A descriptive and functional study of zebrafish Connexin43

by

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Connnexin43(Cx43) encodes a gap junction protein. Cx43 is specifically expressed in blood vessel endothelial cells in the zebrafish embryo as demonstrated by in situ hybridization. When Cx43 is knocked down with antisense morpholinos, the zebrafish embryos have defects in the intersegmental vessel branching and vascular perfusion. These phenotypes reflect problems in endothelial cell migration. Further analysis with microangiography revealed that the endothelial tubes or lumens fail to form normally. The co-injection of Cx43 morpholinos and p53 morpholinos rescued the vascular defects to limited degrees. The co-injection of Cx43 morpholinos with VE-Cadherin morpholino enhanced the effects observed with the injection of Cx43 morpholino alone. The transgenic zebrafish expressing the fusion protein of Cx43 and egfp under control of flk1 promoter (Tg[flk1: Cx43-egfp]) has been generated for studying the dynamic regulation of Cx43 protein in zebrafish endothelium. From live imaging of this transgenic line, green fluorescent puncta from Cx43-gfp can be found in the endothelial cells. Those puncta could be patches of gap junctional channels or undocked hemichannels on the cell-to-cell borders. The existence of hemichannels could support a role of Cx43 in cue sensing for migration or in the endothelial lumen formation. Immunohistochemistry of VE-Cadherin in Tg[flk1: Cx43-egfp] line shows that not all the Cx43-egfp puncta are co-localized with the VE-Cadherin. The endogenous Cx43 appears on the putative surface of the endothelial cells of the sprouting intersegmental vessels at 22 hours post fertilization (hpf), as determined by staining with an antibody against Cx43 in transgenic fish with gfp-highlighted endothelial cells. This suggests a potential role of Cx43 in endothelial migration. It is possible that
Cx43 forms gap junction channels with other cell types or functions as hemichannels. To test the function of Cx43 in vascular formation, Cx43 dominant negative mutants (DN) are expressed in an endothelium-specific manner. In order to control the expression of those DNs during the generation of the transgenic zebrafish, a Loxp flanked fluorescent protein expression cassette is inserted between the promoter and DN expression cassette, which will be excised when Cre is introduced.
CHAPTER 1. GENERAL LITERATURE REVIEW

Most metazoan cells are connected via gap junction channels except blood and skeletal muscle cells (Kumar 1996). Gap junctions are a unique kind of the transmembrane channel in that they span the two layers of the cell membrane and connect the cytoplasm of two cells while being sealed against the extracellular space. Gap junction channels formed by different gap junction proteins that have different permeabilities. The gap junction channels may allow the formation of the polarized distribution of signal molecules over a range of cells connected by gap junctions. This could be important in cell differentiation or physiology (Levin, M., 2007).

Connexin genes encode the gap junction proteins and are unique to the chordates. Gene targeting experiments in mice and the association of mutations in connexin genes with many human disease states have demonstrated crucial roles for these proteins in both development processes and homeostasis of adult tissues (Wei, C., 2004). Connexins may also function without forming gap junctions. For example, the hexamer of connexins forms a connexon, also known as a hemichannel, which may allow the passage of certain signal molecules between the intra- and extracellular environment (Spray, D., 2006). A recent study examining neuron migration shows that the adhesion force between two connexons is required for neuron migration (Elias, L. 2007).

Zebrafish Cx43 is prominently expressed in the notochord and the endothelial cells (Chatterjee, B. 2005). We have shown that after knockdown of Cx43 using morpholinos in zebrafish, the vasculature is malformed. In addition, the notochord cells fail to adopt a differentiated state. Since signals from the notochord can sequentially affect somitogenesis
and vascular morphogenesis, it is unknown whether the endothelial Cx43 plays an important role in endothelial migration and lumen formation. To begin to address the role of Cx43 in vascular morphogenesis, some descriptive and functional studies of Cx43 in endothelial cells have been carried out in this thesis.

1.1 Thesis Organization

This thesis is organized into four parts. The first part provides the general outlines of the thesis, followed by a more detailed literature review on the gap junction proteins, Connexin 43 and vasculature morphogenesis. This includes consideration of endothelial migration and tube formation. The second chapter is organized in the format of a paper, including its own introduction, methods, results and discussion. The third chapter presents the general conclusions of this thesis, following by the references.

1.2 Literature Review

1.2.1 Gap junction

The term of gap junction refers to the junction patch between two cells on the cell membrane. These are plaques of close aggregates of the intercellular channels that go across two membranes of adjacent cells. This allows the direct connection of their cytoplasms. The gap junction exists between cells in almost all the multi-cellular animals (metazoan), including invertebrates and vertebrates. Gap junctions mediate the intercellular communication in almost all cell types except skeletal muscle cells and blood cells (Kumar 1996, Wei 2004).
Two unrelated gene families were found to encode the gap junction proteins. They are pannexins (previously as innexins) existing in both invertebrate and vertebrate species, and connexins that are only found in vertebrates. They are all 4-pass transmembrane proteins, having 2 extracellular loops and 1 cytoplasmic loop with both the amino (N) and carboxyl (C) terminal ends inside the cytoplasm. They all form hexamers in the membrane, which constitute a hydrophilic channel across the cell membrane. Two such channels from two adjacent cells dock with each other to form a gap junction that allows the free diffusion of molecules under 1000 daltons between the two cells. Previous studies describe differential selectivity of the channels composed of different gap junction proteins (Wei, C., 2004). The probability of the open state of the channels is also affected by the transmembrane voltage, pH, and Ca$^{2+}$ level (Panchin, 2006). There are 21 different connexins in human, 20 in mouse and 37 in zebrafish. Different connexins display different spatiotemporal expression patterns in an organism.

As mentioned above, gap junctions are phylogenetically ancient, coupling almost all the metazoan cells, and the long evolutionary history enables them to adapt diversified functions. One of the remarkable functions of gap junction is to electrically couple the excitable cells in to allow the cell-to-cell transfer of action potentials (Bennett, 1978). In addition, gap junction mediated intercellular communication can be detected at very early stages of embryogenesis. This indicates that gap junction communication is competent to affect early development (Caveney, 1985). Cells associated via gap junctions constitute a communication compartment that is often co-localized with developmentally significant domains. Such cellular compartments may allow the generation of signal gradients (Wei, 2004).
Connexin expression profiles suggest functions in early development. During murine preimplantation development, Cx30, Cx31, Cx36, Cx43, Cx45 and Cx57 are expressed from the two- to four-cell stage while Cx30.3, Cx31.1 and Cx40 are expressed from the eight-cell stage (Houghton, 2005). In human preimplantation embryos, Cx43 is predominantly expressed. Cx43 protein level increases throughout development to the blastocyst stage. However, it is still controversial whether the gap junction proteins are required functionally for embryogenesis. For example, mice with homozygous mutations in Cx37, Cx32, Cx40, Cx46 or Cx50 are viable (Nicholson, 1997). But some other connexins seems to be indispensable for the development and functions of a variety of tissues and organs (Willecke, 2002). For example, Cx37 is expressed in both the oocyte and the granulosa cells that surround the oocyte and is required for the maturation of the oocyte (Nicholson, 1997). During the progress of organogenesis, Cx43 is expressed in discrete spatiotemporal regions coinciding with formation of organ rudiments (Lo, 1996). Mice with null mutant Cx43 die after birth because of conotruncal heart defects (Reaume, A., 1995).

Many genetic diseases are linked to mutations in the connexin genes. Mutations in Cx37 are associated with Charcot-Marie-Tooth disease, and Cx26 mutations cause skin problems and deafness (Wei, 2004; Laird, 2006). It was found that oculodentodigital dysplasia (ODDD), a congenital developmental disorder associated with abnormalities in face, eyes, limbs, and dentitions, is correlated with dominant mutations in Cx43 (Gong, X. 2007). The mutated sites in Cx43 are distributed almost randomly along the coding sequence of Cx43. Mutations distributing throughout the protein sequence imply not only the channel domains but also the nonchannel domains contribute to the normal functions of the
connexins. Given the broad distribution of Cx43, it is remarkable that most human patients of ODDD usually live long lives in relatively good health (Laird, D., 2006).

The turnover of the gap junctions is rapid in comparison to the other ionchannels or to the components of the tight junctions and desmosomes (Beardslee, 1998). The half-life of some connexins is only a couple of hours. Cx43 has a half-life of 1.3 hrs in the heart (Beardslee, 1998). The rapid turnover of the connexins offers quick adjustment of the specificity and the level of communication by regulation of connexin gene expression in response to physiological changes.

Consistent with being an integral membrane protein, connexins are thought to be co-translationally threaded into ER (Laird, D., 1995). It is reported that the connexin oligomerization is also accomplished in the ER (Koval, M. 2006). Phosphorylation is an important post-translation modification occuring in different stages of the life cycle of certain connexins (Sosinsky, G., 2005; Laird, D., 2006). The C-terminal region of Cx43 appears to be a primary region that becomes phosphorylated. Phosphorylation of Cx43 by PKC leads to closing of the hemichannel or channels (Solan, 2005).

Connexin43 is the most widely expressed connexin gene and plays important roles in neural and cardiovascular development in mouse (Ewart, J., 1997). However, it is still unknown how Cx43 functions in those development processes. There are three possible functional modes that have been roughly established for Cx43. First, the formation of canonical gap junction channels may allow signal transmissions within cellular compartments. Second, the formation of Cx43 hemichannels may participate in paracrine regulation by mediating signal exchanges across a single layer of cell membrane (Goodenough, D., 2003). Third, when Cx43 connexons dock through their extracellular loops
the adhesion force generated between two Cx43 connexons is required in the radial migration during mouse neocortex development (Elias, L. 2007).

1.2.2 Zebrafish *Connexin43*

Zebrafish Cx43 shares considerable sequence identity with the mouse, rat, bovine and human Cx43 orthologs (Chatterjee, B. 2005). As revealed by the whole mount *in situ* hybridization, the zebrafish Cx43 mRNA appears in a conserved pattern during zebrafish development similar to the expression profile in the mouse (Yancey, S., 1992; Chatterjee, B. 2005). Zebrafish Cx43 is expressed in the nervous system and in the cardiovascular system, including the heart and blood vessels (Chatterjee, B. 2005). The cardiac expression of zebrafish Cx43 appears at 48 hours post fertilization (hpf), possibly in cardiac neural crest cells situated in the developing heart. Both major axial vessels and the intersegmental vessels (ISV) express Cx43 at 24 hpf. This expression is coincident with the stage when the endothelial tubes are forming in the axial vessels and ISVs are migrating dorsally, indicating Cx43 is competent to participate in vascular morphogenesis. The conserved expression pattern in zebrafish to that in mice may reflect the conserved functional role for Cx43 during the embryonic development of the vertebrates.

Zebrafish Cx43 mutants have been associated with the short fin phenotype (Iovine, K., 2005). The original allele of Cx43, called *short fin* (*sof*\(^{bl23}\)) is a mutant in non-coding sequence, showing reduced Cx43 expression and short fins. This allele was regarded as a hypomorphic allele. Three ENU-induced mutants have been identified that display no complement to *sof*\(^{bl23}\). These have single mis-sense mutations in the first transmembrane domain, the second extracellular loop and the fourth transmembrane domain. Since none of
these alleles shows a more severe phenotype than \( sof^{b123} \), they may all be the hypomorphic alleles. Morpholino knockdown of Cx43 in zebrafish results in pericardial edema at 50-60 hpf and reduced hematopoiesis (Iovine, K., 2005). This implies that Cx43 is also required during zebrafish cardiovascular development.

### 1.2.3 Cell migration

During zebrafish vasculogenesis, the endothelial precursors migrate from lateral plate mesoderm to the midline where they coalesce into a solid cord (Jin, SW, 2005). The cord of endothelial cells is remodeled into a tube shape structure. Slightly later on, the new branches sprout out from the major vessels during the first wave of embryonic angiogenesis (Jin, SW, 2005).

Cell migration is a common phenomenon during development and morphogenesis. Cell migration involves two important processes: 1) determination of direction and 2) movement leading to spatial displacement. Similar to how we travel by road, cells often follow a paved road, such as certain types of extracellular matrix (ECM), to get to their destinations (Kirfel, G., 2004). Sometimes this process involves the immobilized or diffusible signal molecules in the ECM that guide the direction of migration. The cell senses attractive and repulsive cues from the surroundings to make an integrated decision on where to go. In endothelial cells, the mechanical force produced by the blood pressure from the preexisting functional lumens is also an important factor for determining the direction of the new sprouting (Lamalice, L., 2007). Migration is fulfilled through the cytoskeleton machinery, and changes in the cytoskeleton are initiated by the polarity decisions of the cell in response to the external cues. Generally, the migration can be hypothetically divided into
the 6 steps (Lamalice, L. 2007). First, the cell extends filopodia to sense the signals unevenly distributed in a gradient, by which polarity changes are induced in the cell (Lamalice, L. 2007). Second, the polarity changes are reinforced as the protrusions form, such as lamellipodia or pseudopodium (Lamalice, L. 2007). Third, the protrusion are stabilized by focal adhesions (Lamalice, L. 2007). Fourth, the cell body is translocated forward with the help of the stress fibers attached to the focal adhesions. Fifth, the rear part of the cell is released from extracellular matrix and sixth, the focal adhesion components on the rear part are recycled (Lamalice, L. 2007).

From the work on the social amoeba Dictyostelium discoideum, researchers have found the polarized accumulation of Ptdins(3,4,5)P$_3$ (Phosphatidylinositol (3,4,5)-trisphosphate) at the cell membrane in response to the signal gradient (Charest, P., 2007). The localized generation of Ptdins(3,4,5)P$_3$ is determined by the polarized activation of phosphoinositide 3-kinase (PI3K) and of the phosphatase and tensin homolog (PTEN) (Charest, P., 2007). The cell senses and transforms external cues into cell polarity changes, which leads to the cytoskeleton changes for movement.

Strong evidence indicates that Cx43 plays an important role in cardiac neural crest cell migration in mice (Huang, G. 1998; Sullivan, R. 1998; Lo, C. 1999; Xu, X. 2001). The rates of cell migration were estimated during neural crest outgrowth from pieces of dorsal neuroepithelium tissue dissected from the mouse embryo (Huang, G. 1998). From 24 to 48 hours of the culture, the outgrowth area was measured as an index of the migration activity. It was found that the index is correlated to Cx43 gene doses. The index is the highest in the Cx43 over-expression outgrowth culture, and the lowest in the Cx43 knockout ones, while there is no difference in cell proliferation rate (Huang, G. 1998).
A transgenic mouse was generated that expresses a Cx43/β-Galactosidase fusion protein under control of elongation factor-1α promoter (Lo, C. 1997). The fusion protein exhibits a dominant-negative effect as shown by the inhibition of the dye coupling among cells in the neural crest outgrowth culture (Lo, C. 1997). The migration index is reduced compared to the wild type. A 6.5k mouse Cx43 promoter was found to express only in neural crest cells and was used to drive Lac-Z expression (Lo, C. 1997). Using this promoter to mark neural crest cells, less neural crest cells arrived in the heart when Cx43 was depleted or functionally disturbed. By tracing individual cells, it was also found that the Cx43 knockout (KO) cells show no obvious change in speed but display reduced directionality (Lo, C. 1997).

The most recent report from the same group shows that in the Cx43 KO mouse neural crest outgrowth, the β1-integrin is reduced and consequently the adhesion to the fibronectin is reduced (Xu, X. 2006). Likewise, the expression of vinculin is also decreased in the Cx43 KO cells. Apparently, the depletion of Cx43 affects the abundance of focal adhesions on cell surface, which then influences the motile behavior. Cx43 is known to colocalize with F-actin and several actin-binding proteins such as vinculin, ezrin, and α-actinin along the cell protrusions in the regions of cell-cell contacts, suggesting a role in the cytoskeleton organization (Wei, C., 2004; Xu, X. 2006). In Cx43 KO cells, the actin stress fibers extend in polygonal shape that is different from the parallel bundles appearing in the wild type cells, indicating defects in cell polarity (Xu, X. 2006). This is consistent with the directional change of the protrusions in individual cells captured by the time-lapse images (Xu, X. 2006). Both the KO and over-expression Cx43 cell lines exhibit more active protrusions compared to the wild type, but cells that are over-expressing Cx43 can still
maintain a constant direction while the KO cells display defects in directionality (Xu, X. 2006).

These studies demonstrate a strong correlation between the cardiac neural crest mobility and Cx43. Since neural crest cells migrate in groups and maintain cell contacts while migrating, Cx43 gap junctions may function in mediating the coordinated locomotion of a group of cells by allowing signals being shared among the group. On the other hand, Cx43 may participate in cytoskeleton changes and regulate the focal adhesion distribution and cell polarity. Endothelial cells also migrate in groups, both during vasculogenesis and angiogenesis. Thus it is possible that Cx43 participates in endothelial migration in a similar way to its role in the migration of cardiac neural crest cells.

Cx43 is also reported to be required for normal neuronal migration in the neocortex. Researchers have found that neocortical neuronal migration was significantly delayed in Cx43 null mutant mice (Fushiki, S. 2003). During the development of neocortex, the progenitor cells residing in ventricular zone give rise to radial glial cells that serve two important functions: stem cells that produce neurons and guides for neuronal migration to the layers in the cortex. Cx43 is expressed both on radial glial fibers and the neurons (Wiencken-Barger, A. 2007). Cx43 may function between these two cell types, possibly by mediating neuron migration on the glial cells (Wiencken-Barger, A. 2007). Radial glial specific depletion of Cx43 causes disorganization of cortex resulting from migration and proliferation defects (Wiencken-Barger, A. 2007). Recent results not only provide evidence for the requirement of Cx43 in neuron migration along radial glial fibers but also demonstrate that the adhesion force between the two Cx43 connexons is required for the normal neuronal migration (Elias, L. 2007). Cx43 puncta are shown to localize on the cell contacts between
the radial glial fibers and the neurons. Cx43 puncta are also immediately next to the actin puncta, which are apparently reduced when Cx43 is reduced (Elias, L. 2007). This is similar to the co-localization of Cx43 with actin and the actin associated components in neural crest cells (Xu, X. 2006). In addition, the neurons with reduced Cx43 extend more unstable protrusions than the wild type, reminiscent of the active random protrusions in Cx43 KO mouse neural crest cells. The dominant protrusions that are stabilized along the migration path may be predicted by the presence of Cx43 puncta (Elias, L. 2007). The movement of Cx43 puncta in the cell body shows strong correlation with the nuclear translocation, implying the association with the centrosome and a potential role of Cx43 in nuclear translocation. This is coincident with the involvement of Cx43 in the interkinetic nuclear migration in chick retina development (Becker, D. 2007). The above studies on neuronal migration seem to support a role of Cx43 in the locomotion machinery of the neurons.

1.2.4. Lumen formation

The cellular lumen structure formed in an organism serves to separate the space for different functions and topologically it has two distinct surfaces, facing the inside and the outside respectively. Once the two distinct surfaces are defined, the lumen structure is established geometrically. The acquisition of apical-basal cell polarity is at the center of lumen formation. Biologically, most tube or lumen structures are formed in one of the following 5 ways: 1) wrapping, as in neural tube formation in vertebrates; 2) budding, as in branching morphogenesis; 3) cavitation, involving apoptosis of the inner cells in a cord or ball of cells; 4) cord hollowing, which has no cell death, as in zebrafish vasculogenesis or gut
formation; 5) cell hollowing, as the cell reorganizing its membrane, typically exemplified by capillary formation (Lubarsky, B., 2003).

During zebrafish embryonic development, endothelial tube formation in vasculogenesis is related but different from that in angiogenesis. When the major axial vessels form, the angioblasts migrate from lateral plate mesoderm and coalesce into a cord at the midline. The cord hollows from its center along the midline into a tube (Jin, SW., 2006). The sprouting of the intersegmental vessels from the axial vessels, a typical example of angiogenesis, is a budding process in which the tip cells move away from its original position and pull up the connected endothelial cells that are called stalk cells (Blum, Y., 2008). Those stalk cell may proliferate during the new branch formation. It is possible that the lumen of the new branch is directly extended from the lumen of the axial vessel and expanded while the cells migrate and divide. A simple lumen can be formed within one endothelial cell, as exemplified by the capillary, through the cell hollowing process (Lubarsky, B., 2003).

Examples of multi-cellular tubes formed through cord hollowing without cell death include the zebrafish gut, the zebrafish axial blood vessels and zebrafish neural tube (Bagnal, M., 2007; Jin, SW., 2006; Horne-Badovinac, S. 2001). Forming a solid cord may be a prerequisite for the cells to coordinate with one another for setting up the apical-basal polarity. They closely contact with one another to form cell junctions at the multi-cell borders. Those junctions are then remodeled into a line in the center of the cord. The two-dimensional apical surface starts to develop and expand from the line. The difference between apical and basal surface is embodied by the different protein or lipid components, which must be achieved by biosynthetic sorting and directional transportation that rely on the polarized cytoskeletons. Two mechanisms are proposed for the formation of the central
extracellular lumen: i.e. directed exocytosis and water secretion. By directed exocytosis, when the water is secreted to the central extracellular space, the apical membrane is also expanded with apical specific proteins deposited (Lipschutz, J., 2000; Lubarsky, B., 2003).

Some of the apical or basal proteins are ion channels that may function to establish electrochemical or osmotic differences across cell membrane or para-cellular space, which in turn drive the fluid influx to expand the lumen (Lubarsky, B., 2003). The refined balance among the ion or water pores can also sense the mechanical force and perhaps regulate the lumen size (Lubarsky, B., 2003). In zebrafish gut morphogenesis, claudin15 is required for the formation of a single gut lumen in a Na⁺-K⁺ ATPase dependent way (Bagnal, M., 2007). This suggests the paracellular fluid movement driven by electrochemical gradient generated by ion channels may play an role in normal lumen formation.

The delivery of certain proteins or lipids to distinct apical or basal domains is accomplished via the cytoskeletons in polarized cells. Cell-to-cell contact may be required as an initial cue for establishing the cell polarity. This may explain why a solid cord is a prerequisite before a lumen expands. In worms, flies, and mammals, Par3, a component of the cell polarity complex, is recruited to the tight junction domain through junctional adhesion molecules JAM (Ebnet, K., 2001). Par3 recruits Par6/aPKC, in which aPKC is an atypical protein kinase C isoform. The aPKC is the only catalytic component within the formed complex and will be activated by the binding of Cdc42 to Par6 (Itoh, M., 2001; Henrique, D., 2003; Yamanaka, T., 2001). The downstream effect of the activated aPKC includes deposition of the apical or basal determinants (Henrique, D., 2003). Recent work also shows the phosphoinositides, PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂, define apical and basal plasma membranes of epithelial cells, respectively. PTEN is exclusively distributed on the
The apical deposition of PtdIns(4,5)P₂ is sufficient to recruit the Par6/aPKC for the further polarization (Martin-Belmonte, F., 2007, 2008; Comer, F., 2007).

The research on endothelial cell cultures shows that the interaction between the extracellular matrix and integrins may initiate the polarized vesicle transportation in a single cell. This leads to the fusion of the vesicles in the center of the cell and eventually the formation of the lumen (Davis G, 1996, 2002; Kamei, M., 2006).

The permeability of the aqueous channels or hemichannels of Cx43 for water and ion influx and its localization with other cell junctional or cytoskeletal components suggest a possible function in the lumen or tube formation. The Cx43 channels could allow the water influx down an osmotic difference. This is a suggested mechanism for how Cx43 is required for the expansion of the aqueous humor in the mouse eyes (Calera, M., 2006). For mediating the influx of fluid and ions, Cx43 gap junctions coordinate well with other ion channels, including Na⁺/H⁺ and Cl⁻/HCO₃⁻ antiporter, Na⁺/K⁺ ATPase, Cl⁻ channels, and the water transporter of aquaporin4 across multiple layers of cells (Calera, M., 2006). In addition, gap junction channels are implicated in the mechanosensitive Ca²⁺ signaling transduction in renal cell cilia to restrict the size of renal tube (Lubarsky, B., 2003). These observations suggest Cx43 may function in the biological lumen expansion processes.

During murine organogenesis, it is intriguing to note that Cx43 is almost exclusively gathered on the apical side of the epiphysis as it wraps and pitches off as an outgrowth from the diencephalon. This suggests a potential role of Cx43 in the apical-basal polarity establishment during the lumen formation of the epiphysis (Yancey, S., 1992). The involvement of Cx43 in lumen expansion and in cell polarity establishment would be consistent with a role for Cx43 in endothelial lumen formation.
1.2.5 Significance of Cx43 function in endothelial migration or tube formation

The vascular morphogenesis is of vital importance for the normal development and physiological functions in vertebrates. Abnormal angiogenesis is often associated with a variety of pathological conditions, including cancer, asthma, diabetes, multiple sclerosis, and certain ocular disorders (Carmeliet, P., 2005). The understanding of the molecular mechanisms of vascular formation will lead to the development of the new therapies for the relevant disorders or diseases. With the advantages that the zebrafish provide for in vivo study, we hope to gain an understanding of the fundamental mechanisms of vascular formation.
2.1 Introduction

The gap junction channel connects most metazoan cells and allows the direct sharing of ions and small cytoplasmic molecules. Recent studies show that short stretches of shRNAs or peptides can wiggle through gap junction channels (Neijissen J, 2005, Valiunas V, 2005). This intercellular communication is believed to be important for the development and the normal physiological function of organisms. Gap junction channels may allow the formation of the signal gradients across a range of cells. This model has been proposed for the initiation of left-right asymmetry in chicks and amphibians (Levin, M., 2007). There are three possible function modes that have been roughly established for gap junction proteins: 1) canonical gap junction channels that allow signal transmission within cellular compartments, 2) hemichannels that mediate exchange of signals as a part of paracrine regulation across a single layer of cell membrane (Spray, D., 2006), and 3) adhesion force between two connexons when they dock via their extracellular loops, as required in radial migration during mouse neocortex development (Elias, L. 2007).

Gap junction proteins all have a 4-pass transmembrane topological structure with both their N-terminal and C-terminal ends inside the cell membrane. The C-terminal region of gap junction proteins is subject to regulation by phosphorylation and is capable of interacting with other membrane associated proteins or cytoskeletal proteins, such as ZO-1 and F-actin (Wei, C., 2004). The gap junction channels or hemichannels composed of
different connexins may have different permeabilities toward certain ions or small molecules, providing refined constrictions to the communications among the connected cells (Harris, A. 2007). These molecular properties of connexins may support their possible functions in the finely regulated developmental processes.

Connexin43 is the most widely expressed connexin and plays important roles in neural and cardiovascular development in mice (Yancey, S., 1992; Lo, C., 1997; Wei, C., 2004). Zebrafish connexin43 is prominently expressed in notochord and the blood vessel endothelial cells (Chatterjee, B. 2005). The mRNA of zebrafish Cx43 appears in notochord from 10 hpf when the notochord is developing and signaling to surrounding tissues. At 24 hpf when both vasculogenesis and angiogenesis are underway in zebrafish, Cx43 mRNA appears in both axial and intersegmental vessels (Chatterjee, B. 2005). This suggests a possible role of Cx43 in vascular development.

The zebrafish provides an excellent system to study in vivo blood vessel development. Zebrafish embryos are optic transparent, external fertilization and relatively normal early development till day 5 independent of a functional circulation (Isogai, S., 2001). The object of this research is to determine the molecular identity and the function of Cx43 in the processes of endothelial migration and tube formation.

2.2 Methods

2.2.1 Zebrafish husbandry

Zebrafish were maintained as described previously (Westerfield, M. 2000; Lawrence, C., 2007). The embryos were harvested from the pair-wise crosses of the wildtype or transgenic zebrafish, raised and staged at 28.5 °C. The transgenic lines used in this study
include Tg[fli1:egfp] (Kamei, M., 2006), Tg[flk1:Cx43-egfp] (see below), and Tg[flk1:lxp-mcherry-lxp-Cx43DN-egfp] (see below).

### 2.2.2 Morpholino injection

Morpholino Phosphorodiamidate Oligonucleotides (MO) stock solutions were made by dissolving 100 nmoles of the solid MO in 33.3 μl of milli-Q grade water. This is approximately 25 mg/ml concentration. The stock is aliquoted and kept at -20 °C. The working solutions are made by diluting the stock solution to 2 mg/ml with water and aliquoted into 20 μl each, stored at -20°C. MOs were injected into the yolk of the zebrafish embryo at one to two-cell stage (Nasevicius, A., 2000). For the co-injection of MOs, the embryos were injected twice with the same needle as used for single injection instead of being injected with MO mixtures. The doses of MOs were determined through dose response.

The Cx43 MO has the sequence of 5’-AGGGAGTTCTAGCTGGAAA GAAGTA-3’ and is complementary to a stretch of Cx43 5’UTR including the translation start codon AUG. Similarly, the sequence of Cx43.4 MO is: 5’-TCAAGAAGTACCACCCTCAGTCC-3’; that of Cx44.2 MO is: 5’-AACGTGTCAAGAAGCTCCAACTCAT-3’; p53 MO is 5’-GCGCCATTGCTTTGCAAGAATTG-3’ (Robu, M., 2007); Cdh5 MO is 5’-ATATCCTCCTCAGTTTCAGGTCAGC-3’.

### 2.2.3 Microangiography

Microangiography was carried out as previously described (Weinstein, B., 1995). 10mg/ml of dextran, Alexa Fluor® 568 1,000,000 MW (Invitrogen) was injected to the
zebrafish heart at 36 hpf or older. The injected fish were visualized under a Zeiss V12 fluorescent dissection microscope.

### 2.2.4 Generation of transgenic zebrafish

The promoter of zebrafish *flk1*, also called *kdra* or *kdr-like* (Bussmann, J. 2008) was amplified by PCR from zebrafish genomic DNA, from -6627 to +350 of the gene. The forward primer is 5’-GTACTTTCAGGAAATGAGTGAGCTGATGT-3’ with an Xmal site tagged to its 5’ end. The reverse primer is 5’-TTTGTCTGTAAAAATAACGTCCCGAATGATAC-3’ with an EcoRI site tagged to its 5’ end. The PCR product was cloned into the pBluescript II KS(-) vector between XmaI and EcoRI. The coding sequence of egfp was connected to the C-terminal of Cx43 in frame, followed by a SV40 poly (A) signal. This fusion gene was cloned downstream of *flk1* promoter in the above engineered pBluescript II KS(-) vector between EcoRI and XhoI.

The fragment of flk1: Cx43-egfp was excised by the restriction enzymes XmaI and XhoI. The concentration of the DNA fragment was adjusted to approximately 200 pg/ul. About 300 pg of this DNA fragment was microinjected into one-cell stage embryos to generate transgenic lines as previously described (Jin, S. 2005). The injected embryos were screened for gfp puncta in the vasculature and then raised to adulthood. Those fish were then crossed to wild type fish to identify the founder fish that had a germline integration of the injected DNA fragments. The fluorescent offspring of the founder fish are the stable transgenic zebrafish. Among about 40 fish screened two founders were obtained.
2.2.5 Immunohistochemistry

Cx43 antibody staining was conducted as follows: zebrafish embryos at 20-50 hpf were dechorionated manually in fish water and fixed in 2% paraformaldehyde (PFA) in 25 mM sodium phosphate buffer (Na$_2$HPO$_4$, NaH$_2$PO$_4$, pH 7.4) for 30 min at RT. The fixed embryos were washed in 25 mM sodium phosphate buffer containing 10% sucrose for 1 hour at RT before being blocked for 1 hour (RT) in TBSTx (50 mM Tris-HCl, pH 7.4, 1.5% NaCl, 0.3% Triton X-100) with 4% sheep serum. The embryos were incubated overnight at 4°C with a rabbit-anti-zebrafish Cx43 polyclonal antibody (provided by Dr. Kathryn Iovine) at 1:1000 dilution in blocking solution. The embryos were washed for one hour in the block solution before being incubated for 2 hours at RT with in goat anti-rabbit Alexa Fluor 546 secondary antibody (Invitrogen) at 1:200 dilution in block solution. The embryos were washed in TBSTx before mounting in Gel/Mount (Biomeda). The embryos were visualized on a Leica TCS NT confocal microscope system with 63x water lens and a step size of 0.2 μm.

The VE-Cadherin (Cdh5) antibody staining was performed as previously described (Blum, Y., 2008). Embryos from 22-36 hpf were fixed in 4% PFA for 2 hours at RT, and then washed for 5 minutes in PBST (PBS + 0.1% Tween20) 5 times and another 5 minutes in PBSTX (PBS + 0.1% Tween20 + 0.1% Triton X100) before being blocked in the block solution (PBSTX + 10% BSA + 1% NGS) for 2 hours. The embryos were then incubated with primary antibody (rabbit-anti-zebrafish-Cdh5 polyclonal serum, at 1:1000 dilution) in the diluted block solution (PBSTX + 1% BSA + 0.1% NGS) overnight at 4°C. Embryos were washed 6 times for 1 hour in the diluted block solution (PBSTX + 1% BSA + 0.1% NGS). The embryos were incubated with secondary antibody (Alexa-568 goat-anti-rabbit IgG
antibody, at 1:1000 dilution) in block (PBSTX + 1% BSA + 0.1% NGS) overnight at 4°C. The embryos were washed in PBST for several times before mounting for visualizing. The head and yolk was removed, and the trunk was mounted in Gel/Mount (Biomed). Images were taken on Leica TCS NT confocal microscope system with 63x water lens and a step size of 0.25 μm.

**2.2.6 Endothelial expression of Cx43 dominant negative mutants**

The construct of pTol2(flk1:lxp-mcherry-lxp-Cx43DN-egfp) was engineered as schematically shown in figure 9 using pTol2-msc (made by Maura McGrail) as the backbone vector. The minimal Tol2 transposon elements are included in the vector to increase the frequency of the incorporation of the engineered DNA fragment into the genome (Kawakami, K., 2007). Two Loxp sites were added downstream of flk1 promoter by the synthesized linkers, which were annealed from the forward oligos, 5’-AATTCGCTAGCATAACTTCGTATAGCATACATTATACGAAGTTATACGCGTACCTTGATGCATATAAC TTCGTATAGCATACTGTTATGAAGTTATA-3’ and the reverse oligo, 5’-GATCTATAACTTCGTATAATGTATGCTATACGAAGTTATGCTAGCGTATAACTTT CGTATAATGTATGCTATACGAAGTTATGCTAGCG-3’. The linker has MluI and NsiI sites between two Loxp sites. Mcherry coding sequence with SV40 poly (A) sequence was inserted between MluI and NsiI. The Cx43 dominant negative mutants were made via the site-specific mutagenesis PCR from pT7TS-Cx43-egfp. The latter one is obtained by inserting Cx43-egfp fragment between BglIII and SpeI sites in pT7TS vector (constructed by Andy Johnson and Paul Krieg). The mutant Cx43 (T154A) has its amino acid 154 Threonine converted to Alanine using the primers of 5’-TGTAGGCGGCAGGCT -3’ and 5’-
CTGC CGGC CTACATCTTCAGCAT -3’ while the mutant Cx43 (C61S) has its extracellular amino acid 61 Cysteine converted to a Serine with the primers of 5’-CTCGCTACCAGGCTGCT-3’ and 5’-CTGGTAGCGAGAATGTCTGCT-3’. The C-terminal truncated Cx43 has the deletion of C terminal end from amino acid 238, made with the primers of 5’-GTATCCATGG CGTCCTTGATTCGTTTAAGA-3’ and 5’-GAATCCATGGTGAGCAAGGG-3’.

In the advanced construct II (Figure 9), pTol2(flk1:lxp-egfp-lxp-Cx43DN-2A-TAG) and construct III (Figure 9), pTol2(flk1:lxp-egfp-lxp-Cx43DN-egfp-2A-TAG), 2A peptide linker (Provost, E., 2007) is used to tag TAG rfp (Evrogen) to the C-terminal ends of Cx43DN or Cx43DN-egfp.

The Tol2 transposase mRNA was generated by mMESSAGE mMACHINE® T7 ULTRA Kit (Ambion). The Tol2 transposase plasmid is pBD600 (a gift from Dr. Stephen Ekker at University of Minnesota) (Balciuas, D., 2006) and was linearized with BamHI. T3 polymerase was used to generate mRNA of Tol2 transposase.

The mixture of 150 pg Tol2 transposase mRNA and 30 pg Tol2 vector DNA with Tol2 mini transposon elements (the vector is a gift from Dr. Stephen Ekker at University of Minnesota) in 2 nl was injected into the 1-cell stage embryos. The steps for screen transgenic zebrafish are the same as above described for generation of Tg[flk1:Cx43-egfp].

The Cre mRNA was made the same way as Tol2 transposase. The pT3TS-Cre plasmid (a gift from Karl Clark at University of Minnesota) was linearized with XmaI and transcribed with T3 polymerase. 1 pg and 10 pg of the Cre mRNA was injected into the one-cell stage embryos of Tg[flk1: lx-mcherry-lx-Cx43DN-egfp].
2.3 Results

2.3.1 Morpholino knockdown

Dependent on the dose injected, all three connexin morpholinos (Cx43 MO, Cx43.4 MO and Cx44.2 MO) cause similar morphological defects during somitogenesis (Table 1). While the high doses cause early death that happens before or during gastrulation, the relative low doses result in the general malformation of the trunk, such as the disrupted somites, round cells in the notochord, thin yolk extensions and the upward bent tails as shown in Figure 1 and Figure 2. The intersegmental vessels (ISVs) are delayed in their migration compared to the uninjected zebrafish (examples shown by Figure 1B, F and G). According to the pigmentation in the retina and the formation of the yolk extension, the general development of the MO injected embryos is delayed compared to the uninjected ones. The numbers of individual morpholino injections were listed in Table 1.

2.3.1.1 Vascular phenotypes in the morphants

Tg[fli1:egfp] zebrafish were used in morpholino injections for studying the vascular phenotypes. At 36 hpf (Figure 1) in the uninjected fish, most of ISVs in the trunk have reached the position where dorsal longitudinal anastomotic vessel (DLAV) has formed and show a well-aligned chevron shape (Figure 1B). In contrast, some ISVs in either the Cx43 morphant or Cx43.4 morphant have branched earlier or extended toward wrong directions while they are migrating dorsally towards the position where DLAV forms (Figure 1F and J). These morphological differences may reflect migration defects in the direction determination.
At 2 dpf in the uninjected fish, the parachordal vessel (PAV) forms at the level of the turning points of ISVs, parallel to the dorsal aorta (Figure 2Q). Most of the ISVs in control embryos have formed patent vessels and possessed circulation inside. Functional vessels can be observed by microangiography. In the tail, the caudal vein extended into a vascular plexus, which is continuously remodeled. In the morphants (Cx43, Cx43.4 and Cx44.2) at 2 dpf, some of the defects in ISV branching still exist (Figure 2R and 2S). The ISVs do not have a typical chevron shape and the PAV is absent at the corresponding position. The tail plexus of the caudal vein was also underdeveloped. In addition, the dextran-Alexa Fluor® 568 does not perfuse into some of the ISVs in the morphants (Figure 2V and 2W), indicating the tubes are not properly formed. Some of the migration defects and the tube formation defects persist on 3 dpf (Figure 3).

The severity of the vascular phenotypes is positively correlated to the injected dose of the connexin morpholinos in most of experiments, and it varies between individual experiments (Figure 4). For the convenience of calibrating the general vascular phenotype, the absence of blood flow in the axial vessels on 2 dpf is used as the indicator of vascular defects (percentage=1-nf/ni, where nf is the number of fish having the axial flow, and ni is the total number of fish injected). Figure 4 shows dose responses of the connexin morpholinos (Cx43, Cx43.4 and Cx44.2). Among these 3 connexin morpholinos, the Cx43 MO is of the strongest dose efficiency and the Cx44.2 MO is the least efficient one. But by increasing the dosage, Cx44.2 MO can produce the same phenotypes as Cx43 MO does.

As shown in Figure 4A, it is clear that four out of five independent dose response experiments of Cx43 MO showed positive dose dependence of the phenotypes. Figure 4B
shows one positive dose dependent responses out of three independent experiments for Cx43.4 MO. There are two positive dose dependent responses of Cx44.2 MO shown in Figure 4C.

The dose causing less than 15% absence of the axial flow on 2 dpf is regarded as a low phenotype dose. Likewise, the dose causing more than 85% loss of axial flow is a high phenotype dose. Low phenotype doses were used for co-injections of different morpholinos.

2.3.1.2 Synergistic effects with VE-Cadherin morpholino

Cx43 has been reported to interact and co-localize with adhesion junction components (Wei, C., 2004). We tested whether the endothelial Cx43 has interactions with VE-Cadherin in zebrafish blood vessel endothelium. The knockdown synergy is expected between these two genes if Cx43 affects the vascular morphology in a cadherin-dependent way.

When a low phenotype dose of VE-Cadherin (Cdh5) morpholino is co-injected with a low phenotype dose of connexin morpholinos (Cx43, Cx43.4 or Cx44.2), the severity of the vascular phenotype as indicated by the absence of axial flow at 2 dpf are mostly more than the additive results caused by single doses as shown in Figure 5.

The synergistic effect between Cx43 MO and Cdh5 MO can be seen from Figure 5D. The injection of 1.5ng of Cx43 MO caused 17.1% absence of axial flow, and the injection of 6ng Cdh5 MO caused 21.6% absence of axial flow. But the combination of 1.5ng of Cx43 MO and 6ng Cdh5 MO caused 83.7% absence of axial flow. In four independent experiments, the combination of the low phenotype doses all produced synergy (Figure 5 A-D).
In case of Cx43.4 and Cdh5, the double knockdown is less dramatic than that of Cx43 and Cdh5. In one out of two independent experiments the double knockdown produced a little more than the sum of two single knockdown results (Figure 5E). The injection of 4ng of Cx43.4 MO caused 40% absence of axial flow, and the injection of 6ng Cdh5 MO caused 10% absence of axial flow. The combination of 4ng of Cx43.4 MO and 6ng Cdh5 MO caused 62.5% absence of axial flow.

In one out of two independent experiments, injection of the Cx44.2 MO showed synergism with the Cdh5 MO (Figure F). 56% of the embryos displayed an absence of axial flow after the co-injection of 6ng Cdh5 MO and 6ng Cx44.2 MO, while the two single doses only produced 10% and 2% absence of axial flow, respectively.

### 2.3.1.3 Rescue effects by co-injection with p53 morpholino

The overall developmental defects of the connexin morphants may be partially due to the undesirable off-target effects of the morpholinos. The major off-target effects are mediated through p53 activation (Rubu, M., 2007). Therefore, we co-injected p53 MO with each of the connexin MOs to estimate the off-target effects of each connexin MO.

The rescue effects on the absence of axial flow are apparent as shown by Figure 6. The p53 MO was injected at 4 ng per embryo. When p53 MO combined with 4ng Cx43 MO, the absence of axial flow is 54.3% while single injection of 4ng Cx43 MO achieved 75%. For 6ng Cx43 MO, the p53 MO decreases the absence of axial flow from 87.5% to 64.1% (Figure 6A).

For 4 ng Cx43.4 MO, co-injection with 4 ng p53 MO decreased the percentage of embryos without axial flow at 2 dpf from 29% to 10.3% in one experiment. In another
experiment p53 MO decreased the percentage from 40% to 25.8% for 4 ng Cx43.4 MO, and decreased it from 87.5% to 68.8% for 6 ng Cx43.4 MO (Figure 6B).

The same trend was observed for Cx44.2 MO. Co-injection with 4 ng p53 MO decreased the absence of axial flow at 2 dpf from 26.7% to 24.1% for 9 ng Cx44.2 MO in one experiment and from 18.0% to 7.7% in another experiment (Figure 6C).

### 2.3.2 Dynamic distribution of Cx43

#### 2.3.2.1 Live imaging of Tg[flk1: Cx43-gfp] in the vasculature

In order to correlate sub-cellular events involved in endothelial migration and lumen formation with the spatial-temporal distribution of Cx43 in the endothelial cells, we have generated transgenic zebrafish expressing Cx43-egfp fusion protein under control of *flk1* promoter (Jin, SW., 2005), called Tg(flk1:Cx43-egfp) zebrafish.

Two founders were obtained for Tg[flk1: Cx43-gfp]. The offspring with brighter gfp signals were raised and used in all the imaging experiments, named as Tg[flk1:Cx43-egfp]$^{\text{br}}$. The dim line is named Tg[flk1:Cx43-egfp]$^{\text{dm}}$. Tg[flk1: Cx43-gfp]$^{\text{br}}$ zebrafish were crossed with the Tg[flk1:lxp-mcherry-lxp-Cx43(C61S)-egfp]$^{\text{br}}$ zebrafish that expresses mcherry in the endothelial cells to label the endothelial cells. Gap-junction-like gfp puncta can be seen in the endothelial cells from 14 hpf (data not shown), and distributed in the vasculature faithfully as the *flk1* promoter should be activated. For example, the gfp signal is more intense in the dorsal aorta than in the posterior caudal vein (Fig 7D). An interesting characteristic of this line is that gfp signal intensity is different among the intersegmental vessels (ISVs) in one zebrafish. For instance, in Figure 7D the ISV on the left shows less intense signals than the one on the right. At 22 hpf when the ISVs just start to sprout, the
Cx43-egfp puncta have already appeared on some of the sprouting tip cells (Fig. 7A). At 28 hpf, the Cx43-egfp puncta appeared on the migrating ISV endothelial cells (Fig. 7C).

### 2.3.2.2 IHC

In order to test whether Cx43 interacts with Cdh5, the embryos of Tg[flk1: Cx43-gfp] were fixed and stained with Cdh5 antibody at 22 hpf and 30 hpf. Most of Cx43-egfp puncta appears along the junctions of the Cdh5 (Figure 7A and 7B). However, there are patches of Cx43-egfp signals that are not co-localized with Cdh5. The co-localization signals show yellow color.

Since the ectopic expression of egfp tagged Cx43 may not reflect the endogenous state of Cx43, we have also studied Cx43 distribution with Cx43 antibody. When using the Tg[fli1:egfp] line to label the endothelial cells, Cx43 protein was shown to distributed on the surface of the endothelial cells (Figure 8). At 22 hpf there are many Cx43 puncta on the sprouting ISVs, either between the endothelial cells or outside the endothelial cell-to-cell contacts. At 30hpf when ISVs have already remodeled, it is hard to detect Cx43 puncta on the ISVs though the axial vessels show the existence of Cx43.

### 2.3.3 Tissue specific expression of Cx43 dominant negative mutants

In order to circumvent the limitations of morpholino knockdown for genes with pleiotropic functions, such as Cx43, we utilize Cx43 dominant negative mutants. Three basic mutants have been made and combined to assay the requirement of Cx43 in endothelial cell for the normal vascular morphogenesis.
By injecting the DNA fragments of construct Ia, pTol2(flk1: lxp-mcherry-lxp-Cx43DN-egfp) (figure 9), two founders were established with the C61S mutation. Over 50% of the offspring of one founder are stable transgenic zebrafish showing bright red fluorescent mcherry expression in their endothelial cells (Figure 9A). That line was named Tg[flk1:lx-mcherry-lx-Cx43(C61S)-egfp]br. This line was used to cross with Tg[flk1: Cx43-egfp] to label endothelial cells (Figure 7D).

After the Cre mRNA was injected to the 1-cell stage embryos of Tg[flk1:lx-mcherry-lx-Cx43(C61S)-egfp]br zebrafish, the Mcherry expression is abolished from some of the endothelial cells (figure 9B and 9C). However, under the compound microscope no gfp signal can be discerned from those endothelial cells that have lost their Mcherry expression.

### 2.4 Discussion

#### 2.4.1 Morpholino knockdown

The injection of Cx43 MO at 1 to 2-cell stage causes the defects in the vascular development in zebrafish. However, the mRNA of this gene is not only maternally deposited in the oocyte but also expressed prominently in the notochord from about 10 hpf. When the earlier expression is knocked down, early embryonic development may be affected. This could lead to the defects in the vascular formation. The limitation of the morpholio knockdown for investigating the genes of pleiotropic functions needs to be circumvented.

The mRNA of zebrafish Cx43 can be detected at 64-cell stage by in situ hybridization (Chatterjee, B., 2005). If it is required in the early embryogenesis, it may have a consequence on the later somitogenesis and vasculogenesis. To avoid knocking down the maternal contribution of Cx43 mRNA, a splicing morpholino was designed and injected. Though it
results in severe general dysmorphic phenotype, the RT-PCR shows no significant reduction of the spiced mRNA of Cx43 (data not shown). The reason may be that the coding sequence of Cx43 including the AUG is all in one exon. Therefore, the Cx43 mRNA may be able to be translated into functional proteins without the mRNA being properly spliced. The severe dysmorphic phenotype then could also be caused by nonspecific toxicity of that morpholino.

The rescue of the morpholino knockdown can show a specific requirement of Cx43 during the normal development. For this purpose, the mRNAs encoding Cx43 or Cx43-egfp have been co-injected with the Cx43 MO at 1-cell stage. However, no significant rescue was observed (data not shown). Several different DNA constructs of Cx43 have been engineered to express Cx43 in a tissue specific way. The flh promoter is used to drive notochord expression, flk1 promoter for endothelial expression, and β-actin promoter for ubiquitous expression. None of these constructs was able to rescue the knockdown phenotypes (data not shown). For most of the rescue injections, the morpholino was injected first using the same needle calibrated only once to minimize the variations of the doses injected and the rescue constructs or mRNAs were injected separately. The second injection could cause the extra stress to the embryos, which may counteract any rescue effect. To solve this problem, the transgenic zebrafish Tg[flk1:Cx43-egfp] has been tested for the rescue effect in the vascular system. However, no difference between the fluorescent and non-fluorescent siblings can be observed after injection of Cx43 MO (data not shown). This may result from the fluorescent tag of the fusion protein, which may jeopardize the normal function of Cx43. Without a rescue, the specific tissue requirement of Cx43 for vascular development cannot be established by morpholino knockdown. Furthermore, the specific knockdown effect of this Cx43 morpholino needs to be determined. The use of the Cx43 endogenous promoter that is
mutated to avoid the morpholino binding could test this. Rescue of vascular phenotypes by this construct will tell us the requirement of Cx43 for the vascular development, but we would still not know in which tissue its function is most relevant for the normal vessel formation.

Similar knockdown vascular phenotypes of these different connexins, Cx43, Cx43.4 and Cx44.2, were observed. According to in situ hybridizations at 24 hpf, both Cx43 and Cx43.4 mRNAs only appear in the tip of the tail. Cx43 is more concentrated in the notochord cells while Cx43.4 is also expressed in the surrounding somites (Essner, J., 1996; Chatterjee, B., 2005). On the contrary, Cx44.2 is more broadly expressed in the trunk somites (Thisse, B., 2004). The antibody staining of the Cx43.4 shows consistent results. Cx43.4 is absent in most of the trunk but present in the tail (unpublished data from our collaborators). Differently, Cx43 protein persists in the notochord intensively till later stages, even after 48 hpf. The vascular phenotype of Cx43.4 knockdown is more likely a consequence of its earlier function since Cx43.4 protein is cleared from the trunk when blood vessels start to form there. The same phenotype caused by Cx44.2 knockdown could be caused by the abnormal somite development since Cx44.2 is quite intensively expressed in the somites. Only Cx43 is distinctly expressed in the blood vessel endothelial cells when vascular morphogenesis is occurring. The similar knockdown results from these different connexins reflect the limitation of morpholino knockdown in determining the direct or secondary requirement of genes for the development of specific tissues or organs. It is possible that Cx43 is required for vascular formation in the endothelial cells.

Morpholino knockdown can also produce the off-target effects (Robu, M., 2007). The injected embryos show dark color in their heads, which could be cell death in the central
nervous system, and general abnormalities in their somites and notochords. These all can be observed in the connexin morphants (Cx43, Cx43.4 and Cx44.2). Studies have shown that most of these off-target effects are mediated through p53 signaling (Robu, M., 2007). The co-injection with p53 MO all rescued the vascular phenotypes to the limited degrees for these three connexin MOs (Figure 6). This means the apoptosis related off-target or on-target effects contribute to the vascular phenotypes that we observed.

Cx43 has been reported to interact and co-localize with adhesion junction components (Wei, C., 2004). It is interesting to test whether the endothelial Cx43 functions in the zebrafish blood vessel endothelium in a VE-Cadherin related pathway. The synergy is expected between these two genes when they are knockdown together if Cx43 affects the vascular morphology in a cadherin-dependent pathway. Moreover, since VE-Cadherin is exclusively expressed in the endothelial cells (Larson, J., 2004), its deficiency may amplify the effect of Cx43 knockdown so that only small dose of Cx43 MO is required to produce vascular defects. At those small doses, the early defects will be decreased. Our co-injection results fit this expectation. The combination of low phenotype doses of Cx43 and VE-Cadherin MOs produced synergy, likely because they both are expressed in the endothelial cells. Cx43.4 displayed the least synergy with VE-Cadherin, likely because Cx43.4 is almost cleared from the trunk at that stage. Interestingly, Cx44.2 also achieved high synergy with VE-Cadherin. This suggests a potential role for Cx44.2 in the endothelial cells or defects in the somites may elevate the defects of vascular development.
2.4.2 Dynamic regulation of Cx43 in endothelial cells

The transgenic line Tg[flk1:Cx43-egfp] are generated for studying the sub-cellular distribution of Cx43 in endothelial cells during migration and tube formation. The gfp is attached to the C-terminal end of Cx43 protein. Studies in tissue culture have shown that the C-terminal gfp-tagged Cx43 and the endogenous Cx43 have comparable intracellular and cell membrane localization and assemble into similar-sized gap junction puncta (Thomas, T., 2005). Between the activities of flk1 promoter and the Cx43 endogenous promoter, the transgenic line with bright gfp signals may have an increased level of Cx43-egfp in the endothelial cells. The over-expression could result in the protein distribution different from its endogenous state. The Cx43 promoter may be activated at a time different from when the flk1 promoter is activated. Therefore, the stage-related dynamic distribution of Cx43-egfp may not reflect exactly the endogenous state either. Since the transgenic line can be maintained normally from one generation to another, it appears that the ectopic expression of Cx43-egfp by flk1 promoter in endothelial cells does not jeopardize the general normal physiology of the fish.

By crossing Tg[flk1:Cx43-egfp]br with another transgenic line expressing Mcherry under control of flk1 promoter, Tg[flk1:lxp-mcherry-1xp-Cx43(C61S)-egfp]br zebrafish, most of the Cx43-gfp puncta correlated with assumed borders of endothelial cells. For example, as shown in figure 7D, at 2 dpf, most of the green fluorescent spots on the axial vessels align into circle shape, and the green puncta on the ISV align into lines. When using VE-Cadherin as a cell-cell junction marker, not all of the Cx43-egfp puncta co-localize with VE-Cadherin. It is unknown whether those Cx43-egfp puncta not localized to the VE-Cadherin junctions also exist on the cell border or not. A better way to show the whole cell border may be to
create mosaic endothelial cells that will express different fluorescent proteins. With the red fluorescent endothelial cells as contrast, the question whether the Cx43-egfp exists on the luminal surface of the vessel tube would be addressed by taking the single section of confocal image.

In Tg[flk1: Cx43-egfp]br, the expression level of gfp is not always consistent among different fish. It is also interesting to notice that there is more Cx43-egfp signal on the ISVs at 22 hpf than at 30 hpf. As shown in figure 7B, at 30 hpf, the most of ISVs seem to be cleared of the gfp signals. In figure 7D, the right ISV has a much higher level of gfp signal than the left one but the overall expression in the ISVs is much lower than at early stage (7A). These different expression levels of Cx43-egfp may be due to regulated promoter activity, increased turnover of the ectopic expressed protein or shutdown of the transgenes by methylation. However, the general expression of Cx43-egfp in ISVs seems to be down regulated through some mechanism. Such regulation may suggest the involvement of Cx43 in the stage-critical developmental processes. Nevertheless, with this line, the sub-cellular distribution of Cx43-egfp can be investigated with the proper markers highlighting the sub-cellular structures of interest, such as the filopodia for migrating endothelial cells and the apical membrane of the forming endothelial lumen.

The IHC of endogenous Cx43 provides important complementary information about the dynamic regulation of Cx43 in endothelial cells. With the contrast of gfp-highlighted endothelial cells in Tg[fl1: gfp], the puncta of Cx43 antibody staining likely exist on the surface of the endothelial cells, as shown in Figure 8. At 22hpf, the Cx43 antibody stains outside the endothelial cell-to-cell contacts, which may suggest a role of Cx43 in endothelial migration. Those spots could possibly be the gap junction channels formed between
endothelial cells and other cell types or be hemichannels that sense cues for the migration. At 30hpf, no staining of the Cx43 antibody was observed in ISVs, supporting the existence of a down regulation for the Cx43 in ISVs. The electron micro-image may be necessary for the decisive proof at the desired resolution. Despite of the possible cross-talk signals from the gap junctions of other connexins and the possible tissue specific background signals, Cx43 IHC represents relatively the true endogenous distribution of Cx43, by which earlier stages of vasculogenesis and angiogenesis should be investigated. A new antibody against the extracellular loops of Cx43 would distinguish the gap junction channels from hemichannels.

2.4.3 Tissue specific loss-of-function by dominant negative mutant

The transgenic lines obtained with pTol2(flk1: lxp-mcherry-lxp-Cx43DN-egfp) are made by injecting DNA fragments. Therefore the bright Mcherry expression may be caused by large concatemers inserted in the genome. When Cre is introduced, most of the repeated concatemers may be excised together, leaving a rearranged locus or a few copies of Cx43 dominant negative mutant to be expressed. The low level of expression of a dominant negative mutant is not expected to have an effect on the endogenous Cx43. The adequate expression level of ectopic Cx43 (as a negative control) or its mutant is critical for this experiment. By using Tol2 vector, single insertions at different loci will be achieved. The expression level of the dominant negative mutants still needs to be gauged in comparison to the endogenous Cx43 and the negative control.

The efficiency of the dominant negative mutants in abolishing the normal functions of the Cx43 protein depends on both the intrinsic structural characteristics of the mutant and the relative ratio of the dominant negative mutant to the endogenous Cx43 protein in one
functional unit, i.e. in a gap junction channel or a hemichannel. Even though it is difficult to determine the precise *in vivo* ratio of the introduced mutants and the endogenous Cx43, such a value can be estimated through RT-PCR and the variations should be obtainable through the screen for the transgenic zebrafish.

In comparison to the negative control transgenic zebrafish expressing wild type Cx43 with *flk1* promoter, at the similar expression levels, any defects in the vascular morphogenesis should be attributed to the structural difference between dominant negative mutant and the wild type Cx43, i.e. either the single amino acid mutation or the C-terminal truncation. This would indicate the requirement of normal function of the functional unit formed by Cx43 in the vascular morphogenesis. On the other hand, a negative result, i.e. the intact vascular morphogenesis, at an adequate expression level may argue against the necessity of endothelial Cx43 for the vascular morphogenetic processes, at least when Cx43 is in the form of the predicted functional unit.

If the gap junction channel is required for both the migration and tube formation, the Cx43 DN (T154A) is expected to lead to defects in both the processes since it closes the gap junction channels. The gap junction channel may function to coordinate the movement when a group of cells migrate altogether, Cx43 DN (T154A) will de-couple such coordination, and probably reduce both the speed and directionality of the cell groups. This could be reflected as the delayed or failed arrival of angioblast to the midline and a corresponding ISV migration problem. Though the gap junction channels between endothelial cells may function to allow the transmission of the vascular wall pressure in the endothelial cells and facilitate the mechanical pushing for the tube extension, it may not show an obvious phenotype when the channels are closed since the vessels forms in the silent heart fish mutant (Isogai, S.,...
2003). As for the Cx43 DN (C61S), which is unable to dock and form an intact gap junction channel, the signals that need to transmit through endothelial cells will leak to the extracellular space but those signals may still be able to diffuse into other cells to continue the transduction. This means if the spatial constriction of the signal is not important, the cell-to-cell communication defects caused by Cx43 DN (C61S) may be less severe than Cx43 DN (T154A). Similarly, the double mutant conceiving both (T154A) and (C61S) may produce the defects no more severe than Cx43 DN (T154A).

If the hemichannels instead of the gap junction channels are required for the migration or tube formation, then Cx43 DN (C61S) isn’t expected to produce any defect since it still forms open hemichannels but the mutant conceiving T154A change will lead to defects. If the hemichannels function in cue sensing in migration, the cell with closed hemichannels may be unable to react to external signals and thus cannot initiate the morphological changes. If the tube expansion requires the hemichannels to allow fluid flux, the closed hemichannel will results in the tube defects due to the blocked water influx to the lumen.

If neither the hemichannel nor the gap junction channel but only the adhesion force between the two connexons is required, then Cx43 DN (T154A) will look normal while all the C61S change conceiving mutants will give out the phenotypes. If such forces between the endothelial cell and other cell type are required for the migration, the speed may be affected. The adhesion force may also be important for the formation of the junctions preceding the polarity establishment for the lumen formation. Since the Cx43 DN (C61S) interfere with docking, it may cause the cell to loose their contact with one another at gap junctions. This may effect the formation of other cell-cell junctions.
If the C-terminal of the Cx43 is the only part required for the migration or tube formation, we wouldn’t expect many defects in either (T154) or (C61S) single mutant but in all the C-truncated mutants.

Such expression of the dominant negative mutants can be knockdown by specific morpholinos, which will constitute the rescue for the loss-of-function effect of the dominant negative mutants. This can support the requirement of the functional Cx43 in the studied processes.
2.5 Figures and Tables

Figure 1. Morpholino knockdown phenotypes at 36 hpf in Tg[fli1:gfp] zebrafish

A, B, C and D are images of an uninjected Tg[fli1:egfp] embryo; E, F, G and H are images of an embryo injected with Cx43 MO 4.5 ng; I, J, K and L are images of an embryo injected with Cx43.4 MO 4 ng. Red arrow points to the DLAV and blue arrow points to an ISV; yellow arrows point to the ISVs that have only migrated half way, and purple arrows point to the early branches in the ISVs. The black asterisks indicate the notochord.
Figure 2. Morpholino knockdown phenotypes at 2 dpf in Tg[fli1:gfp] zebrafish

At about 58 hpf, patent blood vessel lumens are shown by microangiography. A, E, I, M, Q and U are images of an uninjected embryo; B, F, J, N, R and V are images of an embryo injected with Cx43 MO 4.5 ng; C, G, K, O, S and W are images of a Cx43.4 morphant at 4 ng; D, H, L, P, T and X are the images of a Cx44.2 morphant at 6 ng. The white arrow points to the parachordal vein (PAV); the purple arrows point to the early branches; the green arrows points to the ISVs that cannot be filled with Dextran-Alexa Fluor® 568. The black asterisks indicate the notochord.
**Figure 3. Morpholino knockdown phenotypes at 3 dpf in Tg[fli1:gfp] zebrafish**

At about 78 hpf, patent blood vessel lumens are shown by microangiography. A, B, C and D are images of an uninjected embryo; E, F, G and H are images of a Cx43.4 morphant at 12 ng; I, J, K and L are images of a Cx43 morphant at MO 9ng. The white arrow points to PAV; the purple arrows point to the early branches; the green arrows point to the ISVs that cannot be filled with Dextran-Alexa Fluor® 568; the green arrow head points to the unfilled axial vessels.
Figure 4. Dose responses of the connexin MOs

Dose responses of the connexin MOs were determined by counting the number of embryos without the axial flow at 2 dpf. There are injections listed on Table 1. Each color represents the injections done with the same batch of embryos.
Figure 5. Co-injection of connexin MOs with Cdh5 MO

The co-injections of Cdh5 and connexin morpholinos produced synergistic effects on the vascular phenotypes. Values are based on the injections listed on Table 1. Numbers below are the n values for individual injections.
Figure 6. Co-injection of connexin MOs with p53 MO

The co-injection with p53 MO rescued the connexin knockdown phenotypes to limited degrees. Values are based on the injections listed on Table 1. Numbers below are the \( n \) values for individual injections.
Figure 7. Tg[flk1:Cx43-egfp] zebrafish and Cdh5 antibody staining.

The Cdh5 antibody staining in Tg[flk1:Cx43-egfp] fish: A, 22 hpf and B, 30 hpf. White arrows points to the Cx43-egfp puncta that are co-localized well with Cdh5, which are yellow spots; blue arrows point to the Cx43-egfp puncta that are not localized on the Cdh5 lines, green spots. The live images from Tg[flk1:Cx43-egfp; flk1:mcherry] obtained by crossing Tg[flk1: Cx43-egfp]$^\text{br}$ and Tg[flk1: lxp-Mcherry-lxp-Cx43C61S-egfp]$^\text{br}$: C, 28 hpf and D, 50 hpf.
Figure 8. Cx43 antibody staining in Tg[fli1:egfp] zebrafish.

A, the 22 hpf embryo; B is the high magnification of the inset of A; C, the 30 hpf embryo. Blue arrows point to the Cx43 puncta between endothelial cells and white arrows point to the Cx43 puncta outside the endothelial cell junctions.
Figure 9. Constructs of Cx43 dominant negative mutants.

The constructs that allow the Cre-loxp controlled expression of Cx43 dominant negative mutants in zebrafish endothelial cells. Construct Ia, pTol2(flk1:loxP-Mcherry-loxP-Cx43DN-egfp) is the first construct used to make the transgenic zebrafish. A, the Tg[flk:loxP-Mcherry-loxP-Cx43(C61S)-egfp]$^{br}$, expressing Mcherry under control of the flk1 promoter. When Cre was introduced, the Loxp (loxP) flanked Mcherry expression cassette was excised, as shown by B and C. Then the construct Ia is converted to construct Ib, pTol2(flk1:loxP-Cx43DN-egfp), allowing the expression of Cx43 dominant negative mutants under control of the flk1 promoter. Construct II and III are the advanced versions, in which 2A is the peptide linker; TAG, the rfp; CAAX, the membrane-targeting sequence; Tol2, the minimal components of Tol2 transposon.
Table 1. Morpholino injections grouped by MOs and doses

mo: morpholino injected
ni: number of embryos injected
nd: number of dead embryos at 1 dpf
nl: number of living embryos at 1 dpf
nf: number of embryos with axial flows at 2 dpf
aaf: percentage of embryos with absence of axial flow at 2 dpf

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CHAPTER 3. CONCLUSIONS AND PERSPECTIVES

Gap junction proteins are required for migration (Elias, L. 2007) or involved in lumen expansion in certain tissues (Calera, M., 2006; Lubarsky, B., 2003). Research on the function of endothelial Cx43 in endothelial migration or lumen expansion will not only shed light on the general mechanisms of cell migration and lumen formation but also disclose how Cx43 affects angiogenesis. Therefore this will provide potential directions of development of therapies to angiogenesis-related diseases.

In mice, endothelial specific knockout of Cx43 does not cause severe vessel defects and the mice survive to maturity. At adulthood, hypertension and bradycardia are observed, which means Cx43 is involved in the normal vascular function (Liao, Y., 2001). However, the mice may have a different gene requirement for Cx43 in endothelial cells. It is possible that the endogenous Cx43 plays a role in both vascular development and normal function but its absence can lead to the immediate activation of other gap junction proteins to compensate its loss. Since the pilot morpolino knockdown in zebrafish has produced the vascular defects, zebrafish may provide an additional in vivo system to understand the function of gap junction proteins in vascular development. The optic transparency of fish embryos is advantageous for descriptive studying of the dynamic distribution of certain proteins by tagging the fluorescent proteins. The presence of the Cx43 in the sub-cellular structures important for migration or lumen formation in endothelial cells will support its involvement in those processes. On the other hand, the absence of the Cx43 at the relevant places at the relevant time may argue against its direct involvement. From both the live images of the Tg[flk1:Cx43-egfp]^{br} and the whole mount IHC of the endogenous Cx43 in Tg[fli1:egfp], it appeared that Cx43 exists
between endothelial cells, which may be important to keep the continuity of the communications among the endothelial cells while they undergo migration. Those cell-to-cell junctions may also be relevant to the establishment and maintenance of apical-basal polarity of the lumens. The IHC signal of Cx43 on the surface of the endothelial cells outside endothelial cell-cell contacts may support a role in endothelial migration no matter whether as a hemichannel sensing the cue from the surroundings or a gap junction channel formed between endothelial cell and other cell types since at that stage the endothelial cells moves over the relative still surroundings. It would also be interesting to check closely whether the hemichannel of Cx43 exists on the luminal side of the endothelial cells since that would support a role of Cx43 in lumen formation, expansion or maintenance. The higher magnification may be required for a better study of the sub-cellular distribution of the Cx43 in the endothelial cells.

However, the available technique for functional study of certain gene in zebrafish is rather limited. The antisense morpholino cannot be applied in a tissue specific way. For genes of pleiotropic functions, such as Cx43 that is expressed earlier during embryogenesis than when the vasculogenesis starts, the vascular defects caused by morpholino injection can be a secondary event due to its impaired earlier functions. The morpholino knockdown phenotypes need to be validated through rescue experiments. The failure to rescue by mRNA or DNA injection does not mean the morphant phenotype is not a result from loss-of-function of certain gene. In order to find out how the endothelial Cx43 could possibly function in the endothelial migration or endothelial lumen formation, techniques other than morpholino injection should be developed.
There are well-studied dominant negative mutants that affect the channel opening or the docking between the two hemichannels (Elias, L., 2007), which will lead to the impairment of the function of either the hemichannel or the gap junction. If endothelial Cx43 is required for vascular morphogenesis in the form of the hemichannel or the gap junction channel, the introduction of the channel-blocking or docking-blocking dominant negative mutant to the endothelial cells is expected to produce phenotypes in blood vessel formation. In order to test this, we have generated the transgenic zebrafish that expresses Cx43 dominant negative mutant (DN) only in endothelial cells in a controlled manner with the use of the Cre-Loxp system. After the DN mutant is introduced to the endothelial cells the phenotypes in the endothelial migration or tube formation can be scored according to the criteria used in the knockdown experiments. The different results from the different DN mutants can be informative about how Cx43 functions in those processes. The technique of introducing dominant negative mutant in a tissue specific way may also be applied to other genes that may function in the vascular formation.
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