeability regulation. The well known example is the blood-brain barrier (BBB). BBB is one of most important protection mechanisms in the central nervous system; also it is one of the most significant functions for the TJ. BBB can prevent material flow away from the brain; meanwhile it can prevent hazardous material from entering nervous system. Disorder of the BBB can introduce many diseases such as brain edema (Yang, 1994). Claudin-5 plays a very important role in the molecular selection in the BBB. Claudin-5 deficiency mouse show peculiar abnormalities (Nitta, 2003). A claudin-5 knockout in the mouse results in alterations of the blood–brain barrier and death within 10 days of birth (Nitta, 2003). Thus a claudin-5 deficiency in the BBB has severe consequence. Interestingly, some experiments reveal that claudin-5 deficiency mouse result in loss of normal regulation against small molecule (<800Da) but not large molecule (Nitta, 2003). This may tell us claudin-5 not only manages molecular movement in the blood brain barrier but only has special selection according to the molecular size. Moreover, some authors even suggest that there are two kinds of TJs in BBB and one of them is composed of claudin-5 only. The claudin-5 TJ used to restricts the paracellular permeability of small and large molecules, another TJ restricts only larger molecules (>800 Da) (Ohtsuli, 2007). In addition to the brain, claudin-5 also has strong expression in the heart (This work). It has been found that claudin-5 protein level decreases in the cardiomyopathic mouse models (Sanford, 2005). Claudin-5 is a critical component of

TJ in the cardiac muscle lateral cell membrane. Dystrophin and utrophin knockout mouse can breakdown the connection between submembranous action protein and transmembrane protein- claudin-5 thereby causing cardiomyocyte disorder (Sanford, 2005). Previous experiments from the Grubb lab have revealed that claudin-5 RNA injection in xenopus produces beating tissue similar to the heart (Xie & Brizuela, 2005). This interesting find shows that the TJ such as claudin-5 is critical in determining heart development and should be the first step in constructing the heart structure of Xenopus. Recently, many experiments have revealed a lot of new functions for claudin-5. For example, hyperstimulation and Gonadotropin-Releasing Hormone Agonist downregulate claudin-5 mRNA to change ovarian vascular permeability in ovarian hyperstimulation syndrome (OHSS) (Kitajima, 2005). On the other hand, claudin-5 is also responsible for disruption of the barrier of neural blood vessels and other symptoms by hypoxia (Koto, 2007).

1.8 Claudin-12 protein

Claudin-12 protein is new in the claudin family. Little research has been done regarding to claudin-12. It is known that claudin-12 coexpresses with claudin-5 in the BBB. Claudin-12 compensates for claudin-5 loss in the claudin-5 deficiency mouse (Nitta, 2003). Although claudin-5 mRNA expression in the rat brain capillary fraction was 751-fold greater than that of claudin-12 mRNA (Ohtsuli, 2007), claudin-12 protein seems to act as a primary component in the central nervous system. TGF-β signal inhibits endothelial cells proliferation

and integrity through down-regulation of claudin-5 protein but not claudin-12 protein (Watabe, 2003). So BBB may not lose size-selective ability completely in TGF-β signal treatment. On the other hand, Claudin-12 is also a target for vitamin D signaling (Fujita, 2008). It is down-regulated in the vitamin D receptor knockout mouse which has decreases in Ca2+ update in the intestine (Song, 2002).

1.9 BMP4& chordin

BMP (bone morphogenetic protein) is a member of superfamily transforming growth factor-β (TGF-β). BMP is very important in specifying the cell fate in the gastrula stage of Xenopus, which directs ectoderm to form epidermis not neural tissue (Moreno, 2005). BMP is regulated in a concentration-dependent manner and is modulated by the secreted signals from Spemann's organizer: *noggin, chordin* (Wilson, 1997). These signals work as the antagonist to BMP, which direct ectoderm to neural tissue instead of epidermis (Piccolo, 1996; Yamashita, 1995; Zimmerman, 1996). *Chordin* (*chd*) is a primary antagonist for BMP4, it is able to bind BMP4, BMP2 and BMP4/7 heterodimer to prevent them binding with their receptors (Piccolo, 1996). The final cell pattern decision depends on the stage, as well as depends on the concentration ratio between BMP and antagonist.

1.10 Heart development

Xenopus heart development includes four continuous processes: precursor cardiac cell

production, heart tube formation, heart looping, and chamber specification. From late blastula and early gastrula stage, mesoderm specifies into precardiac cells. These cells move anteriorly and ventrally during the gastrula stage. These special precardiac cells can be marked by early heart marker *xNkx2.5*. During the late tail bud stage, a straight heart tube is formed (Kolker, 2000; Mohun, 2000), which will undergo rightward and dorsal looping until the sinus venosus and atrial structures are located on the dorsal side of the ventricle. After looping, chamber specification starts. Ventricle chamber becomes thickener. At the same time, an atrioventricular valve has been gradually constructed. Atria and ventricle separate with each other (Kolker, 2000; Mohun, 2000). Except for the *xNkx2.5*, BMP is another early heart marker in the Xenopus heart development. Over expression of dominant-negative BMP receptors in the gastrula stage will inhibit heart formation and downregulate *xNkx2.5* expression (Shi, 2000). These results reveal that BMP is very important for the normal heart development and is upstream factor of *xNkx2.5*. Many experiments provide information that BMP can induce cardiac myocytes and required for cardiac differentiation. These functions are time and position dependent. Moreover, BMP may induce mesoderm to stimulate cardiogenice markers and produce beating tissues (Shi, 2000). In addition to inducing heart formation, BMP4 is also necessary for right-side development. The BMP/ALK2/SMAD signaling pathway is important in determining the right side (Hyatt, 1996; Hyatt, 1998; Ramsdell, 1999). On the other hand, TGF-β seems to have inverse function, inducing left

side development (Lohr, 2000). The two pathways interact with each other with many regulators, such as *xLefty* (Wright., 2007) and *Xnr-1* (Brizuela, 2001). Although much work has been done in the BMP decision on the right side, less work has involved in the claudin gene in the left-right decision. Xenopus experiment shows that overexpression of claudin-3 and 4 mRNA causes bilateral *Xnr-1* and left–right randomization (Brizuela, 2001). So far, it is still not clear about the mechanism for claudin-5 in the left-right decision.

CHAPTER 2 Main Body

2.1 Introduction

Research reported in the literature has shown that claudin genes are critical in determining cell permeability in endothelia and epithelia tissues. As a major component of the BBB, claudin-5 plays an important role in brain development. Meanwhile, previous work done in Grubb lab has demonstrated that altered claudin-5 expression affects heart development. These have led to the following two hypotheses:

1. Claudin-5 expression is required for normal heart development.

To understand this hypothesis, the objectives below are necessary:

A. Determine where claudin-5 and -12 are expressed in the Xenopus embryo with whole mount *in situ.* To Determine if claudin-12 and claudin-5 is co-expressed in heart as in BBB of mice.

B. Find suitable heart marker genes to examine claudin-5 function in Xenopus heart development.

C. Use claudin-5 mRNA injection experiment to decide what happen to heart development when claudin-5 is over expressed.

D. Use claudin-5 Morpholino injection experiment to decide what happen to heart

development when claudin-5 is reduced.

2. Claudin-5 is involved in a signaling cascade with other genes for normal heart development.

The objectives for the second hypothesis should be

A. Determine if claudin-5 involve in pathway with early markers-BMP4 and *chd*.

B. Determine what other signaling pathways be affected by examining TGF-β and estrogen signaling

All the experiments in this thesis are based on the hypothesis and objectives above.

2.2 Method and material

2.2.1 Synthesis of RNA probe and heart markers

All the plasmids in the experiment were extracted from *E.Coli* with the Qiagen Maxprep kit. Sense and antisense claudin-5 probe were from the plasmid pCMV-SPORT6, while claudin-12 was from the plasmid pBluescript SK minus. The antisense probe can be used as probe in the *in situ* experiment and sense strand was just used as control. 10ug plasmid was used DNA in linearization reaction. The claudin-5 plasmid was linearized with restriction enzyme EcoRI and claudin-12 plasmid with enzyme XhoI at 37 degrees for over two hours. Digestion completeness was checked by running an aliquote on a 1% agarose gel. Template was then extracted with phenol/chloroform once and chloroform /isoamyl twice, precipitated in 100% ethanol overnight and washed in 70% ethanol before use. The DNA was then resuspended in about 10ul TE buffer to a final concentration 0.5-1ug/ul measured by a nanodrop machine (DN-1000) and stored at -80 degrees.

To set up the RNA transcription reaction, 1000ng of template DNA was taken from TE buffer. This reaction must be RNase-free. 1ul RNA guard or RNase inhibitor was used to protect RNA against RNase. T7 RNA polymerase was used for claudin-5 RNA template and T3 was used for claudin-12 template. Labelling mix was Digoxigenin labeled. Distilled water was added up to 20ul. The transcription reaction was incubated at 37 degrees about two hours

in order to get enough RNA probe. Unincorporated probe was then removed; free nucleotides were spun down with Quick Spin Columns (Roche). High speed spin removed the unbound RNA and collect mRNA. All the spins were carried out at 4 degrees in order to prevent mRNA from degradation. Probe mRNA was aliquoted at -80 degrees until used for the *in situ* experiment. The procedure was based on the protocol from Dr Robertis laboratory.

http://www.hhmi.ucla.edu/derobertis/protocol_page/XenopusWholeMountInSitu.pdf

Heart markers *xNkx2.5*, cardiac *Troponin*, *xMsr* and *xTbx*, also BMP4 and *chordin*(*chd*) probe mRNA were synthesized through the similar procedure. The difference was that *xNKx2.5* with pGEM plasmid, *Troponin* with pSK, *xMsr* with pSK a, *xTbx* with pGEM T-easy, BMP4 with pCS2 and *chd* with pCS2.

2.2.2 Preparation of Xenopus embryos

The Xenopus fertilization procedure was based on the protocol from Grubb laboratory (Brizuela, 2001).

Female frogs were primed a week before fertilization with human chorionic gonadotropin (hCG) (Sigma Inc, Cat.9002-61-3) in about 30-40 units. Also, HCG can be used in male frog to induce sperm development. Night before fertilization, female frogs were induced for ovulation with about 300-400 units HCG. Female frogs were kept in 1X high salt Barth

(NaCl 0.1095M, KCl 0.001M, $NaHCO_{3,}$ 0.0024M, HEPES 0.01M, $MgSO_4 \cdot 7H_2O$ 0.0008M, Ca $(NO_3)_2 \cdot 4H_2O$ 0.0003M, $CaCl_2 \cdot 2H_2O$ 0.0004M) until fertilization starts. Sperm can be isolated from male frog testis and saved in about 1 mL of Steinberg's solution (NaCl 3.4g, KCl 0.05g, Ca $(NO_3)_2 \cdot 4H_2O$ 0.08g, $MgSO_4 \cdot 7H_2O$ 0.2g, Kanamycin 0.01g, $C_4H_{11}NO_3$ (Tris-HCl) 0.6g). Fertilization was started after the eggs were dried as well as possible. 2% and PH 7.8 cysteine (Fisher, Cat.376-100) was used to remove the jelly coat. After removing jelly coat, cysteine was decanted and eggs were washed for several times with 0.1X Barth (NaCl 0.009M, KCl 0.0001M, $NaHCO_{3,}$ 0.00024g, HEPES 0.00999M, $MgSO_4 \cdot 7H_2O$ 0.00008M, Ca $(NO_3)_2 \cdot 4H_2O$ 0.00003M, $CaCl_2 \cdot 2H_2O$ 0.00004M) solution. About one and half hour, eggs went to two-cell stage. Fertilized eggs can be kept at room temperature to different stages. Eggs were fixed to stage we want according to Nieuwkoop normal table (Nieuwkoop, 1994). MEMFA (37% Formaldehyde, 10X MEMFA, and DEPC H2O) was always used to fix fertilized embryos. After that, embryos were needed to be dehydrated in a continuous wash from 25% PBSw/Methanol (Fisher Cat.A433-4), 50% PBSw/Methanol and 75% PBSw/Methanol. Finally, embryos were washed with 100% PBSw/Methanol for two times and stored in -20 degrees freezer.

2.2.3 RNA microinjection

Claudin-5 mRNA was injected into embryos resulting in overexpression of the gene. While, antisense morpholino oligomers were designed to inhibit claudin-5 expression. Morpholino

oligomers are targeted to block translation by choosing sequences extending from 5' cap to the start 25 bases against the upstream 5' untranslated region and near the initiation sequence in the gene promoter (Figure 2.2-1).

XCLA5-MO1

TGA GCGGTGATAA CTGTAGAGAAGCCCTGCTCT CGAGGTGACT GGAAATGGCA TCCGCCGCAA

XCLA5-MO2

TGA GCGGTGATAA CTGTAGAGAAGCCCTGCTCT CGAGGTGACT GGAAATGGCA TCCGCCGCAA

XCLA-MO$^{\infty}$

TGA GCGGTGATAA CTGTAGAGAAGCCCTGCTCT CcAGGTcACT GcAAATcGCA TgCGCCGCAA

Figure 2.2-1 Claudin-5 morpholino sequences injected to block gene expression.

Microinjection experiments were carried out by pneumatic pressure (PL-100, picoinjector, Harvard Apparatus, Cambridge, MA) with Narishige manipulators (Medical Systems Corporation) and microscope (Leica Cat.Mz75). Glass micropipettes were heated and pulled through puller (Model P-97 micropipette puller, Sutter instrument).

GFP (green fluorescent protein) was used as a reporter and marker of expression in the microinjection experiment (Rosorius, 1999). The mRNA was co-injected with GFP. GFP can mark the location of mRNA and show whether mRNA has been injected successfully. The original concentration for the claudin-5 mRNA and GFP were 180 ng/ul and 250 ng/ul. The claudin-5 RNA and GFP were diluted to 1:1 ratio. The mRNA was co-injected with GFP in 2 cells of 4-cell stage, about 1 nl in each cell, also in 4 cells of 8-cell stage.

Microinjection was done in 1X Barth (NaCl 0.09M, KCl 0.001M, $NaHCO_{3,}$ 0.0024g, HEPES 0.0999M, $MgSO_4 \cdot 7H_2O$ 0.0008M, Ca $(NO_3)_2 \cdot 4H_2O$ 0.0003M, $CaCl_2 \cdot 2H_2O$ 0.0004M) plastic dish because 1X Barth can heal injected embryos. The eggs were then moved to 0.1 X Barth plastic dishes and incubated at room temperature until they were at the stage for *in situ* experiment. Uninjected embryos and embryos injected only with GFP were used as controls.

2.2.4 Whole mount in situ hybridization

The Xenopus whole mount *in situ* procedure was based on the protocol from Dr Robertis laboratory.

http://www.hhmi.ucla.edu/derobertis/protocol_page/XenopusWholeMountInSitu.pdf

The whole mount *in situ* hybridization experiment took as long as about 3-4 days. In the first day, the embryos were digested with 10ug/ml Proteinase K (Roche, Cat.1413783) in PBSw and stopped by washing with 2mg/ml glycine (Sigma) in PBSw. Embryos were refixed in 5ml of 4% paraformaldehyde (ICN Biomedical Inc) /0.2% glutaraldehyde (Electron Microscopy Science Inc) in PBSw for 15 minutes. The probe was denatured in hybridization solution at 95ºC for 5 minutes; following washes three times with PBSw. Before starting the second day, the antibody buffer (10% heat inactivated goat serum, 10% Boehringer blocking stock solution and 80% PBSw) was prepared. Embryos were washed in several solutions that day, from 2x SSC, pH4.5 for 5 minutes at 70ºC for three times, 2x SSC, pH7/0.1% CHAPS

at 70ºC for 30 minutes to 2 times wash in MAB at room temperature and 70ºC for 30 minutes. Before adding antibody, embryos were blocked by 1ml Antibody Buffer (without antibody) at 4ºC, rocking, for at least 2 hours. The antibody was blocked at same time. The antibody was Anti-Dig - Alkaline Phosphatase dilutes 1:7,000 from a stock of 150units/200ul (Enzo Company, Cat. 1093274). The pre-blocked antibody was then added to embryos and the embryos were kept rocking in 4 degree overnight. In the last day, the embryos were washed more than 5 times with PBSw. The AP1 Buffer wash was necessary for BM Purple staining. Finally, the AP1 Buffer was replaced with 1ml BM Purple **(**Roche, Cat.904152**)**. The embryos were covered with foil and incubated with rocking at 4 degree. Cold condition and longer rocking overnight help get less background (Xu, 1997). When purple color was strong enough, staining reaction was terminated through stop solution wash and dehydrated with a series of methanol wash (25%, 50%, 75%, and 2x 100%) and stored in 100% methanol in -20 degree for a long time.

2.2.5 Embryo paraffin section after whole mount in situ hybridization

After whole mount *in situ* hybridization, the embryos were incubated for an extended time in metal before paraffin embedding. First, embryos were dehydrated in isopropanol **(**Acros Organics, Cat.67-63-0**)** at room temperature for 15 minutes and incubation with heat (62 degrees) for another 15 minutes. The embryos were then incubated in 50% isopropanol/ paraplast (Fisher) for half an hour. The embryos were always incubated in pure paraplast

for more than two hours. The embryos were rapidly oriented under a dissecting microscope (Fisher Histo-center II) when they were solidified slowly on the heat platinum wire. The solidified embryos were cool down gradually in the cold plate of the machine and were stored at 4 degree for use. The embryos were cut on the section 820 microtome (Spencer**)** at 25μm thickness and put on the microscope slides **(**Fisher**)**. The slides were then dried on the slide warmer at room temperature (Fisher).

2.2.6 Exposure

Embryos were treated with TGF-β, estrogen and estrogen inhibitor (Coenzyme Q-10) (Jolliet, 1998) to examine the effect of these signaling pathways on claudin-5 gene expression. TGF-β was set up as 0.01ug/ml in 0.1X Barth from 2ug/ml **(**Sigma, Cat. 35H0952**)** stock. 0.1X Barth only was the control in the experiment. TGF-β was added to embryos from about stage 16 until embryos fixed in MEMFA around stage 36. Control embryos were fixed at the same stage without exposure. The exposure experiment was done at the room temperature.

Estrogen and inhibitor exposure were performed in three petri dishes containing about 100 embryos per treatment group. Control embryos were placed in 0.1X Barth dishes. In another dishes, embryos were exposured to 20μm 17 beta Estradiol. In the other dishes, embryos exposed to estrogen inhibitor with 20μm concentration. Embryos were left to

grow and remained in solution for three days before data was collected.

2.3 Result

2.3.1 Characterization of Claudin-5 and claudin-12 expression by whole mount *in situ* followed by paraffin sections.

Claudin-5 is a critical component for the TJ primary in the epithelial and endothelia tissue. The results presented here support previous findings that claudin-5 is expressed in the brain and participates in the Blood Brain Barrier (BBB) (Nitta, 2003). In this project, claudin-5 expression is further characterzed. Claudin-12 is another important member in claudin gene family. However, its location in Xenopus development is still not clear. Claudin-12 coexpresses with claudin-5 in the BBB of mouse and compensates claudin-5 loss in the claudin-5 deficiency mouse (Ohtsuki, 2008). The next objective was to determine the expression pattern for claudin-12 in Xenopus development also.

First, whole mount *in situ* hybridization for claudin-5 in embryos around late tail-bud stage was performed. From the claudin-5 whole mount *in situ* figure 2.3-1 (shown as arrow), claudin-5 is strong expressed in mid-brain, heart region and eye. Also, weakly in somite.

claudin-5

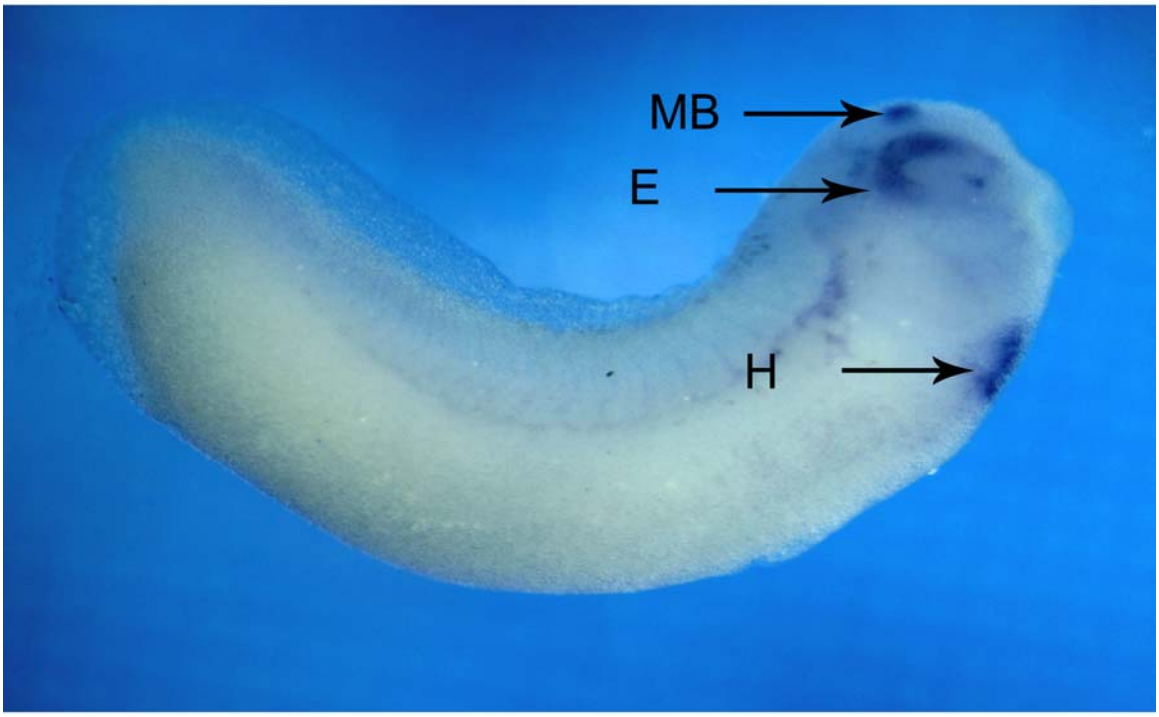

Figure 2.3-1 Claudin-5 whole mount *in situ* in the Xenopus late tail bud stage.
The BM purple color represents claudin-5 expression and darker color means more expression. BM purple can be seen in mid-brain(MB), eyes(E) and heart(H).

To confirm expression of cladin-5, paraffin section was done next. From the paraffin section (Figure 2.3-2), strong expression of claudin-5 RNA can be seen in midbrain and heart. For eyes expression, claudin-5 RNA is localized in the retina. In the heart, myocardial tissue encompasses the endocardial tube in this stage. The myocardial tissue remains a thin, single –cell layer. Claudin-5 is expressed mostly in myocardial (shown as B, C); expressions can also be seen in endocardial in the anterior section (shown as A). With posterior section, claudin-5 is shown in neural tube (shown as D). No claudin-5 expression in liver primordium. Cladin-5 is appears to be the only one found in the claudin gene family so far with expression in myocardium and endocardium. Claudin-5 also has a very complex expression pattern in heart. These results taken together with previous findings in the lab by J.Xie (Xie & Brizuela, 2005) lead us to investigate claudin-5 function in Xenopus heart development.

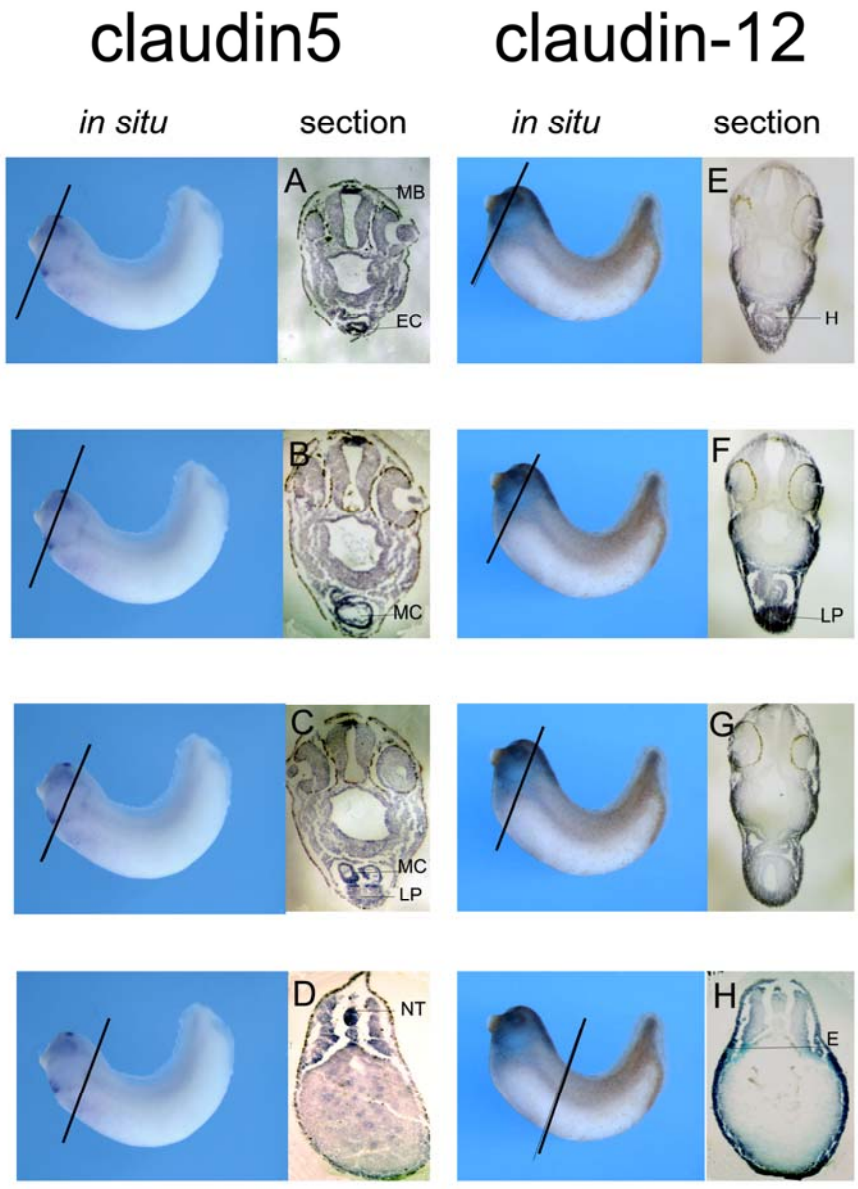

Figure 2.3-2 Paraffin sections on claudin-5 (A-D) and claudin-12 (E-H) whole mount *in situ* embryos. Claudin-5 expression can be seen in brain, eyes and heart. In heart, most of staining is in myocardium. None or just little staining is in the liver primordium. Claudin-12 has little expression in heart but very strong expression in the epidermis. MB; mid-brain, EC: endocardium, MC:myocardium, LP: liver primordium, NT: neural tube, H:heart, E: epidermis.

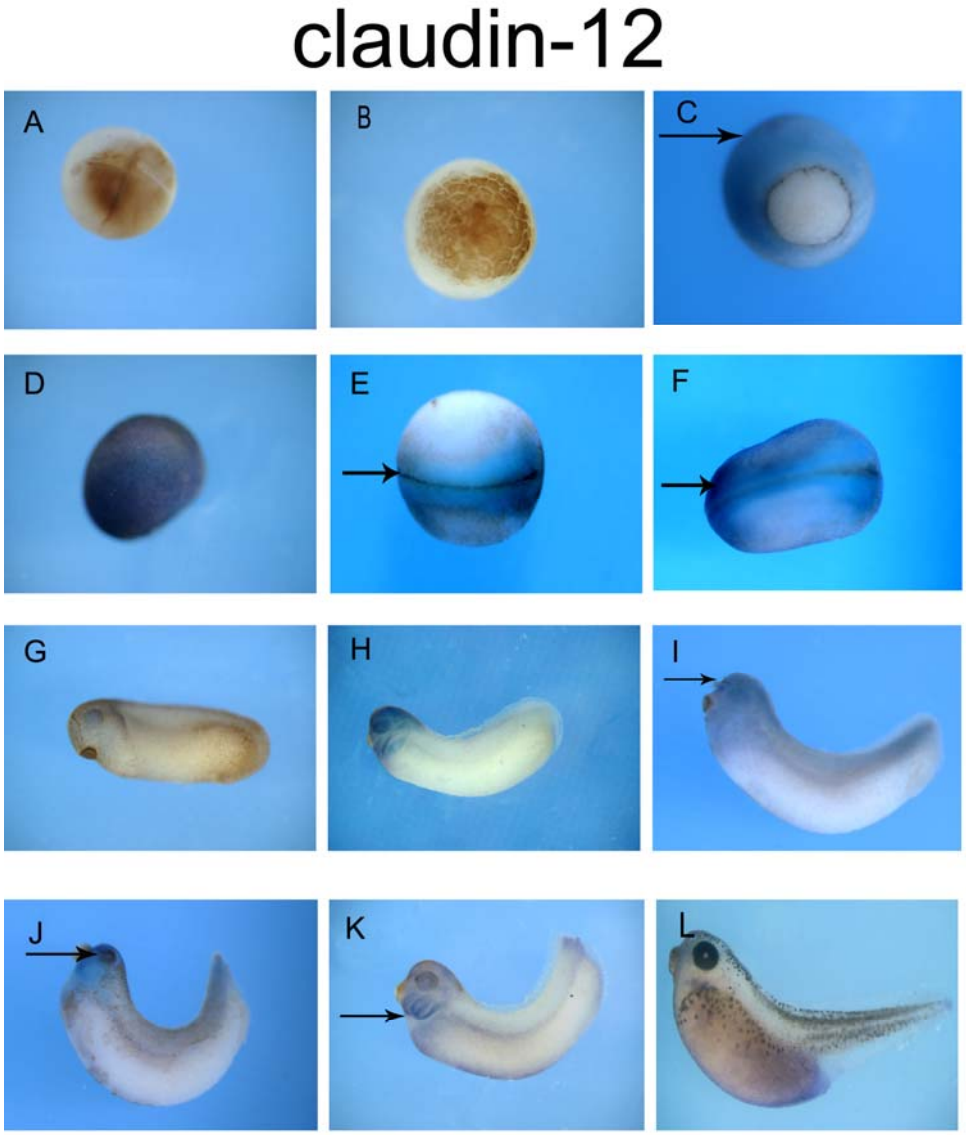

Figure 2.3-3 Expression pattern for claudin-12 with whole mount *in situ* hybridization. From four cell stage(A),blastula stage(B), gastrula stage (C) , early neural stage(D), late neural stage(E,F), early tailbud stage(H, I, G), late tail bud stage (K,) to tadpole stage(L). Claudin-12 started to express in gastrula stage (C). In neural stage, claudin-12 expression is around neural tube (shown as arrow, E, F). In tail bud stage, claudin-12 expression can be seen in eyes, heart and branial arch (H, I, J, K) also in somite (J,K,I). In the tadpole stage, expression is in heart and posteior skin (L).

The claudin-12 expression pattern can be seen from whole mount *in situ* hybridization

figures (Figure 2.3-3). There is no staining in four cell stage. Claudin-12 started to express in gastrula stage. In gastrula stage, claudin-12 is expressed in ventral side. Claudin-12 expression is strong around neural tube in both of the early and late neural stages (shown in E and F). In tail bud stage, claudin-12 expression can be seen in eyes, neural tube and brachial arch also a little in heart. In the tadpole stage, expression is in brain and epidermis. Overall, claudin-12 has complicated expression pattern in Xenopus development. In section figures (Figure 2.3-2), claudin-12 is expressed very weak in the heart region. It has strong expression in neural tissue, where claudin-12 is well known for BBB composition. However, claudin-12 has no similarity in heart region with claudin-5. So we can conclude that although claudin-12 and claudin-5 have same expression in somewhere such as brain but they still have very different expression pattern such as heart.

In conclusion, claudin-5 has much better and more special expression pattern in the heart so our future experiments will focus only on claudin-5.

2.3.2 Identification and description of suitable heart markers genes in Xenopus tailbud stage.

Strong expression of claudin-5 in Xenopus heart structure during development was characterized by whole mount *in situ* and section. To determine if claudin-5 is important in heart development, over expression and knock down of claudin-5 gene expression was used.

It is required to find good markers for heart development in Xenopus. So the second task was to make RNA probes and demonstrate expression of these markers in Xenopus embryos. Four markers were shown to expressed in the developmental stage of interest and in the heart tissue: *xNkx2.5, Troponin*(*Tro*), *xMsr* and *xTbx*. These marker genes are been popular used (Cleaver, 1996: Allen, 2005; Miskolczi-McCallum, 2005). The goal in this part is to screen the best genes which co-localize with claudin-5.

The methods are whole mount *in situ* hybridization and paraffin section in this part.

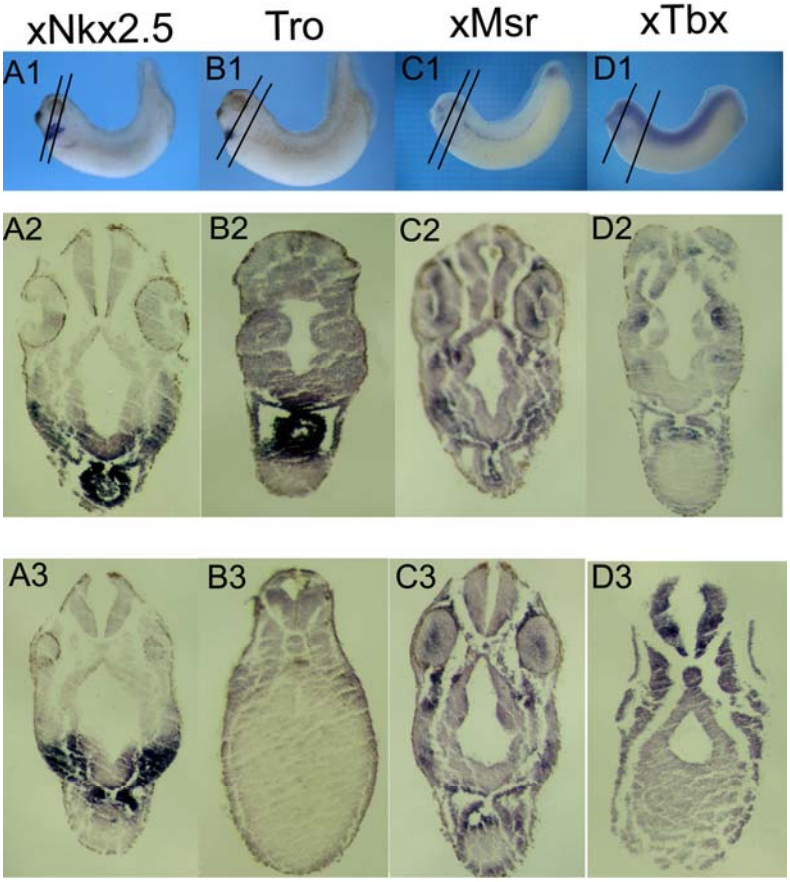

Figure 2.3-4 Whole mount *in situ* hybridization and paraffin sections for heart marker genes.
The whole mount *in situ* results(A1,B1,C1,D1) and sections (A2-D2, A3-D3) for heart markers--xNkx2.5 (A1-A3), Tro (B1-B3), xMsr (C1-C3) and xTbx (D1-D3). Figures A2, B2, C2 and D2 are sections in heart region while A3,B3,C3 and D3 are section figures in more posterior. Lines in the *in situ* show the dissection site.

Paraffin sections were done on pigment embryos chosen from late tail bud stage and stained darkly according to the whole mount *in situ* hybridization protocol (Figure 2.3-4). All of the heart markers have bilateral expression in Xenopus embryos. The *xNkx2.5* and *Tro* genes have similar expression pattern which is strong in the myocardium. The expression domain is also seen in claudin-5, however, much stronger can be seen in the

xNkx2.5 and *Tro* section figures. The *xMsr* gene has a complicated expression pattern in Xenopus. From *in situ* figure, expression can be seen in four regions such as brachial arch, somite, heart and tail. Sections of *xMsr* show expression in the neural tube, eyes, and epithelia of midgut and endocardium of heart. More attention should be paid to the difference between *xMsr* and other marker genes. Only *xMsr* expresses in endocardium in this paper. The next heart marker is *xTbx*, *xTbx* is not only specially fixed in heart. Instead, *xTbx* is widely expressed from head to neural tube and notochord. Compared with other markers, *xTbx* expression is continuous in the dorsal of embryo. This may be due to *xTbx* expression along the neural tube which is in dorsal side of embryo. Because *xMsr* and *xTbx* have multiple expression regions in the Xenopus embryo, they were not selected as markers for the experiment below. The x*Nkx2.5* and *Tro* expression coincides with claudin-5 location and were therefore the best two candidates for heart makers for these experiments.

2.3.3 Claudin-5 over expression experiments

Claudin-5 mRNA injection following heart marker genes whole mount *in situ*

Injection of synthetic claudin-5 mRNA can result in over expression. GFP was coinjected, which has been applied as a microinjection tracer in Xenopus research (Sobkow, 2006). Green fluorescent was checked by fluorescent microscopy (Figure 2.3-5). This must be done on live embryos fixed for in situ experiment. The green spots in figure indicate

successful injection of the RNA and also the location of the injection. For instant, from figure 2.3-5, it is easy to see that claudin-5 RNA was injected in the heart and head. Only the embryos with heart injection were picked for future experiment such as whole mount *in situ* hybridization and paraffin section.

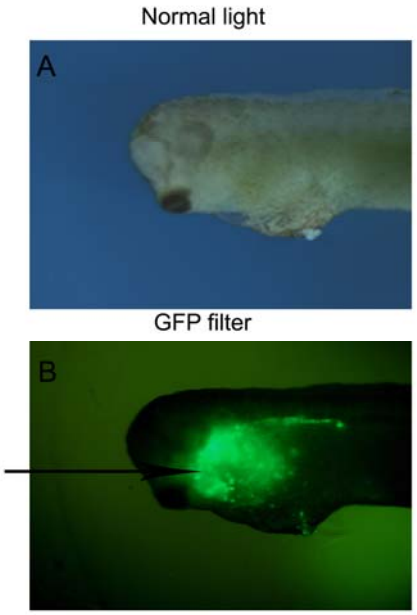

Figure 2.3-5 Claudin-5-GFP co-injected embryos under fluorescent microscope.
The embryos are taken pictures after co-injected with GFP RNA and claudin-5 RNA. A is under normal light and B is under GFP filter. GFP is green color under filter as arrow shown. The green spots mark location of claudin-5 and means successful injection (shown as arrow).

The methods in this part are microinjection, whole mount *in situ* hybridization and paraffin section. One hundred embryos were set up in each plastic dish for control (GFP injected only) and over expression (claudin-5 mRNA+GFP).

The Xenopus heart is formed from bilaterally symmetrical patches of mesoderm which fuse

at the ventral midline to form a linear heart tube (Miskolczi-McCallum, 2005). The myocardium or heart muscle is derived from the mesoderm of embryo also. Claudin-5 expression starts in blastula stage and dorsal lip in gastrula stage (Xie & Brizuela, 2005). From former student work, over expression of claudin-5 mRNA causes ectopic heart in tadpole stage (Xie & Brizuele, 2005).

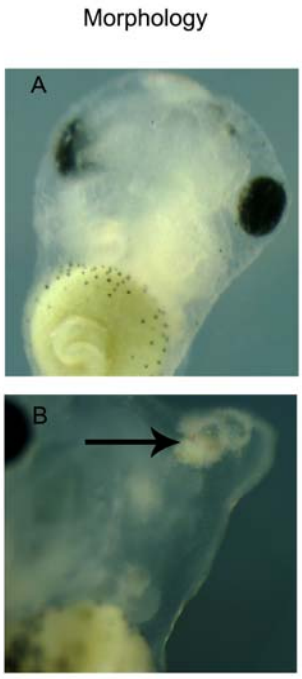

Figure 2.3-6 Ectopic heart found after claudin-5 over expression (Xie & Brizuela, 2005).
A is control embryo while B is claudin-5 mRNA injected embryo. The arrow shows the ectopic heart.

The ectopic heart is labeled by *xNkx2.5* in tailbud stage (Figure 2.3-7, C and D) in my study. The tinman homolog *Nkx-2.5* is expressed in the precardiac mesoderm prior to heart formation and essential for normal heart development (Bartlett, 2007). The *Tro* expression shown by whole mount *in situ* hybridization after claudin-5 over expression (Figure 2.3-8)

illustrates that injected embryos have larger heart compared with control embryos. The region of heart enlarges a little bit and vessels include arteries extend from anterior ventral side to posterior dorsal side. The ectopic expression for *xNkx2.5* is found about half (about 35 out of 70) in the survival embryos and 57% (about 40 out of 70) for *Tro*. 100% (98 out of 98) of control group show the normal heart development.

Ectopic expressions of heart marker gene-*xNkx2.5* and beating tissue in tadpole stage have proved that claudin-5 can induce ectopic heart, which demonstrates claudn-5 may play a very important role in heart formation. In addition of main component of TJ, claudin-5 may be a strong regulator for heart formation and can initiate ectopic heart. These results mean claudin-5 should be work in heart formation as early as gastrula stage to initiate ectopic heart and involve in a complex pathway.

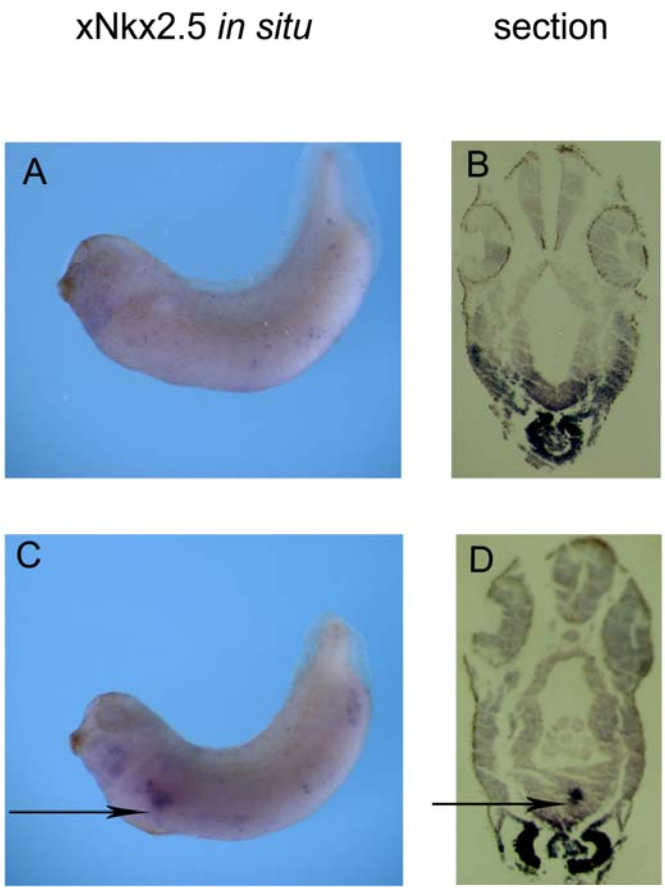

Figure 2.3-7 *XNkx2.5* whole mount *in situ* hybridization from claudin-5 mRNA injection experiment. A and B are control embryos and C and D are injected embryos. Extra xNkx2.5 expression can be seen in claudin-5 mRNA injected embryos (shown as arrows in C and D).

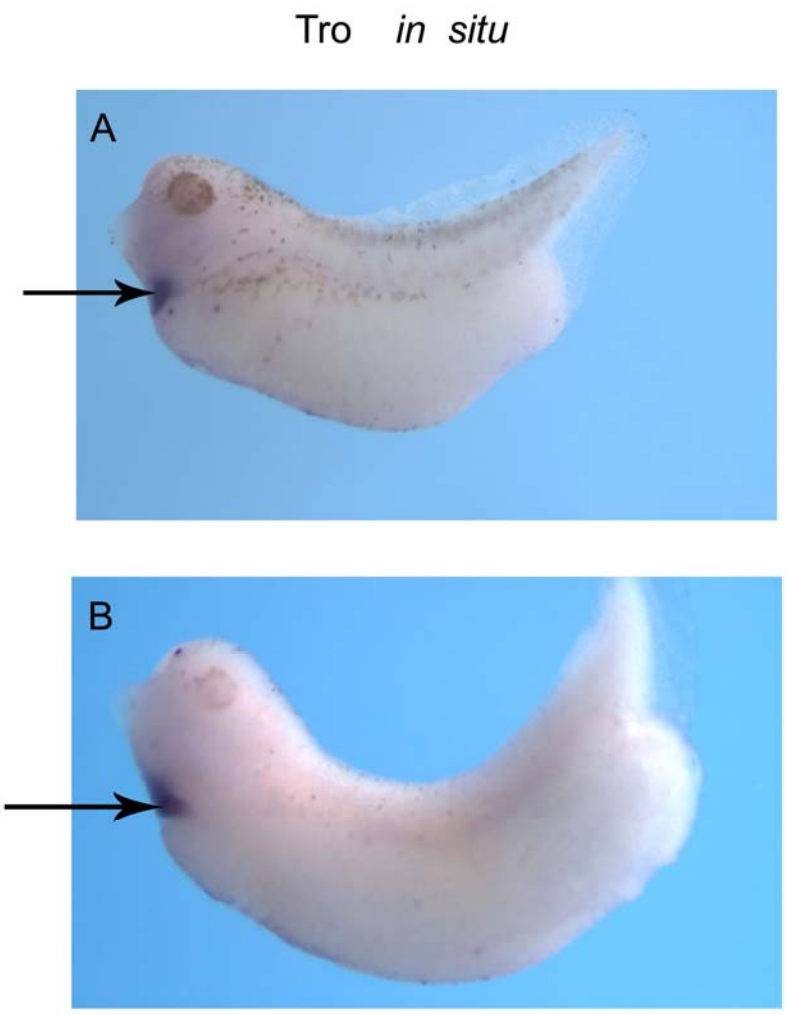

Figure 2.3-8 *Tro* whole mount *in situ* hybridization after claudin-5 over expression.
Control embryo is without injection (A) while injected embryo is after claduin-5 RNA injection (B). Tro expresses in heart in both of embryos although expression of claduin-5 over expression is a little bit stronger (shown as arrow in B).

2.3.4 Knock down by claudin-5-morpholino causes abnormal heart development

To further investigate claudin-5 function in heart development, claudin-5 knock down

experiment was done with Morpholino Oligomers (MO). MO is targeted to block translation (Method and material) and is useful in knocking down claudin-5 expression. The methods are microinjection and whole mount *in situ* hybridization in this part. Same as last part, one hundred embryos set up for control (without injection) and knock down group (MO injection).

From the figure 2.3-9 and 2.3-10, the injected embryos obviously show much weaker expression. For the *xNkx2.5 in situ* after claudin-5 MO injection, the region of heart reduces and *xNkx2.5* mRNA level is depressed in the injected embryos. For the *Tro in situ* after MO injection, no *Tro* expression is found in injected embryos (shown in Figure 2.3-10 B). Loss of claudin-5 expression by claudin-5 MO results in reduced heart tissue and a tube fail to loop. All of these results demonstrate *xNkx2.5* and *Tro* mRNA activities are downregulated after claudin-5 knock down and heart development is abnormal. The light color in injected embryos may attribute to loss of cell layers and cell number after claudin-5 RNA knock down. The weaker expression for *xNkx2.5* and *Tro* can be seen in 80 % (about 60 out of 75) and 75% (60 out of 80) of survival embryos. While the normal heart development can be found 100% from control dish (99 out of 99).

Combining claudin-5 over expression and knock down experiments, it is very clear that

claudin-5 affects heart formation and plays an important role as early as heart initiation. Over expression of claudin-5 causes ectopic heart while knocking down claudin-5 induce heart defect. This can explain why claudin-5 protein less could cause TMVCF and Digeorge syndrome in human being.

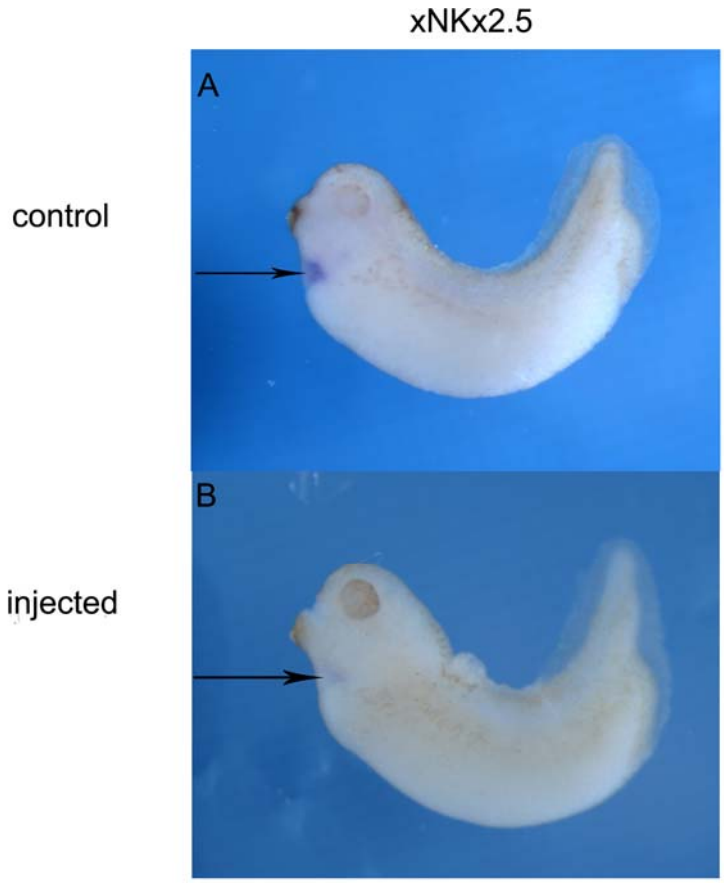

Figure 2.3-9 *XNkx2.5* whole mount *in situ* hybridization after claudin-5 knock down.
A is control embryo. B is embryos after Morpholino injection. Morpholino was used to knockout claduin-5 RNA in the embryos. The morpholino injected embryos has much weaker expression for *xNkx2.5* (shown as arrow).

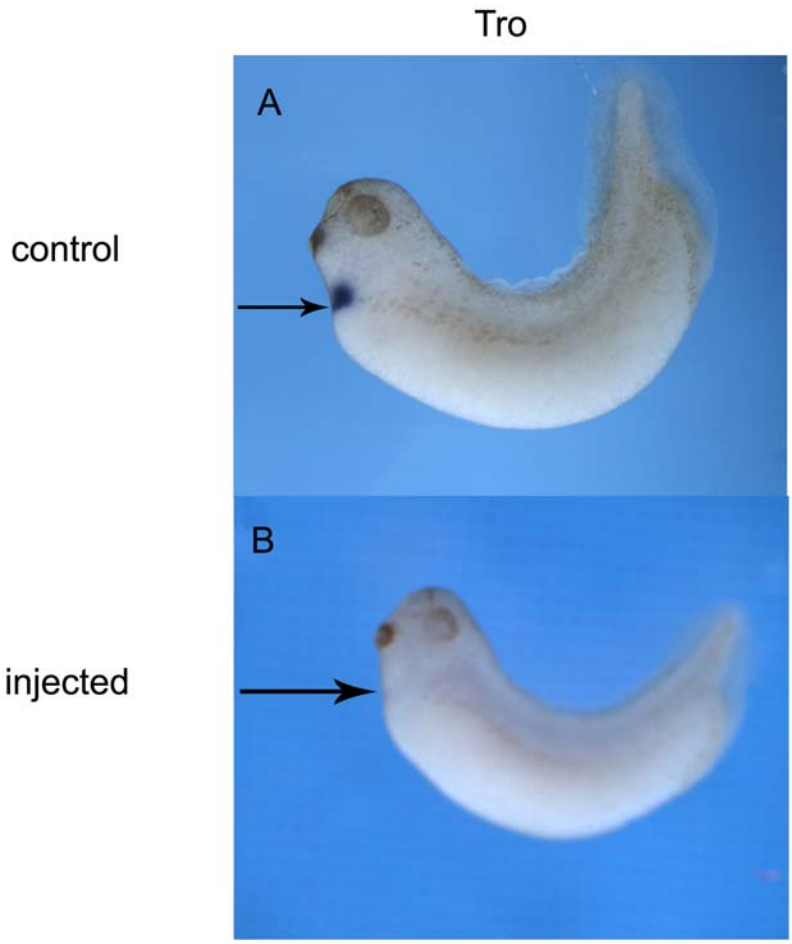

Figure 2.3-10 *Tro* whole mount *in situ* hybridization after claudin-5 knock down.
A is control embryo. B is embryo after Morpholino injection. The morpholino injected embryo has little or no expression for *Tro* after claudin-5 mRNA knockdown (shown as arrow in B).

2.3.5 Investigation possible signaling pathways for claudin-5

A Over expression of claudin-5 causes ectopic expression of *chordin* and reduced expression of BMP4

In last part, morphogenesis of heart in late stage with heart marker gene is investigated. Next object will be further investigation of possible pathway for claudin-5. First, to find exact claudin-5 function in heart development, it is important to see what happen as early as cardiac precursor initiate----gastrula stage. Meanwhile, the claudin-5 over expression is dose dependent. The embryos with 2nl in 4-cell injection could survive to late stage as last chapter shown. Other embryos with 4nl in 4-cell injection all died in gastrula stage. What happen in gastrula stage is critical for understanding claudin-5 function in heart development. The object in this part was to analyze claudin-5 function in gastrula stage with early markers.

Embryonic patterning is established as early as gastrula stage through a complex network with many molecules. Dorsoventral patterning of vertebrate embryos has been well studied and is one of important paradigms to understand morphogen function (Moreno, 2005). The gradient of BMP4 activity in extracellular determines dorsoventral axis. The final gradient of BMP4 depends on BMP4 concentration (Moreno, 2005) and antagonist proteins including *chordin* (*chd*) (Xie, 2005), *noggin* (Piccolo, 1996), *Xolloid* (*Xld*) (Moreno, 2005), also some bilateral function protein such as Twisted gastrulation (*Tsg*) (De Robertis, 2000; Wills, 2006). *Chd* is the central regulator of BMP4 and it can bind BMP4 through conserved cysteine-rich domains (CRs) and blocks binding of the growth factor to its cognate receptor (Moreno,

2005). Compare with other factors such as *noggin*, *chd* can gain detectable dorsalizing and neutralizing activity in lower amount and has much stronger blocking function for BMP4 (Piccolo, 1996). Except for dorsal axis determination, BMP4 is also important in causing a specific region of mesoderm to form heart tissue while this function is dose dependent. Recently, it been found that BMP4 implantation can lead to increase expression of *Nkx2.5* in chick and induce robust cardiac myocyte differentiation (Schuhheiss, 1997). The antagonist of BMP signaling inhibits cardiac myogenesis. Furthermore, the regulation is time and position dependent which is in the anterior paraxial mesoderm. The effect of BMP4 on *Nkx2.5* is by stabilizing the existing precursors against inhibitory signals arising from nearby tissues or by recruiting additional cells to the specified state (Sater, 1990). In conclusion, BMP4 is critical in cardiac myocyte specification through regulation of *Nkx2.5* and is an appropriate candidate for early marker in claudin-5. Meanwhile, since *chd* is a primary blocking factor for BMP4 (Xie, 2005), we shoose it as the main antagonist for BMP4 in this paper.

The methods in this part are claudin-5 mRNA microinjection, whole mount *in situ* hybridization. One hundred embryos were set up each dish: claudin-5 mRNA injection for *chd in situ* and BMP4 *in situ*; control (without injection) for *chd* and BMP4 *in situ*.

BMP4 control *in situ* results show BMP4 mRNA expression in the ventral side (Figure 2.3-11 C). Meanwhile, *chd* control mRNA expression is in the dorsal lip (Figure 2.3-11 A). Due to *chd* inhibition on BMP4, their expressions are complementary.

After claudin-5 RNA injection, cells movement pattern has been changed by over expression of claudin-5. In injected embryos, the shape is abnormal. On the other hand, injected embryos have big yolk plug compare with control group and this dues to a loss of ingression at the dorsal lip and cells fail to move inward. For chd *in situ* after claudin-5 microinjection, about 93 percentages of survival embryos (75 out of 80) show the cells expressing *chd* move to the marginal of embryos instead of dorsal lip (compare arrows in A and B). Ectopic expression of *chd* can be seen in injected embryos and expression region extends from dorsal lip to ventral side. Due to larger region of *chd* mRNA, BMP4 activity decreases to ventral point. This can be seen about 82% (70 out of 85) in survival embryos. It is very clear that *chd* mRNA level enhances while BMP4 mRNA expression is depressed after claudin-5 mRNA microinjection. 100% embryos in control dish show the normal expression for *chd* and BMP4.

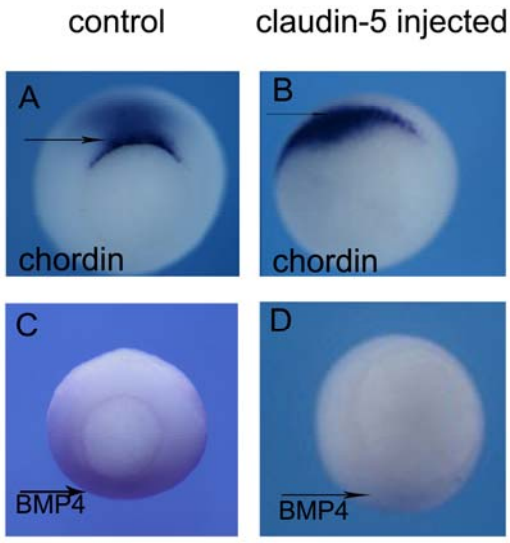

Figure 2.3-11 BMP4 and *chd* whole mount *in situ* hybridization after claudin-5 mRNA injection. All the embryos are from gastrula stage. A and C are control embryos while B and D are embryos after claudin-5 mRNA injection. A and B are *in situ* with *chd*; C and D with BMP4. Ectopic expression of *chd* is shown after injection (shown as arrow in B).

Furthermore, cell spots with *chd* expression which are migrating from dorsal lip to marginal (shown as arrow in Figure 2.3-12) can be seen. In summary, claudin-5 affect BMP4 and *chd* expression pattern in embryos through changing cells migrating pathway especially in the gastrula stage. Over expression of claudin-5 mRNA disturbs normal cells movement.

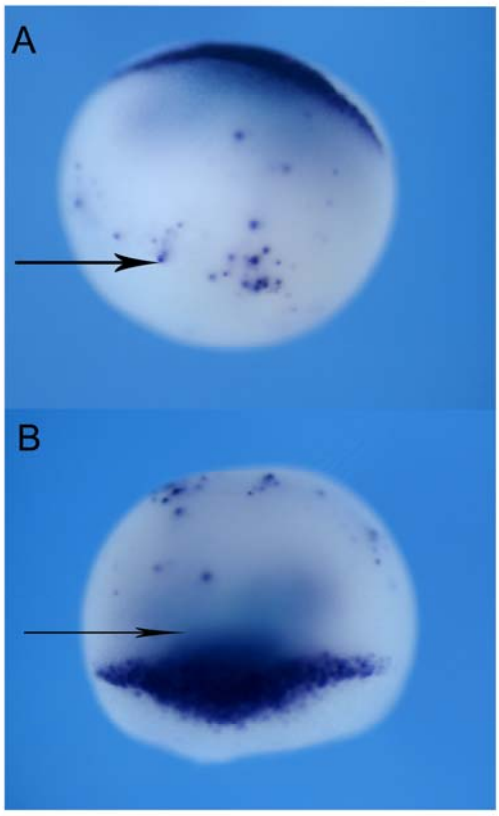

Figure 2.3-12 *Chd* whole mount *in situ* hybridization after claudin-5 mRNA injection. Migrating cells with *chd* expression can be seen in the figures (shown as arrow).

2.3.6 Determine what other signaling pathways be affected by examining TGF-β and estrogen signaling

A Analysis claudin-5 expression in embryo exposed to TGF – β

BMP4 is a member of the transforming growth factor (TGF) – β. To decide if other member such as TGF-β interacts with claudin-5, embryos were exposed to TGF-β to determine the in vivo effect on claudin-5. Watabe show in his paper that TGF-β suppresses the expression of claudin-5, while SB-431542 (a synthetic molecule that inhibits the kinases of receptors for

TGF-β) strongly enhances claudin-5 expression. At the same time, TGF – β does not change expression of claudin-12 or VE-cadherin (a component of adherens junctions) (Watabe, 2003). The inhibition of TGF – β depends on ALK-5-Smad 2/3 pathway while TGF – β has ALK-1-Smad1/5 which can induce cell proliferation. BMP4 activates Smad1/5 through AlK-1 so it may increase claudin-5 activity although not proved yet.

In this paper, Xenopus embryos were exposed to TGF-β at about stage 16, with 0.01ug/ml concentration about 100 embryos in each dish, the embryos without TGF-β treatment were used as control. After exposure, embryos were checked for claudin-5 RNA expression level with paraffin section. From the section result (Figure 2.3-13), Brain and heart are the two locations where claudin-5 has strong expression. For mid-brain, claudin-5 expression of exposure treatment has prominent difference with control ones. Claudin-5 is down-regulated in mid-brain especially for the right side. The expression of claudin-5 on the right side is almost invisible (shown as arrow in B). At the same time, the effect of TGF-β on claudin-5 is one-side also. The left side of myocardium seems to be the same whether in control or in exposure. While, the right side of myocardium is down-regulate after exposure. Part of cell layers loses claudin-5 expression becoming weaker in the right side. In conclusion, TGF-β has asymmetry effect in controlling claudin-5 expression in heart. The can be seen from about 80% (8 out of 10) from section slide in TGF-β treated dish.

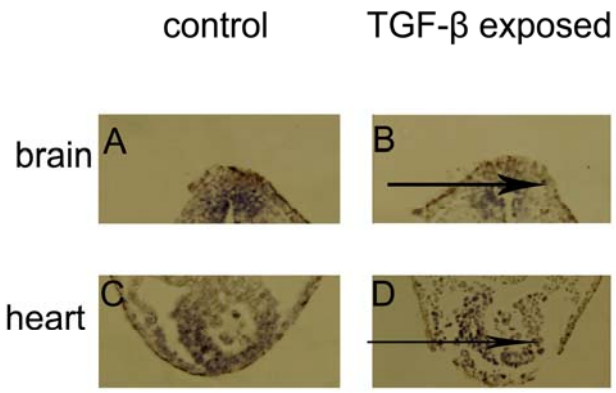

Figure 2.3-13 Section for claudin-5 with and without TGF-β exposure.
Figure A and C are figures for control. B and D are section after exposure. Figure A and B are for brain section while C and D for heart. Exposed embryos seem to have weaker expression in right side of brain and heart (B, D) compare with control (A, C). Right side of myocardium loses claudin-5 expression after exposure (shown as arrow).

C Analysis of claudin-5 expression in the presence of estrogen & estrogen inhibitor.

Estrogens are a group of steroid compounds, named for their importance in the estrous cycle and functioning as the primary female sex hormone in mammal. It has reported that estrogen is a negative regulator for TGF-β signal transduction pathways (Pinkas, 2006). This work suggests that TGF-β downregulates claudin-5 expression. Next objective was to ask if estrogen hormones can enhance claudin-5 level by inhibiting TGF-β signal. Estrogen

and estrogen inhibitor were used in this paper to address this question. Estrogen inhibitor is Coenzyme Q-10 in this project (Jolliet, 1998). The methods in this part are chemical compound exposure and whole mount *in situ* hybridization. One hundred embryos were selected in three dishs: control, estrogen treated and estrogen inhibitor treated. Same as expectation, estrogen can activate claudin-5 expression. From claudin-5 in situ results (Figure 2.3-14), embryos with estrogen treatment have very strong staining especially for brain, heart and eyes. Focus on anterior head region; claudin-5 expression is not just in mid-brain but also reaches to hind-brain area. Meanwhile, claudin-5 expression can be seen in whole eyes from estrogen treatment embryos, comparing with control groups which has staining only in retina. In the heart region, ventricle and myocardium enlarge in estrogen exposure embryos (arrow in E); also extra vessels can be identified. This can be found almost hundred percentages in survival embryos. Claudin-5 shows a much weaker expression in estrogen inhibitor embryos. The heart of embryos treated with inhibitor loses the function of looping (shown in arrow in F). The phenotypes in estrogen and estrogen inhibitor treated embryos can be found almost hundred percentages.

Overall, estrogen is a positive regulator for claudin-5. The conclusion is also supported partly from ELISA work done together with Carmel. Martin-Fairey(data not shown). The

regulation may be through depressing TGF-β signal transduction pathways or upregulating claudin-5 directly. This need further work to prove this signaling pathway.

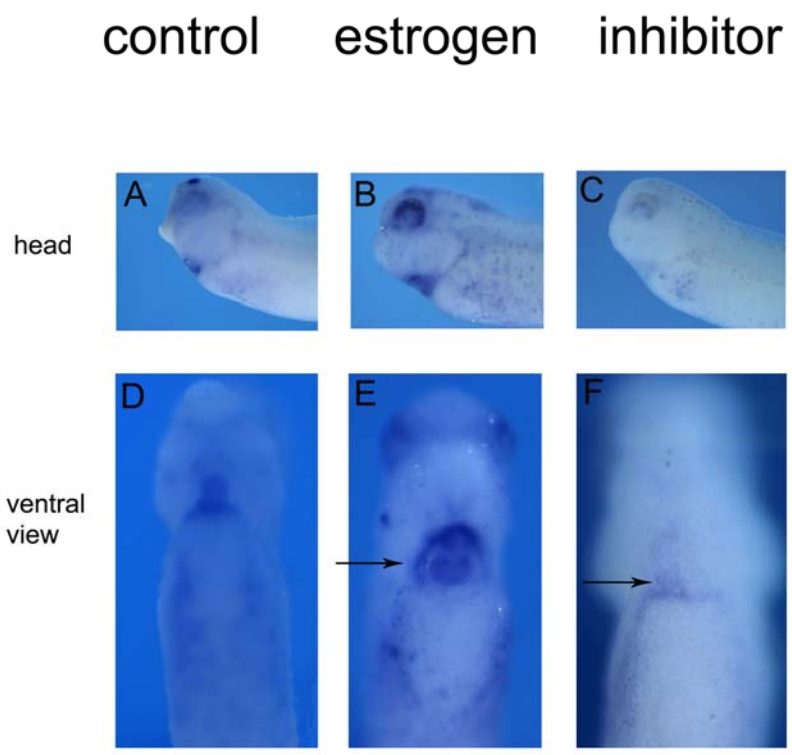

Figure 2.3-14 Whole mount *in situ* hybridization for claudin-5 after estrogen and inhibitor exposure. Control (A, D,), estrogen exposure (B, E) and estrogen inhibitor (C, F). A, B, C are focus on head area. D, E and F are from ventral view and in heart region. Estrogen treated embryos have darker staining in brain, eyes and heart also staining in after-brain (B). Ventricle and myocardium enlarge in estrogen exposure embryos (arrow in E), also extra vessels can be identified. Claudin-5 show much weaker expression in estrogen inhibitor embryos. The heart of inhibitor is without looping (shown in arrow in F).

2.4 Discussion

Cardiac development is a relatively complex process and understanding the heart morphogenesis is important for treating heart defects. In the process of heart development, differentiation of cardiac muscle is a critical event and TJ proteins must exert an essential function in this process. From whole mount *in situ* and cross section results, claudin-5 has special expression in myocardium and part of endocardium; it should be main composition of TJ in Xenopus heart development. The ectopic heart and morphology change induced by claudin-5 mRNA injection show the claudin-5 function in heart development. Claudin-5 may not only be an important part of the TJ junction but also have the ability to initiate heart formation. Over expression of claudin-5 may disturb normal the TJ network, which impacts on channels for some molecules such as *chd*. More molecules flow may ultimately lead to ectopic heart. Alternatively, claudin-5 could affect cell-cell interactions and cell migration.

At the gastrula stage, where heart development initiates, over expression of claudin-5 changes cell movement especially for the cell expressing *chd*. *Chd* is well known as a molecule secreted from the Spemann's Organizer. The interaction between claudin-5 with organizer genes can result in two things. First, upregulation of organizer molecules pushes mesoderm formation. Ectopic random *chd* expression may induce ectopic mesoderm tissue such as heart. Secondly, larger expression region of *chd* decreases BMP4 expression. This

may be another explanation for claudin-5 function in heart development. It is known BMP4 is regulator for the *xNkx2.5* gene and heart formation. Over expression of claudin-5 may upregulate *xNkx2.5* and causes ectopic heart through decreasing BMP4 activity (Schuhheiss, 1997). It was concluded by Schuhheiss that BMP4 implantation can lead to increase expression of *Nkx2.5* in chick and induce robust cardiac myocyte differentiation (Schuhheiss, 1997). His work seems to be different from result in my paper. How to interpret the difference? First, xenopus is not same animal model with chick although they share some similarity. Secondly, the function of BMP4 depends on concentration. Low dose of BMP4 stimulate muscle. Intermediate dose can induce kidney while high does direct cells form blood. Combine my result with Schuhheiss', it can be suggested the effect of BMP4 on *xNkx2.5* or heart formation may be different depending on the time in development and region of the embryos. Downregulation of BMP4 on the dorsal side or upregulation of BMP4 on the anterior and ventral domains stimulate *xNkx2.5* activity to enhance initial heart formation at gastrula stage.

Claudin-5 may affect multiple steps in heart formation (Figure 2.4-1). Over expression of claudin-5 could alter BMP4-*chd* pathway in gastrula stage; induce ectopic heart marker gene expression and ectopic heart during late stage. The ectopic heart may be the result of interaction with extracellular matrix and *xNkx2.5*. On the other hand, reducing the level of

claudin-5 could result in less heart tissue or a tissue fail to loop.

With exposure experiment, other possible pathways have been suggested for claudin-5. Estrogen exposure increases claudin-5 activity while estrogen inhibitor causes a decrease. Further investigation is needed to decide the mechanism of this effect. TGF – β has an asymmetric effect on claudin-5 function, positive on left side while negative on right side. This function could be through the ALK-5-Smad 2/3 pathway but it is still not clear if left-right signal molecules such as *xLefty* are involved in this pathway. BMP4 is a positive regulator for ALK-1-Smad1/5 which has been shown to increase claudin-5 level in microarray analysis (Ota, 2002). BMP may have a negative feedback on itself by the ALK-Smad1/5-cladin-5 pathway. This will be a new mechanism for BMP4 activity and embryo dorsal-ventral pattern.

In conclusion, claudin-5 is an important factor for Xenopus development especially for heart formation. It may be involved in other critical pathways that regulate many molecules. Enhancing claudin-5 level is good for differentiation of cardiac muscle and may be a candidate gene defective for some heart disease such as TMVCF and Digeorge syndrome in human being. This paper offers some suggested pathways to alter claudin-5 levels. This line of research may provide a new approach to deliver potential drugs across the BBB into

the central nervous system. TGF-β knocks down claudin-5 and decreases cell membrane resistance in BBB to deliver drugs of small molecular size into the central nervous system. Thus downregulation claudin-5 is a potential method for treatment in the adults, while upregulation of claudin-5 may have potential for gene therapy in the developing embryos.

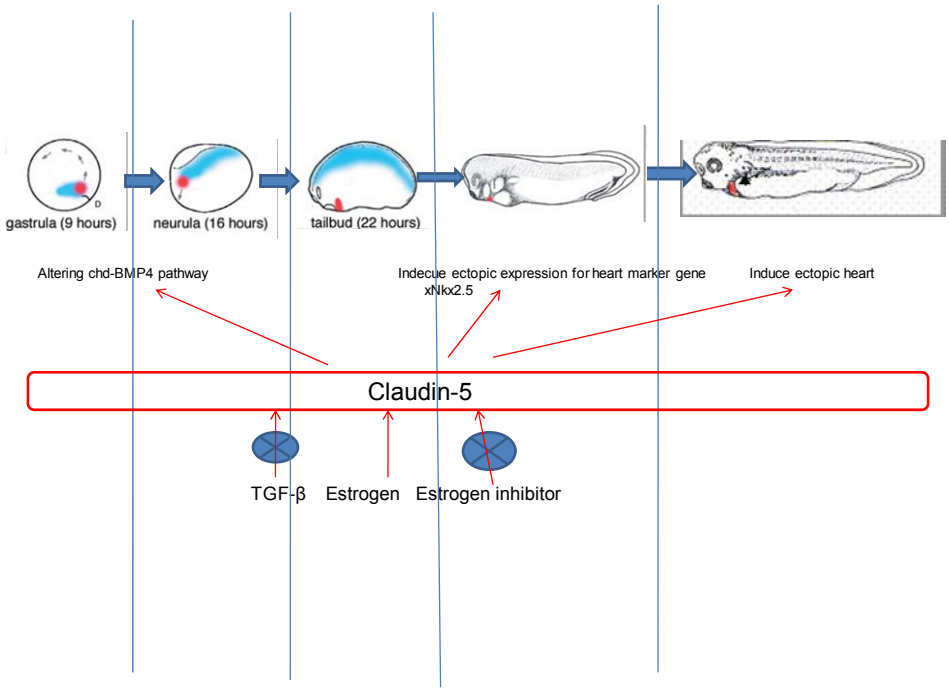

Figure 2.4-1 The claudin-5 may function in multiple steps during heart formation (Mohun, 2003). Xenopus heart formation is a multiple process that begins with specification of cardiogenetic mesoderm, followed by heart tube formation, looping and finally formation of the heart chambers.

REFERENCES

Allen, J. (2005). Narrative Review: Alternative Causes for Elevated Cardiac Troponin Levels when Acute Coronary Syndromes Are Excluded. *American College of Physicians.*

Anderson, J. M., & Van Itallie, C. M. (1995). Tight junctions and the molecular-basis for regulation of paracellular permeability. *AM.J.Physiol , 32*, G467-475.

András, I. E., Pu, H., Tian, J., Deli, M. A., Nath, A., & Hennig, B. (2005). Signaling mechanisms of HIV-1 Tat-induced alternation of claudin-5 expression in brain endothelia cells. *Journal of Cerebral Blood Flow&Metabolism , 25*, 1159-1170.

Balda, M. S., Whitney, J. A., Flores, C., Gonzalez, S., Cereijido, M., & Matter, K. (1996). Functional dissociation of paracellular permeability and transepithelial electrical-resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane-protein. *J.Cell Biol , 134*, 1031-1049.

Bartlett, H. L., Sutherland, L., Kolker, S. J., Welp, C., Tajchman, U., Desmarais, V., et al. (2007). Transient Early Embryonic Expression of Nkx2-5 Mutations Linked to Congenital Heart Defects in Human Causes Heart Defects in Xenopus laevis. *Dev Dyn , 236* (9), 2475-2484.

Belanger, M., Asashima, T., Ohtsuki, S., Yamaguchi, H., Ito, S., & Terasaki, T. (2007). Hyperammonemia induces transport of taurine and creatine and suppresses claudin-12 gene expression in brain capillary endothelial cells in vitro. *Neurochem Int , 50* (1), 95-101.

Brizuela, B. J., Wessely, O., & De Robertis, E. M. (2001). Overexpression of the Xenopus Tight-Junction Protein Claudin Causes Randomization of the Left–Right Body Axi. *Developmental Biology , 230*, 217-229.

Bronstein, J. M., Popper, P., Micevych, P. E., & Farber, D. B. (1996). Isolation and characterization of a novel oligodendrocyte-specific protein. *Neurology , 47*, 772–778.

Bronstein, J. M., Tiwari-Woodruff, S., Buznikov, A. G., & Stevens, D. B. (2000). Involvement of OSP: claudin-11 in oligodendrocyte membrane interactions: role in biology and disease. *J Neurosc Res , 59*, 706-711.

Buhr, J., Weber, G. B., Staub, E., Heinze, M., Klam, I., Pilarsky, C., et al. (2007). Differential expression

of genes encoding tight junction proteins in colorectal cancer: frequent dysregulation of claudin-1.-8 and -12. *Int J Colorectal Dis , 22*, 651-659.

Cereijido, C. (1992). Evolution of ideas on the tight junction. *In Tight Junctions，Boca Raton: CRC Press* , 1-13.

Chen, Y. H., Merzdorf, C., Paul, D. L., & Goodenough, D. A. (1997). COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos. *J Cell Biol , 138*, 891-899.

Cheung, S. T., Leung, K. L., Ip, Y. C., Chen, X., Fong, D. Y., Ng, I. O., et al. (2005). Claudin-10 expression level is associated with recurrence of primary hepatocellular carcinoma. *Clin Cancer Res , 11*, 551-556.

Citi, S., Sabannay, H., Jakes, R., Geiger, B., & Kendrich-Jones, J. (1988). Cingulin, a new peripheral component of tight junctions. *Nature , 333*, 272-75.

Claude, P. (1978). Morphological factors influencing transepithelial permeability: A model for the resistance of the zonula occludens. *J Membr Biol , 39*, 219–232.

Claude, P., & Goodenough, D. A. (1973). Fracture faces of zonulae occludentes from “tight” and “leady” epithelia. *J Cell Biol , 58*, 390-400.

Cleaver, O. B., Patterson, K. D., & Krieg, P. A. (1996). Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in Xenopus embryos results in myocardial hyperplasia. *Development , 122*, 3549-3556.

De Robertis, E. M., Larraın, J., Oelgeschla¨ger, M., & Wessely, O. (2000). The establishment of Spemann’s Organizer and patterning of the vertebrate embryo. *Nat Rev Genet , 1*, 171-181.

Devreotes, P. (Sep. 8, 1989). Dictyostelium discoideum: A Model System for Cell-Cell Interactions in Development Science. *New Series , 245* (4922), 1054-1058.

Enck, A. H., Berger, U. V., Alan, S., & Yu, L. (2001). Claudin-2 is selectively expressed in proximal nephron in mouse kidney. *Am J Physiol Renal Physiol , 281*, F966–F974.

Fanning, A. S., Lapierre, L. A., Brecher, A. R., Van Itallie, C. M., & Anderson, J. M. (1996). Protein interactions in the tight junction: the role of MAGUK proteins in regulating tight junction organization and function. *Curr.Topics Membr , 43*, 211-35.

Fanning, A. S., Mitic, L. L., Melvin, J., & Laura, A. (1999). Transmembrane proteins in the tight junction barrier. *J Am Soc Nephrol , 10*, 1337-1345.

Farquhar, M., & Palade, G. E. (1963). Junctional complexes in various epithelia. *J. Cell Biol , 17*, 375–412.

Fujita, H., Chiba, H., Yokozaki, H., Sakai, N., Sugimoto, K., Wada, ,. T., et al. (2006). Differential Expression and Subcellular Localization of Claudin-7, -8, -12, -13, and -15 Along the Mouse Intestine. *Journal of Histochemistry & Cytochemistry , 54* (8), 933-944.

Fujita, H., Sugimoto, K., Inatomi, S., Maeda, T., Osanai, M., Uchiyama, Y., et al. (2008). Tight junction proteins claudin-1 and -12 are critical for Vitamin D -dependent Ca2+ absorption between enterocytes. *Molecular Biology of the Cell , 19*, 1912-1921.

Furuse, M., Hata, M., Furuse, K., Yoshida, Y., Haratake, A., Sugitani, Y., et al. (2002). Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1–deficient mice. *The journal of Cell Biology , 156* (6), 1099-1111.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., & Tsukita, S. (1993). Occludin - a novel integral membrane-protein localizing at tight junctions. *J. Cell Biol , 123*, 1777-1788.

Furuse, M., Itoh, M., Hirase, T., Nagafuchi, F., Yonemura, S., & Tsukita, S. (1994). Direct association between occludin and ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol , 127*, 1617-26.

Furuse, M., Sasaki, H., Fujimoto, K., & Tsukita, S. (1998). A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J. Cell Biol. , 143* (391-401).

Geiger, B., & Ayalon, O. (1992). Cadherins. Annu. Rev. *Cell Biol , 8*, 307-332.

Getsios, S., Huen, A. C., & Green, K. J. (2004). Working out the strength and flexibility of desmosomes. *Nat Rev Mol Cell Biol , 5*, 271–81.

Gitter, A. H., Bertog, M., Schulzke, J. D., & Fromm, M. (1997). Measurement of paracellular epithelial conductivity by conductance scanning. *Pflu¨gers Arch , 434*, 830-840.

Goldberg, R., Motzkin, B., Marion, R., Scambler, P. J., & Shprint-zen, R. J. (1993). Velo–cardio–facial syndrome: A review of 120 patients. *Am. J. Med. Genet , 45*, 313-319.

Gow, A., Davies, C., Southwood, C. M., Frolenkov, G., Chrustowski, M., Ng, L., et al. (2004). Deafness in claudin 11-null mice reveals the critical contribution of basal cell tight junctions to stria vascularis function. *J Neurosci , 24*, 7051-62.

Gow, A., Southwood, C. M., Li, J. S., Pariali, M., Riordan, G. P., Brodie, S. E., et al. (1999). CNS myelin and Sertoli cell tight junction strands are absent in Osp:claudin-11 null mice. *Cell , 99*, 649–659.

Gumbiner, B. M. (1993). Breaking through the tight junction barrier. *J.cell Biol , 123*, 1631-1633.

Gumbiner, B. (1987). Structure, biochemistry, and assembly of epithelial tight junctions. *Am.J.Physiol , 253*, C749-C758.

Gumbiner, B., Lowendopf, T., & Apatira., D. (1991). Identification of a 160 kDa polypeptide that binds to the tight junction protein ZO-1. *Proc Natl Acad Sci USA , 88*, 3460-3464.

Hart, I. R., Goode, N. T., & Wilson, R. E. (1989). Molecular aspects of the metastatic cascade. *Biochim Biophys Acta , 989*, 65-84.

Hasegawa, S., Furukawa, Y., Li, M., Satoh, S., Kato, T., Watanabe, T., et al. (2002). Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. *Cancer Res , 62*, 7012-7017.

Heiskala, M., Peterson, P. A., & Yang, Y. (2001). The roles of claudin superfamily proteins in paracellular transport. *Traffic , 2*, 93-8.

Hough, C. D., Sherman-Baust, C. A., & Pizer, E. S. (2000). Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. *Cancer Res , 60*, 6281-6287.

Huber, O. (2003). Structure and function of desmosomal proteins and heir role in development and disease. *Cell Mol Life Sci , 60* (9), 1872–90.

Hyatt, B. A., & Yost, H. J. (1998). The left-right coordinator:the role of Vg1 in organizing leftright axis formation. *Cell , 93*, 37-46.

Hyatt, B. A., Lohr, J. L., & Yost, H. J. (1996). Initiation of vertebrate left-right axis formation by maternal vg1. *Nature , 384*, 62-65.

Ishizaki, T., Chiba, H., Kojima, T., Fujibe, M., Soma, T., Miyajima, H., et al. (2003). Cyclic AMP

induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood–brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. *Experimental Cell Research , 290* (2), 275-288.

Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., & Tsukita, S. (1993). The 220-kD protein colocalizing with cadherins in nonepithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells; cDNAcloning and immunolocalization. *J.Cell Biol , 121*, 491-502.

Jiang, W. G., Martin, T., Llaffafian, I., & Mansel, R. E. (2000). Tight junctions, a critical structure in the control of cancer invasion and metastasis. *Cancer Metastasis, Molecular and Cellular Mechanisms and Clinical Intervention* , 195-213.

Jiang, W. G., Puntis, M., & Hallett, M. B. (1994). The molecular and cellular basis of cancer invasion and metastasis and its implications for treatment. *Br J Surg , 81*, 1576-1590.

Johnson, A. H., Frierson, H. F., Zaika, A., Powell, S. M., Roche, J., Crowe, S., et al. (2005). Expression of tight-junction protein claudin-7 is an early event in gastric tumorigenesis. *Am J Pathol , 167*, 577-584.

Jolliet, P., Simon, N., Barré, J., Pons, J. Y., Boukef, M., Paniel, B. J., et al. (1998). Plasma coenzyme Q10 concentrations in breast cancer: prognosis and therapeutic consequences. *Int J Clin Pharmacol Ther. , 36* (9), 506-9.

Kapprell, H., Duden, R., Owaribe, K., Schmelz, M., & Franke, W. W. (1990). Subplasmalemmal plaques of intercellular junctions: common and distinguishing proteins. *Morphoregulatory Molecules, ed. GMEdelman, BACunningham, JP Thiery* , 285–314.

Katahira, J., Sugiyama, H., Inoue, N., Horiguchi, Y., Matsuda, M., & Sugimoto, N. (1997). Clostridium perfringens enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. *J Biol Chem , 272*, 26652-26658.

Katoh, M. (2003). CLDN23 gene, frequently down-regulated in intestinal-type gastric cancer, is a novel member of CLAUDIN gene family. *Int. J. Mol. Med , 11*, 683-689.

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *TIC , 9*, 317-321.

Keon, B. H., Schafer, S., Kuhn, C., Grund, C., & Franke, W. W. (1996). Symplekin, a novel type of tight junction plaque protein. *J. Cell Biol , 134*, 1003–18.

Kitajima, Y., Endo, T., Nagasawa, K., Manase, K., Honnma, H., Baba, T., et al. (2005). Hyperstimulation and a Gonadotropin-Releasing Hormone Agonist Modulate Ovarian Vascular Permeability by Altering Expression of the Tight Junction Protein Claudin-5. *Endocrinology , 147* (2), 694-699.

Kitajiri, S.-i., Furuse, M., Morita, K., Saishin-Kiuchi, Y., Kido, H., Ito, J., et al. (2004). Expression patterns of claudins, tight junction adhesion molecules, in the inner ear. *Hearing Research , 187*, 25-34.

Kolker, S. J., Tajchman, U., & Weeks, D. L. (2000). Confocal imaging of early heart development in Xenopus laevis. *Dev Biol , 218*, 64-73.

Kominsky, S. L., Argani, P., & Korz, D. (2003). Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. *Oncogene , 22*, 2003-1033.

Kooistra, ,. M., Dubé, N., & Johannes, B. L. (2007). Rap1: a key regulator in cell-cell junction formation. *Cell science , 120* (1), 17.

Koto, T., Takubo, K., Ishida, S., Shinoda, H., Inoue, M., Tsubota, K., et al. (2007). Hypoxia Disrupts the Barrier Function of Neural Blood Vessels through Changes in the Expression of Claudin-5 in Endothelial Cells. *The American Journal of Pathology , 170* (4).

Krause, G., Winkler, L., Mueller, S. L., Haseloff, R. F., Piontek, J., & Ingolf, E. (2008). Blasig Structure and function of claudins. *Biochimica et Biophysica Acta , 1778*, 631–645.

Linask, K. K., Yong-Hao, Gui, & Knudsen, K. A. (1997). N-Cadherin–Catenin Interaction: Necessary Component of Cardiac Cell Compartmentalization during Early Vertebrate Heart Development. *Developmental Biology , 185*, 148-164.

Lipschutz, J. H., Li, S., Arisco, A., & Balkovetz, D. F. (2005). Extracellular Signal-regulated Kinases 1/2 Control Claudin-2 Expression in Madin-Darby Canine Kidney Strain I and II Cells. *The journal of Biologucal chemistry , 280*.

Lohr, J. L., & Yost, H. J. (2000). Vertebrate Model Systems in the Study of Early Heart Development: Xenopus and Zebrafish. *American Journal of Medical Genetics , 97*, 248-257.

Long, H., Crean, C. D., & Lee, W. H. (2001). Expression of Clostridium perfringens enterotoxin receptors claudin 3 and claudin 4 in prostate cancer epithelium. *Cancer Res , 61*, 7878-7881.

Luis, R. (1992). Tight junction permeability to ions and water. *Tight junction* .

Matter, M. S., & Balda, K. (1998). Biogenesis of tight junctions: the C-terminal domain of occludin mediates basolateral targeting. *J Cell Sci , 111*, 511-519.

Mclay, R. N., Kimura, M., Banks, W. A., & Kastin, A. J. (1997). Granulocyte-macrophage colony stimulating factor crosses the blood-brain and blood-spinal cord barriers. *Brain , 120*, 2083-91.

Michl, P., Barth, C., & Buchholz, M. (2003). Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. *Cancer Res , 63*, 6265-6271.

Miskolczi-McCallum, C. M., Scavetta, R. J., Svendsen, P. C., Soanes, K. H., & Brook, W. J. (2005). The Drosophila melanogaster T-box genes midline and H15 are conserved regulators of heart development. *Developmental Biology , 278*, 459-472.

Mitic, L. L., & Anderson, J. M. (1998). Molecular architecture of tight junctions. *Annu. Rev. Physiol , 60*, 121-42.

Miyamoto, T., Morita, K., Takemoto, D., Takeuchi, K., Kitano, Y., Miyakawa, T., et al. (2005). Tight junctions in Schwann cells of peripheral myelinated sxons:a lesson from claudin-19-deficient mice. *Cell Biology , 169* (3), 527-538.

Mohun, T. J., Leong, L. M., Weninger, W. J., & Sparrow, D. B. (2000). The morphology of heart development in Xenopus laevis. *Dev Biol , 218*, 74-88.

Mohun, T., Orford, R., & Shang, C. (2003). The origins of cardiac tissue in the amphibian, Xenopus laevis. *TCM, 13, 6.*

Moreno, M., Muñoz, R., Aroca, F., Labarca, M., Brandan, E., & Larraín, J. (2005). Biglycan is a new extracellular component of the Chordin–BMP4 signaling pathway. *The EMBO Journal. , 24*, 1397-1405.

Morin., a. P., Hewitt, K. J., Agarwal, R., & Morin, J. P. (2006). The claudin gene family: expression in normal and neoplastic tissues. *BMC Cancer , 6*, 186.

Morita, K., Furuse, M., Fujimoto, K., & Tsukita., S. (1999). Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc Natl Acad Sci USA , 96*, 511–516.

Morita, K., Sasak, S., Fujimoto, K., Furuse, M., & Tsukita, S. (1999). Claudin-11:OSPbasedtight junctions of myelin sheaths in brain and Sertoli cells in testis. *J Cell Biol , 145*, 579–588.

Morita, K., Tsukita, S., & Miyachi, Y. (2004). Tight junction-associated proteins (occludin, ZO-1, claudin-1, claudin-4) in squamous cell carcinoma and Bowen's disease. *British Journal of Dermatology , 151*, 328–334.

Nieuwkoop, P. D., & Faber, J. (1994). *Normal Table of Xenopus laevis(DAUDIN). A systematical and chronological survey of the development from fertilized egg till the end of metamorphosis.*

Nitta, I., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimot, N., et al. (2003). Size-seletive loosening of the blood-brain barrier in claudin-5-deficient mice. *Cell Biology , 161*, 653-60.

Ohtsuki, S., Yamaguchi, H., Katsukura, Y., Asashima, T., & Terasaki, T. (2008). mRNA expression levels of tight junction protein genes in mouse brain capillary endothelial cells highly purified by magnetic cell sorting. *Journal of Neurochemistry , 104*, 147-154.

Ohtsuli, S., Sato, S., Yamaguchi, H., Kamoi, M., Asashima, T., & Terasaki, T. (2007). Exogenous Expression of Claudin-5 Induces Barrier Properties in Cultured Rat Brain Capillary Endothelial Cells. 210:81-. *Journal of cellular physiology , 210*, 81-86.

Ota, T., Fuji, M., Sugizaki, T., Ishi, M., Miyazawa, K., Aburatani, H., Miyazono, K. (2002). Target of transcriptional regulation by two distinct type I receptors for transformation growth factor-b in human umbilical vein endothelia cells. *J of Cellular P, 193(3), 299.*

Paperna, T., Peoples, R., Wang, Y.-k., Kaplan, P., & Francke, U. (1998). Genes for the CPE Receptor (CPETR1) and the Human Homolog of RVP1 (CPETR2) Are Localized within the Williams–Beuren Syndrome Deletion. *Genomics , 54* (3), 453-459.

Pardridge, W. M. (1997). Drug delivery to the brain. *J.Cereb.Blood Flow Metab , 17*, 713-31.

Pepper, M. S., Spray, D. C., Chanson, M., Montesano, R., Orci, L., & Meda, P. (1989). Junctional communication is induced in migrating capillary endothelial cells. *Cell Biol , 109*, 3027-3038.

Peters, N. S., Severs, N. J., Rothery, S. M., Lincoln, C. H., Yacoub, M. H., & Green, C. (1994). Spatiotemporal relation between gap junctions and fascia adherens junctions during postnatal development of human ventricular myocardium. *Circulation , 90*, 713-725.

Piccolo, S., Sasai, Y., Lu, B., & Robertis, D. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP4. *Cell , 86*, 589-598.

Pinkas, J., & Teicher, B. A. (2006). TGF-b in cancer and as a therapeutic target. *Biochemical Phatmacology , 72*, 523-529.

Ramsdell, A. F., & Yost, H. J. (1999). Cardiac looping and the vertebrate left-right axis: antagonism of left-sided Vg1 activity by a right-sided ALK2-dependent BMP pathway. *Development , 126*, 5195-5205.

Rangel, L. B., Agarwal, R., D'Souza, T., Pizer, E. S., Alo, P. L., Lancaster, W. D., et al. (2003). Tight Junction Proteins Claudin-3 and Claudin-4 Are Frequently Overexpression in Ovarian Cancer but Not in Ovarian Cystadenomas. *Clinical Cancer Research , 9*, 2567-2575.

Rosorius, O., Heger, P., Stelz, G., Hirschmann, N., Hauber, J., & Stauber, R. H. (1999). Direct observation of nucleocytoplasmic transport by microinjection of GFP-taggedproteins in living cells. *Biotechniques , 27* (2), 350-5.

Sáeza, J. C., Retamala, M. A., Basiliob, D., Bukauskasb, F. F., & Bennettb, M. L. (2005). Connexin-based gap junction hemichannels: Gating mechanisms. *Biochimica et Biophysica Acta , 1711*, 215-224.

Saitou, M., Ando-Akatsuka, Y., Itoh, M., Furuse, M., Inazawa, J., Fujimoto, K., et al. (1997). Mammalian occludin in epithelial cells: its expression and subcellular distribution. *Eur J Cell Biol , 73*, 222-231.

Sanada, Y., Oue, N., Mitani, Y., Yoshida, K., & Nakayama, H. (2006). Downregulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. *J Pathol , 208*, 633-642.

Sanford, J. L., Edwards, J. D., Mays, T. A., Gong, B., Merriam, A. P., & Rafael-Fortney, J. A. (2005). Claudin-5 localizes to the lateral membranes of cardiomyocytes and is altered in utrophin/dystrophin-deficient cardiomyopathic mice. *Journal of Molecular and Cellular Cardiology , 38* (2), 323-332.

Sater, A. K., & Jacobson, A. G. (1990). The restriction of the heart morphogenetic field in Xenopus laevis. *Dev. Biol , 140*, 328-336.

Schneeberger, E. E., & Lynch, R. D. (1992). Structure, function, and regulation of cellular tight junctions. *AM.J.Physiol , 262*, 647-661.

Schuhheisis, T. M., Burch, J. B., & Lassar, A. B. (1997). A role for bone morphogenetic proteins in the

induction of cardiac myogenesis. *Genes & Development , 11*, 451-462.

Shi, Y., Katsev, S., Cai, C., & Evans, S. (2000). BMP signaling is required for heart formation in vertebrates. *Dev Biol , 224*, 226-237.

Simons, K., & van Meer, G. (1998). Lipid sorting in epithelias cells. *Biochemistry , 27*, 6197-6202.

Sirotkin, H., Morrow, B., Saint-Jore, B., Puech, A., Gupta, R. D., Patanjali, S. R., et al. (1997). Identification, Characterization, and Precise Mapping of a Human Gene Encoding a Novel Membrane-Spanning Protein from the 22q11 Region Deleted in Velo-Cardio-Facial Sydrome. *Genomics , 42* (2), 245-51.

Sobkow, L., Epperlein, H. H., Herklotz, S., Straube, W. L., & Tanaka, E. M. (2006). Dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration. *Developmental Biology , 290*, 386 – 397.

Song, Y., Kato, S., & Fleet, J. C. (2002).Vitamin-D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and ECa 2+ and calbindin D9k mRNA. *Nutr. , 133*, 374-380.

Staehelin, L. A. (1974). Structure and function of intercellular junctions. *Int.rev.Cyto , 39*, 191-283.
Stevenson, B. R. (1998). ZO-3, a novel member of the MAGUK protein family found at the tight junction, interact with ZO-1 and occludin. *J Cell Bil , 141*, 199-208.

Stevenson, B. R., Silicano, J. D., Mooseker, M., & Goodenough, D. A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol , 103*, 755–66.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulators. *Science , 251*, 1451-1455.

Terris, B., Blaveri, E., & Crnogorac-Jurcevic, T. (2002). Characterization of gene expression profiles in intraductal papillary mucinous tumors of the pancreas. *Am J Pathol , 160*, 1745-1754.

Tsukita, S., Furuse, M., & Itoh, M. (1996). Molecular dissection of tight junctions. *Cell Structure function , 21*, 381-385.

Turksen, K., & Troy, T. C. (2001). Claudin-6: A Novel Tight Junction Molecule Is Developmentally

Regulated in Mouse Embryonic Epithelium. *Development dynamics , 222*, 292-300.

Turksen, T. K., & Claire, T. (2002). Permeability barrier dysfunction in transgenic mice overexpressing claudin 6. *Development , 129*, 1775-1784.

Watabe, T., Nishihara, A., Mishima, K., Yamashita, J., Shimizu, K., Nishikawa, M., et al. (2003). TGF-receptor kinase inhibitor enhances growth and integrity of embryonic stem cell–derived endothelial cells. *The Journal of Cell Biology.*

Willott, E., Balda, M. S., Fanning, A. S., Jameson, B., Van Itallie, C., & Anderson, J. M. (1993). The tight junction protein ZO-1 is homologous to the Drosophila Discs-large tumor suppressor protein of septate junctions. *Proc. Natl. Acad. Sci. USA , 90*, 7834-38.

Wills, A., Harland, R. M., & Khokha, M. K. (2006). Twisted gastrulation is required for forebrain specification and cooperates with Chordin to inhibit BMP signaling during X. tropicalis gastrulation. *Developmental Biology , 289*, 166-178.

Wilson, P. A., Lagna, G., Suzuki, A., & Hemmati-Brivanl, A. (1997). Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. *Development , 124*, 3177-3184.

Wright, E. M., & Diamond., J. M. (1968). Effects of pH and polyvalent cations on the selective permeability of gall-bladder epithelium to monovalent ions. *Biochim.Biophys.Acta , 163*, 57-74.

Www.steve.gb.com/science/cell_communication.html. (n.d.).

Xie, J., & Brizuela, B. J. (2005). Analysis of Xenopus laevis claudin (Xcla) tight junction genes in development. *North Carolina State University ETD* .

Xie, J., & Fisher, S. (2005). Twisted gastrulation enhances BMP signaling through chordin dependent and independent mechanisms. *Development , 132*, 383-391.

Xu, Q., & Wilkinson, D. G. (1997). *In Situ Hybridization, A Practical Approach and In situ hybridization of mRNA with hapten labeled probe.*

Yamashita, H., Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., et al. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J.Cell Biol , 130*, 217-226.

Yang, G. Y., Betz, A. L., Chenevert, T. L., Brunberg, J. A., & Hoff, J. T. (1994). Experimental intracerebral hemorrhage: relationship between brain edema, blood flow, and blood-brain barrier permeability in rats. *Neurosur , 81* (1), 93-102.

Yin, T., & Green, K. J. (2004). Regulation of desmosome assembly and adhesion. *Cell&developmental Biology , 15*, 665-677.

Ylermi, S. (2004). Claudins 2, 3, 4, and 5 in Paget's Disease and Breast Carcinoma.

Yosef, B., Belyantseva, I. A., Saunders, T. L., Hughe, E. D., Kawamoto, K., VanItallie, C. M., et al. (2003). Claudin-14 knock out mice, a model for autosomal recessive deafness DFNB 29, are deaf due to cochlear hair cell degeneration. *Hum Mol Genet , 12*, 2049-61.

Yuki, O., & Christopher, V. E. (2007). Anteriorward shifting of asymmetric Xnr1 expression and contralateral communication in left–right specification in Xenopus. *Developmental Biology , 301* (2), 447-463.

Zessig, S., Bürge, N., Günzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., et al. (2007). Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut , 56*, 61-72.

Zhong, Y., Enomoto, K., Tobioka, H., Konishi, Y., Satoh, M., & Mori, M. (1994). Sequential decrease in tight junctions as revealed by 7H6 tight junction-associated protein during rat hepatocarcinogenesis. *Jpn. J. Cancer Res , 85*, 351–56.

Zhong, Y., Saitoh, T., Minase, T., Sawada, N., Enomoto, K., & Mori, M. (1993). Monoclonal antibody 7H6 reacts with a novel tight junction-associated protein distinct from ZO-1, cingulin and ZO-2. *J. Cell Biol , 120*, 477-83.

Zimmerman, L. B., Jesus-Escobar, J. M., & Harla, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell , 86*, 599-606.