Identifying the Role of Connexin 43 in Transport of microRNAs

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Cardiovascular disease (CVD) is the leading cause of mortality worldwide. Present treatments for CVD rely heavily on surgical interventions and drug therapies that relieve symptoms rather than cure the disease. Therefore, there is active research to find alternative therapeutic approaches for CVD. The small gene regulating molecules, microRNAs (miRNAs) are known to mediate structural changes in cardiovascular diseases. Therapy using miRNAs is a novel approach to restoring function of damaged cardiac tissue in CVD. Global tissue expression of miRNAs is regulated via movement of miRNAs through multiple vesicular transport pathways and gap junctions. Connexin 43 (Cx43) which is the protein constituting the gap junctions specifically between ventricular cardiomyocytes, may provide a potential therapeutic target for regulating the expression of miRNAs in cardiac tissue. The aim of this study was to explore miRNA transport via human Cx43 in a single cell model and then in human cardiomyocytes. I hypothesised that Cx43 will have a role in intercellular miRNA transport and will thus mediate miRNA expression in cardiac cells.

To characterise the function of Cx43 in miRNA transport, without interference from vesicular transport pathways seen in cardiomyocytes, a single cell model of human-Cx43 mRNA injected *Xenopus laevis* oocytes was constructed. The function of exogenously expressed human Cx43 was determined using *in vitro* electrophysiology. No significant increase in current was observed in connexin activating Ca$^{2+}$ free solution for the human-Cx43 mRNA injected oocytes (n=6, N=3) in comparison to the control non-Cx43 expressing oocytes (n=6, N=3). However, consistent with the previous studies, current across the cell membrane of all oocytes from both groups was reduced by 100% or 50% on re-application of Ca$^{2+}$ (2 mM) or non-specific connexin blocker, lanthanum (0.05 mM), respectively. The Ca$^{2+}$ and lanthanum sensitivity confirms that the observed current is mediated by connexins. Western blot analysis on the native oocyte suggests the presence of endogenous connexin, which could be contributing to the lack of significant functional difference for the human cRNA injected oocyte. Hence, an endogenous
connexin knockdown model of oocytes must be considered for testing the permeability of Cx43 to miRNA.

The possibility of transport in adult human cardiomyocytes was tested using AC16 cells derived from human ventricular cardiomyocytes. The transport of transfected miRNA-1 from the cells into its extracellular environment (cell media) was tested using lanthanum (2 mM). No significant difference in miRNA transport was seen upon blocking connexin transport by lanthanum (n=3).

The findings in this thesis represent a first step towards a better understanding of the possibility that miRNA moves through gap junctions in adult human cardiomyocytes.

\[ ^1n = \text{number of oocytes whereas } N = \text{number of frogs oocytes were extracted from.} \]
ACKNOWLEDGEMENTS

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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>A/A</td>
<td>Antibiotic antimiotic</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>eDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complimentary Ribonucleic Acid</td>
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<tr>
<td>C_{T}</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CulOri</td>
<td>Culture oocyte Ringers solution</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region gene 8</td>
</tr>
<tr>
<td>dH\textsubscript{2}O</td>
<td>Coomassie Blue Dye</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>miRNA:</td>
<td>micro RNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular bodies</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pri-miRNA</td>
<td>Primary micro RNA</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polvinylidene difluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RIPA buffer</td>
<td>Radioimmunoprecipitation assay buffer</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>RISC</td>
<td>RNA inducing silencing complex</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse-transcription – quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SNAREs</td>
<td>Soluble N-ethylmaleimide-sensitive-fusion-protein attachment-protein-receptor</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>t-SNARE</td>
<td>Target SNARE</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEVC</td>
<td>Two Electrode Voltage Clamp</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris-buffered saline and Tween20</td>
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<tr>
<td>UTR</td>
<td>3’untranslated region</td>
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<tr>
<td>v-SNARE</td>
<td>Vesicular SNARE</td>
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1. INTRODUCTION

Preface

Cardiovascular diseases (CVDs) are the disorders of the heart and blood vessels. Under such pathological conditions, the cardiac tissue, mainly the myocardium is damaged and drastically remodelled. These structural changes in the cardiac tissue reduce elasticity of the heart and thus impairs the blood pumping function of the organ (Cohn, 1995). As postnatal cardiac tissue is known to have limited regenerative abilities, structural changes in CVD are therefore permanent. In recent years, advances in CVD treatments have been made, however CVD still remains to be the leading causes of mortality and morbidity worldwide (World Health Organization, 2018). Approximately 17 million pre-mature deaths (>70 years old) per year are caused by CVDs (World Health Organization, 2018) making CVD an ongoing problem for the health of the global population and a major burden for the healthcare systems.

It is now known that the structural changes in cardiac tissue are preceded by molecular changes occurring in cardiac cells (Lin et al., 2009). The role of small non-coding RNA molecules, known as microRNAs (miRNAs) in CVD has been brought to light and they are now being researched extensively as potential molecular targets in CVD therapy (Thum et al., 2007). Presently, transport pathways of miRNAs are being investigated in multiple in vivo mouse models to restore lost function of cardiac tissue (Van Rooij et al., 2006; Kosaka et al., 2010). However, the known current miRNA transport pathways lack specificity and hence this reduces the efficiency of miRNA-mediated treatment (Zhu et al., 2012; Ottaviani et al., 2018). This thesis thus investigates a potential new transport pathway for miRNAs between cardiomyocytes that could allow for targeting specific cell-to-cell communication in future research for CVD treatments.
1.1 CVD Pathology

CVDs essentially encompass all the diseases caused by dysfunction of the cardiac tissue and blood vessel. These include left ventricular hypertrophy (increase in cell size), myocardial infarction (apoptotic loss of cardiomyocytes), arrhythmia (abnormal beating of the heart), and heart failure. In all these cardiac pathologies, the myocardial tissue is damaged which results in poor cardiac function. The cardiac output (amount of blood leaving the heart per minute) and stroke volume (cardiac output per heartbeat) are thus decreased due to reduced contractility of the heart muscle (Jackson, 2000). In order to maintain homeostasis and meet the oxygen demand of peripheral organs, the heart undergoes further structural changes such as cardiac hypertrophy to compensate for impaired function of the myocardium (Jackson, 2000). These structural changes are however only beneficial in the short term. Chronically, thickening of the heart muscle caused by hypertrophy, restricts blood flow to the myocardium and this predisposes to myocardial infarction and ischemia, which causes further cardiomyocyte death and dysfunction (Cohn, 1995). There is also increased apoptosis of the cardiomyocytes which are replaced by other non-contractile cells such as fibroblast and as a result, fibrosis occurs. Thus, there is subsequent weakening of the heart wall (Diwan et al., 2007). These overall changes in the structure of the myocardium, interfere with the conductivity across the chambers of the heart; especially fibrosis which can lead to arrhythmia (Tomaselli & Marban, 1999). Therefore, though a specific CVD can occur initially, it can progress to other CVDs initiating a vicious cycle with progressive reduction in function of the heart and eventual heart failure (Conrad et al., 1995; Tomaselli & Marban, 1999; Jackson, 2000). To stop the progression of disease to heart failure, it is vital to identify the new ways to restore function to the damaged myocardium.

1.2 CVD Treatments

The loss of cardiac tissue function in the diseased heart can currently be treated surgically, by implantation of battery-operated pacemakers, coronary arterial stents, and coronary artery bypass grafts. These methods work best to slow the progression of CVDs, but do not stop future
occurrence of CVD incidents (Epstein et al., 2008; Mulpuru et al., 2017). Exercising and taking cholesterol-lowering drugs or beta blockers prescribed by doctors, reduce workload of the heart and risk of CVD but are only capable of slowing rather than stopping the progression of the disease (Greenland et al., 2001). Therefore, in all cases CVD patients are still prone to heart failure. Heart transplantation can restore cardiac function in end stage heart failure, but heart transplant treatment is limited by the availability of donors and relies heavily on recipient-donor matching and this is not always possible (Lund et al., 2017). Hence, at present the major research focus for CVD treatment has shifted towards molecular therapy. Researchers are now focusing on gene regulatory molecules that modulate structural changes in the cardiovascular tissue with the intention of targeting these molecules in future CVD research to restore function of the diseased myocardium (Paiva & Agbulut, 2017; Ottaviani et al., 2018).

1.3 miRNAs as molecular targets

miRNAs are a class of tissue specific small (22 nucleotide) single stranded non-coding RNA molecules (Chen et al., 2006). They regulate gene expression at post-transcriptional level and thus regulate cellular pathways that mediate pathophysiological events in the cells (Bartel, 2004). They form part of the RNA-induced silencing complex (RISC) and interact with target messenger RNA (mRNA) strand to negatively regulate gene expression (Filipowicz et al., 2008). Furthermore, miRNAs can be transported between cells which enables miRNAs to extend their effect on the overall tissue (Kosaka et al., 2010; Boon & Vickers, 2013). They have evolutionary conserved sequences and target multiple cellular pathways (Bartel, 2004). miRNAs are hence an ideal molecular target for controlling pathophysiological events in the tissue, at cellular level.

1.3.1 miRNA biogenesis

A primary microRNA (pri-miRNA) strand is transcribed by RNA polymerase II from miRNA genes or from introns of protein coding genes in the nucleus of the cell. The endonuclease
enzymes Dorsha and the cofactors DiGoerge syndrome critical region gene 8 (DGCR8) cleave pri-microRNA to form ~70 nucleotide long precursor miRNA (pre-miRNA) strand which is transported from the nucleus of the cell and into the cytoplasm via exportin proteins (Han et al., 2004). When in the cytoplasm, pre-miRNA is cleaved by Dicer enzyme to form a functional 22 nucleotide long miRNA strand that is of ~1nm diameter in its linear form; this strand is also referred to as mature miRNA (Grishok et al., 2001) (Figure 1.1).

### 1.3.2 miRNAs mode of action

miRNAs carry out their gene regulatory function in the cytoplasm of the cell. When incorporated into RISC, mature miRNAs interact with the 3’untranslated region (UTR) of multiple mRNA strands. The degree of complementation between these two strands determines the silenced fate of the proteins encoded by the miRNA-complementing mRNA strand. A perfect complementation between the two strands results in miRNA mediated degradation of the mRNA whereas a partial complementation results in translation repression of mRNA (Filipowicz et al., 2008) (Figure 1.1). This flexibility in complementation enables one miRNA to regulate expression of multiple proteins and thus regulate various gene regulatory pathways in a cell (Hutvágner & Zamore, 2002).
**Figure 1.1 miRNA synthesis and mode of action.** A precursor miRNA (pre-miRNA) strand is transcribed in the nucleus of the cell and is later processed into mature miRNA in the cytoplasm. It is the mature miRNA strand that form part of RISC and interacts with mRNA strand to regulate protein expression. [Image adapted from (Ryan et al., 2015)].

### 1.3.3 miRNA in cardiovascular development

There are 526 miRNAs in the human heart (Lewis et al., 2003). These miRNAs collaboratively dictate fundamental cellular biological processes within cardiac tissue, such as proliferation, differentiation, apoptosis and conductivity and are therefore extensively involved in cardiogenesis (Zhao et al., 2007). To list a few, miRNA-1 and miRNA-23 tightly control the expression of mRNA coding for anti-hypertrophic Muscle-specific RING-finger protein whereas miRNA 24 regulates apoptotic protein Bim coding mRNA, to maintain structural homeostasis of the cardiac tissue (Zhao et al., 2005; Lin et al., 2009; Qian et al., 2011).

miRNA-1 which is the predominantly expressed miRNA in cardiomyocytes, interacts with potassium ion channel Kir2.1 and calcium ion channel regulating molecule B56-alpha to regulate conductivity across the heart (Yang et al., 2007). Of the other cardiac miRNAs, miRNA-16 mediates vascular development and miRNA-133 allows differentiation of cardiac stem cells into myogenesis. miRNAs are thus vital for development of the heart and for maintaining its function (Care et al., 2007).
1.3.4 miRNAs and CVD

Dysregulated expression of miRNAs in cardiac cells is correlated with the onset and development of pathology in cardiac tissue. miRNA-1, miRNA-133, miRNA-208 and 63 other miRNAs are reported to be dysregulated in the failed human heart (Thum et al., 2007). In infarcted myocardium, there is downregulation of miRNA 24, miRNA-1 and miRNA-133 (Boštjančič et al., 2010; Qian et al., 2011). Downregulation of miRNA-1 is also associated with hypertrophy and ventricular tachyarrhythmia (Curcio et al., 2013). Contrasting, independent overexpression of miRNA-1 and miRNA-23 are also known to induce cardiac hypertrophy (Van Rooij et al., 2006; Lin et al., 2009). Myocardial dilation, on the other hand, is linked with downregulated expression of miRNA-24 which has been shown to increase apoptosis in murine cardiomyocytes (Qian et al., 2011). Dysregulated expression of miRNAs in cardiomyocytes thus plays a crucial role in modulating CVDs.

Interestingly, restoration of downregulated miRNA expression with miRNA mimics (synthetic RNA duplexes with similar sequence to mature miRNAs) has been shown to restore function of the cardiac cells, especially cardiomyocytes (Vicencio et al., 2015). The in vivo administration of miRNA mimic in CVD mouse models led to significant reduction in infarct size, in hypertrophy and in cell apoptosis in cardiac tissue (Qian et al., 2011; Curcio et al., 2013). Use of miRNA to recover from CVDs is evidently promising. However, post miRNA administration side-affects such as tumour vascularisation have also been reported (Zhu et al., 2012) (Gray et al., 2015).

1.3.5 Challenges with miRNA-based therapy for CVD treatment

As form of a molecular treatment, the miRNA mimics were delivered through the current known miRNA transport route of membrane bound vesicles (Zhu et al., 2012; Gray et al., 2015; Zhang et al., 2016). Details of miRNA transport pathways are described in section 1.3. Here, for the miRNA-based treatment, miRNA mimics were packaged in vesicles which were injected intravenously to reach the cardiac cells via circulation. It was intended that the vesicles would
be taken up by the cardiac cells to restore expression of the dysregulated miRNAs and subsequently improve cardiac function. However, as the miRNA packaged vesicles were administered in the systemic circulation this led to the non-specific uptake of administered miRNA mimics. Hence, off-target effects were observed in the non-cardiac tissue due to disturbance of homeostasis of cells in these tissues.

Therefore, the administration of miRNA mimics is though promising for restoring function of the diseased heart, it is also evident that an improved method of miRNA administration is required to increase miRNA uptake specificity in the cardiac tissue. To improve delivery, it is vital to understand the transport pathways miRNA use to migrate between cells and induce their effect on the overall tissue.

1.4 miRNA transport pathways

miRNA transport between cells are considered vital for cell to cell communication. miRNAs are transported as signalling molecules to maintain synchronicity in the activity of the cells and thus allow the organ to function as a whole (Kosaka et al., 2010; Vicencio et al., 2015; Ottaviani et al., 2018). Understanding the transportation mechanism for these miRNAs is hence of importance in order to add to the knowledge of factors contributing to the dysregulated miRNA expression in a cardiomyocyte which, as previously discovered, initiates cardiac pathology. Further, a deeper understanding of the miRNA transport route would potentially aid in developing a more specific form of miRNA administration in miRNA-based therapy. Current known miRNA transport routes are via membrane bound vesicles (i.e. exosomes and microvesicles) that travel through extracellular fluid (ECF). Recent literature (discussed in section 1.4.4) provides some evidence for transport of miRNAs through connexin proteins that form subunits of the gap junctions. There are, however, no studies of this type in human cardiomyocytes.
1.4.1 Membrane bound vesicle: Exosomes

Exosomes are small (30-100nm) vesicles formed by the inward budding of late endosome and are collectively enveloped within multivesicular bodies (MVB) (Figure 1.2). Alongside proteins, lipids and mRNA, miRNAs are selectively loaded in exosomes in ceramide dependent manner (Kosaka et al., 2010; Wang et al., 2014). The release of exosomes from donor cells and their uptake in recipient cell, both occur via receptor-ligand mediated interaction involving the cell surface marker, SNAREs (Soluble N-ethylmaleimide-sensitive-fusion-protein attachment-protein-receptor). The v-SNARE (vesicle SNARE) on the surface of MVB interacts with t-SNARE (target SNARE) located on the plasma membrane of the recipient cells and initiates fusion of MVB with the plasma membrane of the cell (Fader et al., 2009). Exosomes are thus released in the ECF and are then endocytosed by neighbouring cells or more distant cells. Once in recipient cells, miRNAs from exosome are released in the cytoplasm (Figure 1.2). The miRNAs then bind to the RISC of the recipient cell and interact with mRNA to alter protein expression and thus physiology of the cell (Wang et al., 2014).

However, the exosomal transport of miRNA is subject to limitation induced by the ECF. The rate of release of vesicle and the distance travelled in the ECF to reach the recipient cells are rate limiting steps in the exosomal transport route. Additionally, cell surface markers on all exosomes are similar; regardless of the cell types exosomes are derived from (Kosaka et al., 2010). Lack of variation in surface makers of vesicles thus reduces the specificity of miRNA transport.

1.4.2 Membrane bound vesicle: microvesicles

Microvesicles are comparatively larger in size (110-4000nm) than exosomes. They are created by outward budding and blebbing of the surface plasma membrane of the cell and are released into the ECF (Figure 1.2). Similar to exosomes, microvesicles travel in plasma and carry signalling molecule including miRNAs, to facilitate communication between cells (Hunter et
The miRNAs brought in by microvesicles also interact with RISC in the recipient cells and modulate gene expression (Cantaluppi et al., 2012; Diehl et al., 2012). As the miRNAs transported in microvesicles also follow the ECF transport route, they are subjected to similar rate limiting steps as exosomes and hence microvesicles also lack specificity and efficiency in terms of miRNA delivery.
Figure 1.2 Transport routes/mechanisms of miRNAs from donor cell to the recipient cell between cardiomyocytes. Once the miRNAs are transported from the nucleus and into the cytoplasm of the cells, they can go in either of the following four different directions. 1) Incorporated in RISC to interact with the target mRNA strand. 2) miRNA can be packaged into exosomes via endosomal pathways and be released in extracellular fluid (ECF) to be taken up by recipient cells. 3) Accumulation of miRNA on the cytoplasmic surface of the plasma membrane may result in budding body and transport of miRNA in microvesicles through ECF route. 4) Lastly, miRNAs from the cytoplasm of one cell can be transported across the adjacent cells via gap junctional protein connexin, although this remains to be confirmed in human cardiomyocytes (refer to section 1.4.4). Once the transported miRNAs are taken up in the cytoplasm of recipient cell through exosomes, microvesicels or via gap junctions, they can interreact with the RISC of the recipient cell and regulate gene regulation. [self-made figure].
1.4.4 Connexin mediated miRNA transport

1.4.4.1 Connexin protein

Connexins are a class of transmembrane protein. They have four hydrophobic membrane-spanning domains, two extracellular domains and three cytoplasmic domains which includes the C-terminus and N-terminus end of the protein (Willecke et al., 2002) (Figure 1.3). Hexamers of connexin proteins are assembled in the endoplasmic reticulum or in the Golgi organ of the cell, and then travel to the plasma membrane where they exist as hemichannels. In the plasma membrane of the two adjacent cells, connexin protein hexamers form subunits of gap junctions which are vital for communication between cells (Figure 1.3). It has been long been known that connexin formed gap junctions (pore size of 1.0-1.5nm) provide a pathway for movement of small molecules, ions and gene regulating molecules between adjacent cells (Goodenough et al., 1996; Söhl & Willecke, 2004). Hence, connexins play a crucial role in synchronizing activity between cells thereby maintaining tissue homeostasis and contributing to physiological function of the organ.

As with miRNAs, connexins are also expressed in a tissue specific manner. The gap junctions between ventricular cardiomyocytes are predominantly formed by Cx43 (Vozzi et al., 1999). The connexin channel is opened by acidic pH and lack of Ca^{2+} (Morley et al., 1996; Trexler et al., 1999). The permeability of the channel is therefore pH and Ca^{2+} sensitive. Its carboxyl terminal region acts as the ‘gate’ (Bao et al., 2004; Söhl & Willecke, 2004).
1.4.4.2 Evidence of connexin mediated miRNA transport

There is seminal evidence to demonstrate the connexin mediated transport of miRNAs. Using HeLa cell line, Zong et al., (2016) were the first to demonstrate the permeability of mouse connexin (Cx43, Cx31, Cx30 and Cx26) to miRNA-96 and miRNA-183 which are miRNAs abundantly expressed in the inner ear. Further, they showed that fluorescent tagged miRNA mimics (fluro-miRNA-mimic) were able to be transported from the transfected cells into non-transfected cells following co-culturing of the two cell groups. This was associated with gene silencing in the recipient non-transfected cells. In contrast, when connexin in the mimic transfected cells was blocked, the genes in the recipient non-transfected cells were not silenced.
Connexin thus mediated miRNAs transport between cells and the transported miRNA retained their function and were able to regulate gene expression in the recipient cell. Similar findings of connexin mediated miRNA transport have been reported by (Lemcke et al., 2017) in isolated neonatal cardiomyocytes. In their experiment, a 50% decrease of miRNA mimic transport was observed following Cx43 knockdown.

The accumulated evidence from studies by Zong et al., (2016); Lemcke et al., (2017), for connexin mediated miRNA transport portray connexin as one of the major transport routes for miRNA. As the molecules move through the gap junctions between adjacent cells, they pass directly from the cytoplasm of one cell to another. Therefore, the movement of gene regulating molecule, miRNA, from donor cell to recipient via connexin formed gap junctions avoid any limitations enforced by passage through the ECF. Here, Cx43 mediated miRNA transport is targeted specifically to the adjacent recipient cell and the effects are potentially more precise and circumscribed in comparison to miRNA delivery by membrane bound vesicles.

While it has been shown that connexin is permeable to miRNAs in neonatal mouse cardiomyocytes through mouse isoform of Cx43, it is not known if this is true for adult human cardiomyocytes. Given the tissue specific characteristic of miRNAs and Cx43, it is important to establish whether or not connexin can provide a pathway for cell to cell movement of miRNAs. If so, this would open up the possibility of targeting cell-specific communication in miRNA-mediated CVD therapy.

**Aims and objectives**
Aim: To test if human Cx43 will mediate transport of miRNAs in adult human cardiomyocytes.

This aim will be tested in two stages. First, permeability of human Cx43 to miRNA will be tested in a single cell model. Human Cx43 will be expressed in *Xenopus laevis* oocytes, as their relatively big size (~1mm) allows for easier manipulation. Additionally, *Xenopus* oocytes in the *in vitro* setting do not release membrane bound vesicles. Thus, characterisation of Cx43 mediated miRNA transport in the single cell model of *Xenopus* oocytes, eliminates the possibility of interference from vesicular transport of miRNAs. Second, to test if miRNA can pass through Cx43 in human cardiomyocytes, the aim will be tested in AC16 cardiomyocytes – a cell line derived from the ventricles of the adult human heart.

Objectives:

1. To test the permeability of human Cx43 to miRNAs in a single cell model of *Xenopus* oocytes
2. To test if transport of miRNAs through Cx43 formed channels occurs in AC-16 cardiomyocytes

Hypothesis:

As the pore size of the Cx43 formed gap junction (~1.5nm) conforms with the diameter of linear miRNA molecules (1.0nm), I hypothesise that Cx43 will mediate transport of miRNAs in both single cell model of oocyte and in AC-16 cardiomyocytes.
2. METHODS

2.1 Xenopus laevis oocytes

Use of *Xenopus laevis* for this project was approved by the Environmental Protection Authority, and harvesting of oocytes was approved by the University of Otago Animal Ethics committee.

2.1.1 *Xenopus laevis* housing

The female *Xenopus laevis* frogs were housed in Tecniplast XenoPlus Stand Alone system. This system consists of 12 tanks each designed to hold 17 litres of water and facilitate up to four frogs. The pH and conductivity of the tank water was regulated and monitored by the XenoPlus system. Approximately 10% of the tank water was discharged every day and was replaced with fresh water from rain water tank. The tank had various filters (biological, mechanical and carbon) along with UV disinfectant light to minimise microbial growth and thus ensure flow of good quality water. Each frog had a designated tank and individual frogs were distinguished from one another by their unique skin pattern which was photographed and archived.

2.1.2 Harvesting Oocytes:

Oocyte harvest was performed surgically. Because the surgical procedure requires former experience, the oocyte harvest was mainly performed by Dr. Jan-Peter Baldin and PhD student Sama Mugloo while I observed and learned the technique under their supervision. First, the frog was anesthetised by transferring it into a container with water and Tricaine methane sulfonate (1.3 g/L, pH 7) (Aldrich, Cat. No. E10521-50G) for 15 minutes. To confirm surgical level of anaesthesia, lack of response to painful stimuli was tested by pinching the rear foot of the frog with forceps. The animal was then transferred to a surgical bench and laid on its back on top of a paper towel that was pre-soaked in anesthetising solution. Wet paper towels were used to cover the thorax and lower limbs of the frog and only the abdomen was exposed for the surgery. Povidone-iodine prep pads (Dynarex, Cat No. 1108) were used to sterilise the surgical
sight. Using autoclaved scissors, a ~1cm vertical incision was made through the epidermal layer of the lateral abdomen, just above the groin. A second incision was made through the fascia and muscle layer to access the ovarian lobes. The ovarian lobes were externalised using blunt forceps to gain access to the oocytes. The lobes were cut and placed into a 50mL falcon tube filled with culture oocyte Ringers solution (CulOri) (see Table 5 in appendix for solution components) and the surgical incision on frog was closed in two steps with different needles. First, the incision through the muscle layer was closed by uninterrupted suture using monofilament (B. BRAUN, Cat No. 2021-02-09). Then, using a different suture (DemeTECH, Cat No. PGA184019F4P), the incision through the epidermal layer was closed in the similar manner. As part of recovery, post-surgery the frog was moved into a clean water tank and sporadically monitored for 24 hours before being transferred back to its designated tank.

The maximum number of oocyte harvests from each animal was four and all surgeries were recorded in the animal register. To reduce stress to the animal, each surgery was performed at least 3 months apart. For the fourth harvest, the frog was sacrificed via spinal puncture, and oocytes were then harvested.

2.1.3 Oocyte isolation

Extracted ovarian lobes were transferred into a 5 x 5 cm petri dish filled with culOri buffer. Using forceps, the oocytes were torn from the ovarian lobes. They were then enzymatically defolliculated at room temperature by 90 minutes incubation in collagenase enzyme (1.5mg/ml) (Serva, Cat. No. 17465.02) dissolved in culOri. This was followed by three washes in Ca²⁺ free oocyte Ringers (Ca²⁺ free Ori) to deactivate collagenase and in the last Ca²⁺ free Ori wash, oocytes were incubated on a rocker (Select BioProducts, platform rocker Rock-it) for 15 minutes. A final wash of oocytes with culOri was done. The healthiest stage V-VI oocytes were selected under the dissection microscope and placed in a conical 96 well plate with one oocyte
per well bathed in 200µl of culOri. The 96 well plates containing the oocytes were stored in the incubator at 17°C to be used in experiments later.

2.1.4 Endogenous connexin expression in *Xenopus* oocytes

Before expressing human Cx43 for testing miRNA transport, the expression of connexin coding genes in native *Xenopus* oocytes was quantified. *Xenopus* oocytes are known to express Cx38 (Ebihara, 1996) and thus expression of Cx38 gene was used as reference. RNA was extracted from oocytes and gene reverse-transcription – quantitative polymerase chain reaction (RT-qPCR) was then performed to quantify the expression of *Xenopus* Cx43 genes relative to *Xenopus* Cx38 genes. Human Cx43 gene was targeted as the negative control and 18S was used as the endogenous control for normalising RT-PCR results.

2.1.4.1 RNA extraction

All the procedures using TRIzol were carried out in the fume hood. In a 1.5ml microcentrifuge tube, three oocytes from the same frog alongside three 1.6mm stainless steel beads were added in total of 500 µl of TRIzol reagent. The oocytes were homogenised in bullet blender for 2 minutes at the speed of 8. The tubes were visually inspected for satisfactory homogenisation. Homogenised samples were then centrifuged at 15000g for 15 minutes in 4°C to pellet the debris at the bottom of the tubes. The resulting supernatant was transferred into a new 1.5 ml microcentrifuge tube. Complete dissociation of nucleoprotein complexes in the supernatant was ensured by incubating the samples at room temperature for 5 minutes.

Next, chloroform was added in 1:0.2 ratio of TRIzol : Chloroform and the sample was vortexed for 15 seconds. These samples were incubated at room temperature for 3 minutes and then centrifuged at 12,000g at 4°C for 15 minutes. The top colourless aqueous phase containing RNA was transferred into a new 1.5 ml microcentrifuge tube. To obtain high purity RNA from the samples, extra care was taken to avoid contact with the organic interphase material during aqueous phase collection.
Later, to form an RNA pellet, 100% isopropanol was added to the aqueous phase at a ratio of 1:0.5 of Trizol : Isopropanol. This was followed by a 10 minute incubation at room temperature and then centrifuge step of 12,000g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was washed in 75% ethanol at a 1:1 ratio of Trizol : ethanol. The samples were vortexed for 15 seconds and then centrifuged at 7500g for 5 minutes in 4°C. The supernatant was discarded, and the RNA pellet was air dried at room temperature for 10 minutes. The RNA pellet was resuspended in 40 µL of nuclease free water and incubated in heating block at 60°C for 15mins. Lastly, RNA concentration was measured using the nanodrop reader. Purity ratio of RNA was defined by 260/280 (i.e. RNA to protein) and 260/230 (RNA/phenol) and was recorded along with the RNA concentration (ng/µl) of the samples. RNA samples were then stored at -80°C or were immediately processed for reverse transcription.

2.1.4.2 Gene RT-PCR

Reverse Transcription

The RNA samples were reverse transcribed to form the complimentary DNA (cDNA) strand as cDNA is known to be chemically more stable than RNA. Reverse transcription (RT) was performed according to the supplier’s protocol (High Capacity cDNA Reverse Transcription Kit, appliedbiosystems by Thermo Fisher Scientific., Lithuana; Cat. No. 00568522). To be able to use 18S as the reference gene for normalisation in real-time PCR, random primers were used to generate a cDNA library. As random primers are capable of annealing at multiple points along the RNA script, they can therefore produce a large cDNA yield.

In thermal cycling tubes, 20 µl of RT master mix was prepared per RNA sample with 10X RT buffer, 25X deoxyribonucleotide triphosphate (dNTP) mix (100mM), 10X RT Random Primers and MultiScribe Reverse Transcriptase (PrimeScript™ RT Reagent Kit, Takara Bio., Japan). The volumes of individual RT master mix forming components are listed in Table 1. Then 2000
ng of RNA from each sample was added to the RT master mix. The total volume of RNA sample with RT master mix was made up to 40 µl for each sample with nuclease free H₂O. These reaction mixtures were placed in the thermocycler and run at 25°C for 10 minutes, then at 37°C for 120 minutes to generate cDNA, and lastly at 85°C for 5 minutes to inactivate reverse transcriptase enzyme via heat.

**Table 1. Per reaction volume for components forming RT master mix.** ‘X’ stands for ‘times’.

<table>
<thead>
<tr>
<th>component</th>
<th>Volume for 1 Reaction (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>25X dNTP Mix (100mM)</td>
<td>1.6</td>
</tr>
<tr>
<td>10X RT Random Primers</td>
<td>4.0</td>
</tr>
<tr>
<td>MultiScribe Reverse Transcriptase</td>
<td>2.0</td>
</tr>
<tr>
<td>Nucleases-free H₂O</td>
<td>8.4</td>
</tr>
<tr>
<td>Total volume</td>
<td>20</td>
</tr>
</tbody>
</table>

**Polymerase Chain Reaction**

Cycle threshold (Ministry of Health; National Minimum Data Set *et al.*) values obtained from qPCR were used as a quantitative measure of connexin genes expression in native oocytes. For each gene target, PCR master mix was made up of 5 µl of 2X Syber Premix Ex Taq, 0.2 µl 50X ROX dye, 3.4 µl RNAse free H₂O and 2 µl of each forward (10 µM), and reverse primers (10 µM) of the gene of interest (see appendix 4 for primer details). In a 96 well plate, 9 µl of PCR master mix was added with 1µl of cDNA template. Each sample for each gene was run in triplicate. The plate was sealed and to ensure mixture of all components, the plate was briefly spun down. The 96 well plate was then inserted in the StepOne Plus real time PCR machine.
and was run for 1 cycle of 30 seconds at 95°C to activate the enzyme, followed by 40 cycles of 5 seconds at 95°C and lastly, 1 cycle of 30 seconds at 60°C.

2.1.4 Expressing human Cx43 in Xenopus oocytes
The pTNT™ vector consisting of human Cx43 nucleotide sequence (GenScript, U2946C1120-3) was used. First, the pTNT™ vector was amplified to gain high yield of human Cx43 DNA. This was followed by in vitro transcription to synthesise cRNA from the amplified DNA and the cRNA was injected into Xenopus laevis oocytes using the robot ‘roboocyte’ to allow for human Cx43 expression.

2.1.4.1 The pTNT™ vector
The pTNT™ vector was chosen as the vector for human Cx43 sequence because it contains SP6 and T7 polymerase promoters which allows for efficient in vitro synthesis of RNA (figure 2.1). This vector also contains 5’ β-globin leader and synthetic poly(A)₃₀ tail and these have been reported to enhance protein expression (Wakiyama et al., 1997). Ampicillin resistance gene was present in the vector as a selection marker.
2.14.2 DNA amplification by bacterial transformation

To produce sufficient amount of plasmid with human Cx43 DNA for the reverse transcription to produce cRNA for *Xenopus* oocyte expression system, E. coli strain DH5α competent cells (Cat. No. 18265-017) were used as the transient host for subcloning the plasmid vector. The 100 µl of DH5α cells were mixed with 10 µl of DNA and incubated on ice for 30 minutes. This sample was heat shocked by 45 seconds incubation at 42°C for transforming the plasmid into the DH5α cells and then samples were put back on ice for 2 minutes. The 950 µl of lysogeny broth (LB) was then added to the sample and it was subsequently incubated on shaker at 220g for 1 hour at 37°C. Following incubation, the samples were centrifuged at 10,000g for 1 minute and the resulting supernatant was discarded. Cells were resuspended in 300 µl of LB medium, of which 100 µl was plated on agar plate containing 100 µg/ml ampicillin (Applichem, Cat. No. A0839,0025) to selectively grow the plasmid transfected DH5α cells. The agar plates were incubated for 16 hours at 37°C and a single colony was selected the next day to be transferred
into 500 ml LB medium with 75 µg/ml of ampicillin. This was incubated on the shaker at 220rpm for 16 hours at 37°C and then the steps of plasmid isolation from the bacteria were carried out.

2.1.4.3 Plasmid isolation
The NucleoBand® Xtra plasmid purification kit (Clonetech, Cat. No. 740410.10) was used and the plasmid DNA purification was carried out using the manufacturer’s ‘midipreparation’ protocol. In brief, 100 ml of bacterial culture was harvested from the previously made 500 ml stock in LB medium, by 15 minutes centrifugation at 5000g at 4°C. Collected cells were resuspended in 8 ml of RES (resuspension) buffer and were then lysed by subsequent application of 8 ml of LYS (lysis) buffer. Cell lysis was then neutralised by adding 12 ml of NEU (neutralisation) buffer to the reaction. The lysate was then loaded on to the column filters provided by the manufacturer and the flow-through (driven by gravity) was discarded. Filters were washed using 5 ml of Buffer EQU and discarded. The leftover column was washed with 8 ml of Buffer WASH. Plasmid DNA was then eluted with the application of 5 ml of Buffer ELU. Eluted plasmid DNA was purified by precipitation. The 3.5 ml of 100% isopropanol was added to the elute plasmid DNA, vortexed and centrifuged at 15,000g for 30 minutes at 4°C and the supernatant was discarded. The DNA pellet obtained was washed with 70% ethanol (2 ml) and centrifuged again at 5000g for 5 min at room temperature. Ethanol was removed, and the pellet was air-dried for ~5 minutes. Lastly, the pellet was dissolved in RNase free water and stored at -20°C.

2.1.4.4 Plasmid DNA linearisation
To be able to convert plasmid DNA into cRNA, the pTNT™ vector consisting of Cx43 DNA had to be linearised. As the vector contained BamHI cleaving site, the corresponding restriction enzyme (Roche, Ca. no. 10567604001) was used. The 1 µg of DNA was mixed with 1 µl of
restriction enzyme (BamHI) and 10x reaction buffer, and was incubated for 90 minutes at 37°C. The enzymatic reaction was inactivated by additional incubation at 65°C for 15 minutes.

### 2.1.4.5 In vitro transcription

In order to transcribe DNA into cRNA, SP6 mMessage mMachine Kit (Ambion, Cat. No. AM1340) was used to perform in-vitro transcription. The procedure was carried out according to the supplier’s manual. Components of this reaction consisted of 2x NTP/CAP, 10x reaction buffer and enzyme mix SP6 and were mixed in the ratio of 1 : 0.2 : 2 respectively. To this, 1 μg of linear DNA template was added, and the total volume of reaction was brought up to 20 μl with nuclease-free water. The reaction was incubated for 2 hours at 37°C. TURBO DNase (1 μl) was then added to the reaction and this mixture was incubated at 37°C for 15 minutes.

MEGAclear™ Kit (Ambion, Cat. No. AM1908) was used in accordance to the supplier’s manual to purify RNA. First, 350 μl of Binding Solution was added to the 100 μl of cRNA, followed by addition of 250μl of 100% ethanol. The reaction was loaded onto column filters and centrifuged for 1 minute at 10,000g. Flow-through was discarded and 500 μl of Wash Solution was added. The centrifuge step of 1 minute at 10,000g was repeated. The filter was then transferred into a new Eppendorf tube and 50 μl of pre-heated (95 °C) Elution Solution was then added to the filter and centrifuged again at 10,000g for 1 minute to collect cRNA. The yield (ng/μl) and 260/280 purity ratio of cRNA was measured using nanodrop reader. Finally, the cRNA sample was stored at -80°C.

### 2.1.4.6 cRNA microinjections into oocytes:

The human Cx43 cRNA microinjection into oocytes was done using the roboinject robot (Multi Channel Systems, Germany). The 96 well plate containing oocytes sorted previously was used. In the 96 well plate, each oocyte was placed in an individual well filled with 200 μl of culOri and the plate was placed on the robot platform. A injection pipette (IN-25, Multi Channel
Systems, Germany) was filled with mineral oil to prevent RNA dilution was aligned to pierce the centre of the oocyte. The robot injector was set at the fluid ejection speed of 30nl/second. To express human Cx43, the oocytes were injected with 15 ng of the purified cRNA. These were labelled as Cx43 oocytes. For the control group, equivalent amount of nuclease-free water was injected into the native oocytes. The oocytes were stored in an incubator at 18°C for 24 hours to allow protein expression.

2.1.4.7 Solution suitability test
As a precautionary step to prevent oocyte death in the electrophysiology experiment from oocytes failing to survive in the types of solutions used, the survival of oocytes was tested in Cx43 activating Ca$^{2+}$ free Ori solution and Cx43 blocking lanthanum (La$^{3+}$) solutions. La$^{3+}$ is toxic to the cells, therefore to determine the La$^{3+}$ concentration for optimal oocyte survival, oocytes were incubated with 50 μM, 100 μM, 500 μM and 1000 μM of La$^{3+}$ prepared in Ca$^{2+}$ free Ori. Oocytes bathed in culOri were used as positive control as Xenopus laevis oocytes sustain their structure and function in culOri for at least ~5 days. Oocytes bathed in simply dH$_2$O (distilled water) were used as the negative control. To ensure, difference in survival of oocyte across solution is not affected by varying osmolarity between solutions, the osmolarity of all solution was adjusted to 193 mOsmols/l by the addition of mannitol and detailed components of each solutions are listed in appendix 1, table 5. Oocytes were randomly assigned to the solution groups in a 96 well plate where each well consisted of one oocyte with 200 μl of the assigned solution. Survival was determined by counting the number of dead oocytes under a light microscope for each solution at zero hour, 24hour, 48hour, 72hour and 96hour time points. Only oocytes like the one displayed in image A of figure 2.2 were considered to be surviving whereas oocytes from image B onwards were considered to be dead oocytes.
Figure 2.2 Pictures of Xenopus laevis oocytes. Image A shows a healthy oocyte whereas image B, C and D are dead oocytes. Red circles represent leakage of cytoplasm due to deterioration of the cell membrane. These images were used as a reference to document viability of cells in different solutions. All images are taken from 20x lens under a dissection light microscope.

2.1.5 Connexin 43 expression analysis
The functionality of Cx43 in WT oocytes and in native oocytes (control) was tested using current recordings measured using the two-electrode voltage clamp (TEVC) technique. TEVC is a well established technique used in characterising properties of the exogenously expressed proteins in *Xenopus laevis* oocytes (Wagner et al., 2000; Guan et al., 2013). Ca$^{2+}$ free Ori was used to activate connexin specific current across the oocyte and culOri was used to deactivate the current. Additionally, the effect of connexin blocker Lanthanum (La$^{3+}$) was also tested to confirm the presence of Cx43 mediated current. These solutions were introduced to oocytes in the flow chamber through a pressure regulated perfusion system.

2.1.5.1 TEVC systems set-up
Initially, the flow chamber was filled with culORi and with the help of Pasteur pipette, oocyte was positioned on the concentric circular dent of the chamber (Figure 2.3). To set up flow, solutions in channels 1 to 3 of the perfusion reservoir were filled as follow: culOri, Ca$^{2+}$ free, and 50 µM La$^{3+}$ dissolved in Ca$^{2+}$ free solution. Pressure in the system was adjusted to 3.5 psi to mediate flow through the reservoir and flow of each solution into the recording chamber was controlled by switching on and off button for their respective channel.
The system was then set up to measure whole cell current. A voltage electrode to measure membrane potential and a current electrode were constructed with glass capillary tubes (Hilgenberg, Cat. No. 1103237). These were filled with 18 µl of 3 mM potassium chloride (KCl) for conductance and bleach coated silver wires were inserted to form microelectrodes. The TURBO TEC-05 TEVC amplifier (NPI, Tamm, Germany) was used to record information from both electrodes. Only the electrodes with resistance between 2-4Ω were considered suitable for the experiment. After submerging the electrodes in culOri, the amplifier was used to adjust the offsets for both current and voltage electrodes. Using a micromanipulator, electrodes were then gently advanced through the oocyte membrane into the cell.

The reference electrodes, which were also made of bleached silver wires, were placed in the recording chamber at throughout the experiment. The oocyte was voltage clamped at a membrane potential of -60mV and transmembrane current recorded by amplifier was displayed via PowerLab 4/35 (ADinstruments, Dunedin, New Zealand) on a computer running LabChart acquisition software.
2.1.5.2 TEVC recording Protocol
The whole cell currents for both Cx43 expressing and control oocytes were recorded using the same protocol. First, the oocyte activity was measured without any solution flow for 30 seconds. Then flow of culOri was turned on for 1 minute followed by flow of Ca\(^{2+}\) free Ori for 3 minutes. The oocyte was then washed with culOri for 2 minute and 50 µM La\(^{3+}\) solution was then introduced for 3 minutes. Lastly, the current across the oocyte was brought to baseline with culOri and the recording was stopped.

2.2 AC16 Cardiomyocytes
The AC16 cell line is a commercial cell line derived from adult human ventricular cardiomyocytes fused with fibroblasts to sustain their growth in an in vitro setting. Use of AC16 cell line is becoming common for characterising molecular changes that occur during pathophysiological events in the cardiac cells (Palomer et al., 2008; Palomer et al., 2015; Ram et al., 2017). (Davidson et al., 2005) has shown that AC16 cells retain the nuclear and mitochondrial DNA of the primary cardiomyocytes. As an inherent trait of ventricular
cardiomyocytes, AC16 cells endogenously express Cx43 in their plasma membrane. The gap junctions between these cells have been shown previously shown to be functional in the in vitro setting (Davidson et al., 2005). Hence, AC16 cells were used in this project to test the concept of Cx43 mediated miRNA transport in cardiomyocytes.

2.2.1 Culturing of AC16 cells
AC16 cells for the treatment protocol, were first grown in T75 flasks to ~80% confluency. Advanced Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12, Thermo Fisher Scientific, US) with Fetal Bovine Serum (FBS, at 10%) and antibiotic antimitotic (A/A, at 1%) was added to make up an aliquot of complete DMEM/F-12. The complete DMEM/F-12 media was warmed to 37°C and 8 ml was added to the culture, which was enough to cover the surface of one T75 flask. The flasks were then transferred to the incubator set at 37°C, 5% CO₂ and 95% relative humidity. Cell media was changed every 24 hour and the cells were grown to the confluency of ~80%. The confluency percent of the cells were determined by inspection under a light microscope.

2.2.2 Cell maintenance
Upon reaching ~80% confluency, the cells were passaged in a 1:3 ratio. The growth media, complete DMEM/F-12, was aspirated out and the cells were washed with phosphate buffer saline (PBS) solution at 37°C to remove debris. The trypsinisation agent, TrypLE (2.5 ml) was added to the T75 flask and incubated for 30-50 seconds at room temperature to allow disassociation of the cells from the surface of the flask. Then 8 ml of complete DMEM/F-12 media was added to saturate the trypsin and the cell suspension was collected in a 15 ml falcon tube. To recover cells in the form of a pellet, the cell suspension was centrifuged at 3000g for 5 minutes. The resulting supernatant was aspirated out and cell pellet was resuspended in 3 ml of fresh complete DMEM/F-12 media. Cell counting was then done using haemocytometer (Life technologies, USA). Here, 100 µl of the cell suspension was used to create a 1:5 dilution
and the diluted sample was combined with Trypan Blue dye in a 1:1 ratio. The 10 µl of the trypan blue dyed cells was placed on haemocytometer slide and was used to attain proportion of the live cells to dead cells. The live cell count was used to calculate the volume of cells required to seed a specific cell density.

2.2.3 Experiment design

To test if Cx43 mediates transport of miRNAs, transport of miRNA-1 was specifically tested as it is the predominantly expressed miRNA in the heart and it is highly involved in regulating pathophysiological events in the cardiomyocytes. The presence of U6 and miRNA-16 was also detected as controls. Thus cells were first transfected to overexpress miRNA-1. To test the transport, treatment media of the cells was collected for RT-PCR. This allowed detection of the difference in transport of miRNA between the cells in La³⁺ treated (blocking connexin transport) and control (non La³⁺ treated) medium. Hence, AC16 cells were divided into two groups, miRNA-1 transfected and scrambled transfected (transfection control) and both of these groups contained two sub groups, control and 2mM La³⁺ treatment group (Figure 2.4).
2.2.4 Transfections

Cells at 15th passage were seeded at a density of 800,000 cells/well into a 6-well plate and were grown in complete DMEM/F-12 media in the incubator (37°C, 5% CO₂) for 24 hours. They were either transfected with 25 pmol of mirVana™ miRNA-1 mimic (ThermoFisher, US) or 25 pmol of LNA-Scramble (ThermoFisher, US) depending on the assigned cell group.

Figure 2.4 Experiment design for testing Cx43 mediated miRNA transport in AC16 cardiomyocytes. AC-16 cardiomyocyte image was taken with 20X lens under light microscope.
Lipofectamine™ RNAiMAX (ThermoFisher, US) was used as the transfection agent. First, 9 µl of Lipofectamine RNAiMAX reagent was diluted in 150 µl of serum free DMEM/F-12 media in a 0.65 ml Eppendorf tube. Next, 3 µl of miRNA-1 (10 µM) was diluted in 150 µl of serum free DMEM/F-12 media in another 0.65 ml Eppendorf tube. Identical protocol was carried out for scramble sequence dilution. The diluted Lipofectamine RNAiMAX reagent was then mixed with diluted miRNA-1 or scrambled sequence in 1:1 ratio and the mixtures were incubated for 5 minutes at room temperature. Then 250 µl of the mixed Lipofectamine RNAiMAX and miRNA-1, or Lipofectamine RNAiMAX and scramble mix was added to each of the wells of a 6-well plate. The plates were incubated for 28 hours at 37°C, 5% CO₂.

2.2.5 Lanthanum treatment Protocol
For introducing treatment, SILAC media (ThermoFisher, US) was used to restrict further cell growth during the treatment phase. For this, the transfecting medium was aspirated out and cells in each well were washed with PBS (600 µl/well) twice. For control group, SILAC (ThermoFisher, US) with FBS and A/A was added as 800 µl/well whereas for Lanthanum treatment group, 2mM La³⁺ was also added to the SILAC (A complete supplementation recipe is listed in Table 2). The cells were incubated for 24 hours at 37°C, 5% CO₂ after which media for each cell group was collected for RNA extraction and cells were collected for protein extraction to measure Cx43 expression.
Table 2. Volume of components forming SILAC used in cell treatment

<table>
<thead>
<tr>
<th>Components</th>
<th>SILAC for control treatment (µl)</th>
<th>SILAC with La³⁺ (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>A/A</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>La³⁺</td>
<td>5 µl in RNase free water</td>
<td>2 mM in 5µl RNase free water</td>
</tr>
<tr>
<td>SILAC</td>
<td>4145</td>
<td>4145</td>
</tr>
<tr>
<td>Total</td>
<td>5000</td>
<td>5000</td>
</tr>
</tbody>
</table>

2.2.6 RNA extraction from cell media

The filter column method was used to extract RNA from the media. First, quizol was added to the media (1 : 5; media : quizol) and the samples were briefly vortexed and incubated at room temperature for 5 minutes. Chloroform was then added to the sample (1:1; media : Chloroform). The samples were vortexed again and incubated for 3 minutes at room temperature before centrifuging at 12,000g at 4°C for 15 minutes. The resulting aqueous phase which contains RNA, was transferred to a new Eppendorf tube and 100% ethanol was added to precipitate RNA in the solution. The precipitated solution was immediately transferred into the column filter and centrifuged at 8000g for 15 seconds at room temperature. The flow-through was discarded and each column filter was washed with 700 µl of RWT buffer by 15 seconds centrifugation at 8000g in room temperature. This was followed by two times wash with 500 µl RPE buffer with same centrifugation as RWT. The columns were then transferred into a new tube and dried, again by 8000g centrifugation for 1 minute. Finally, the RNA was recovered by adding 40 µl of RNAase free water to the columns. The columns were incubated at room temperature for 2 minutes and then centrifuged at 8000g for 1 minute at room temperature. The purity of the obtained RNA was measured as described in section 2.1.4.1 and the RNA was stored at -80°C.
2.2.7 microRNA Reverse Transcription

The RNA samples obtained from cell media were transcribed to cDNA as in gene RT-PCR, because the stable structure of cDNA allows for more specific targeting of miRNAs. The RNA samples were thawed on ice and their concentrations were measured. The samples were then diluted in RNase-free water to obtain 20 ng in 5 µl. The RT master mix was then prepared as per TaqMan ® RT kit protocol (components listed in table 3) and mixed with the primers of miRNAs of interest (miRNA-1, miRNA-16 and miRNA-U6) in 70 : 30 ratio of RT master mix : primer. In the interconnected thermocycler tubes, 10 µl of the combined RT master mix and primer was added to 5 µL of the 20 ng RNA. The tubes were briefly spun down in the minicentrifuge to ensure mixing of all components and run in the thermocycler. The thermocycler run consisted of the following four steps: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C on an infinite hold. cDNA samples were stored in -20°C freezer or immediately used in PCR.

Table 3 Volume of RT master mix components required for 1 reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>1 reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM dNTPS</td>
<td>0.15</td>
</tr>
<tr>
<td>Multiscribe Reverse Transcriptase</td>
<td>1</td>
</tr>
<tr>
<td>10x Reverse Transcription Buffer</td>
<td>1.5</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.19</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>4.16</td>
</tr>
<tr>
<td>Total volume</td>
<td>7</td>
</tr>
</tbody>
</table>

2.2.8 miRNA PCR:

A PCR master mix was made of Takara PCR Premix, ROX dye and RNAse free water with specific volume per reaction in table 4. For each sample, in a 96-well plate PCR reaction is carried out in triplicate to increase the validity of the results. The plate was sealed and briefly
spun down to ensure mixing of all components. StepOne Plus real time PCR machine was used to run the samples. The run consisted of three different steps: step one is 30 seconds at 95°C, step two is 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C and step three is an infinite hold at 4°C.

Table 4. Volume of master mix component for PCR on miRNA samples

<table>
<thead>
<tr>
<th>Components</th>
<th>For 1 reaction (µL)</th>
<th>For triplicate reaction (+20% extra) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR premix (Taqman or SYBR)</td>
<td>6.3</td>
<td>22.5</td>
</tr>
<tr>
<td>20X TM primer</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>RT product of each sample</td>
<td>1.0</td>
<td>3.6</td>
</tr>
<tr>
<td>RNAse free water</td>
<td>4.8</td>
<td>17.1</td>
</tr>
<tr>
<td>ROX dye (50X dilution)</td>
<td>0.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Total</td>
<td>13.4</td>
<td>48.3</td>
</tr>
</tbody>
</table>

2.3 Cx43 protein western blot

To test if Cx43 proteins were adequately expressed in WT oocytes and AC-16 cells, western blot was performed. Protein was first extracted from the samples, and protein concentration for each sample was determined using Bradford assay. For negative control, protein extracted from native oocytes was used. A western blot was performed and observed band size for each sample was analysed. The samples were also stained for GAPDH which was used as the loading control.

2.3.1 Protein extraction:

Oocytes:

The oocytes were collected after electrophysiology for western blot. The protein extraction process was performed on ice. The oocytes were homogenised in lysis buffer (Appendix 2, Table 7), used as 10µl/per oocyte. The homogenate was then centrifuged for 10 minutes at
10,000g at 4°C and this resulted in formation of the three following phases, debris pellet at the bottom, protein containing colourless-liquid phase in the middle and lipids located at the top. The colourless liquid layer was extracted and to increase purity of the extracted protein, centrifugation step was repeated. The protein lysate was subsequently frozen at -80°C.

AC16 cells:
To lyse AC16 cells, complete radioimmunoprecipitation assay buffer (RIPA buffer) was used. Protease inhibitor (25X) was added just before use. Complete RIPA was added to the cells in quantity of 150 µl per well of a 6-well plate. The cells were then transferred on ice and were repeatedly passaged through a 24 mm gauge needle to further lyse the cells. Lysed cells were incubated on ice for 20 minutes. They were then transferred into a 1.5ml Eppendorf tubes and were centrifuged at 16,000g at 4°C for 20 minutes. The colourless supernatant, which contains protein was collected and stored and at -80°C.

2.3.2 Bradford assay
The Quick Start™ Bradford protein assay (Bio-Rad, US) was used and the supplier’s protocol for a 96-well plate Bradford assay was followed. In brief, a 200 µg/µl bovine serum albumin (BSA) protein sample was aliquoted to prepare a standard curve for determining sample protein concentration. The standard was developed by step-wise protein dilution of BSA as outlined in the BioRad Microplate Standard Assay (Bio-Rad Laboratories Inc. protocol). Samples were prepared in 1 : 1000 dilution ratio of sample protein : Bradford reagent and vortexed to allow binding of Coomassie Blue Dye present in the reagent with the proteins. For each sample, 100 µl was pipetted in triplicate in a 96-well plate and the absorbance of samples and for standard was read in Multimode Synergy Reader (BioTek ®) at 595 nm wavelength. A standard curve was plotted with the optical density plotted (on Y-Axis) for each standard concentration (X-Axis). The protein concentration of the each sample was calculated from the equation derived from standard curve.
E.g. \( Y = 0.028x + 0.010 \)

Rearranged to \( x = (Y-0.010)/0.028 \)

[ \( Y = \) optical density; \( x = \) concentration]\]

2.3.3 Sample preparation
After determining the protein concentration, the samples were diluted with \( \text{dH}_2\text{O} \) and appropriate amount of loading buffer to the desired concentration and were then denatured by incubation at 95 °C for 4 minutes. The samples were then transferred on ice to be used in the gel electrophoresis stage.

2.3.4 Gel electrophoresis
A 10% resolving gel and 4% stacking gel was used to run the samples (components forming both gels are listed in Appendix 3, Table 9). The electrophoresis tank was set together, and the chambers were filled with SDS running buffer. The ladder was added to the first well (4 µl) to estimate the size of the protein. Samples were then loaded in other wells, initially 20 µg of sample was used but this was optimised to 40 µg loading of samples to obtain easily distinguishable protein bands later in the western blot. The gel was run at 80V for 30 minutes to allow for proteins to run through the stacking gel and then the voltage was increased to 150V and the gel was run for another 1 to 2 hours - until the protein samples reached the bottom of the gel. The SDS, being an anionic detergent, denatures protein resulting in the negative charge of the protein being proportional to the protein length. Hence, the protein movement through the gel allows for size based separation. A band was expected to be detected at \( \sim 43\text{kDa} \) for Cx43 and \( \sim 41\text{kDa} \) for GAPDH protein.
2.3.5 Gel transfer
The proteins from the gel were transferred on the PVDF membrane by wet transfer electrophoretic elution method. The transfer sandwich was prepared with two sponges and four filter paper pre-soaked in transfer buffer. The black side of the plastic cassette was placed at the bottom, first a sponge and then two filter paper were stacked on which the gel was placed. Then the PVDF membrane activated in methanol for 15 seconds and then briefly immersed in transfer buffer, before being placed on top of the gel. The second set of two filter paper and then sponge were stacked on top of the PVDF membrane and transfer sandwich was closed to be loaded into the transfer box. The transfer was set at a constant voltage of 110V and run for one hour 90 minutes.

2.3.6 Staining
The PVDF membrane was washed three times (5 minute each) in TBS. Membrane was then incubated for 90 minutes at room temperature in blocking buffer consisting of 5% Bovine Serum Albumin dissolved in tris buffered saline. This was done to conceal non-specific binding sites on the membrane. The TBS washes were repeated and the membrane was then incubated in cold room for 16 hours in primary antibody, rabbit anti-Cx43 (abcam, USA; Cat. No. ab63851) diluted at 1:1000 ratio in 0.1% BSA. Following this, the membrane was washed four times in TTBS buffer to remove any protein unattached to the primary antibody. The secondary antibody, goat anti-rabbit IgG HRP was then added to the membrane and incubated at room temperature for 2 hours. The membrane was once again washed three times for 5 minutes in TTBS. ECl (enhanced chemiluminescence) substrate (ThermoFisher, US) was added to the membrane in 1:1 ratio and incubated for 5 minutes, following which the membrane was placed between plastic sheet hyperfilm cassette to be imaged by Chemiluminescence PXi Touch imaging system (Syngene, UK).
As the band sizes for Cx43 and GAPDH are similar, the membrane was stripped following Cx43 imaging to proceed towards GAPDH staining. Membrane was first incubated in mild stripping buffer (Appendix 3, Table 8) for 2 hours to strip the Cx43 antibodies and then imaged again to see if the stripping was successful (lack of visible bands). If not, the membrane was incubated in stripping buffer again for another 30 minutes and then imaged. To stain for GAPDH, the steps from membrane blocking were carried out again and rabbit anti-GAPDH antibody (GeneTex US, Cat.No. GT239) was used.

2.4 Data analysis

PCR results
The C_T values represent the number of cycles required for fluorescent signal to reach threshold. A high C_T value represents a lower miRNA or gene expression. The triplicate C_T values for each sample are averaged. Further, to account for variability between groups, C_T values for each sample were normalised to the C_T values obtained for the endogenous control in the same sample i.e. U6 values for miRNA-PCR and 18S values for gene-PCR. The difference in C_T value (DC_T) were calculated using 2^DCT equation. To represent the increase in DC_T as increase in miRNA expression, the equation was inverted and values were graphed as 2^-DCT.

Protein analysis
Protein in each sample visualised on PVDF membrane, was quantified using Image J. Each individual band that represented the protein of interest was selected using a constant area for the region of interest (ROI). The mean grey value within the ROI was measured for Cx43 expression and these values were normalised to the mean grey value of loading control, GAPDH, of the same sample. This was done for western blot data for proteins detected for WT oocytes, native oocytes and AC-16 cells. An unpaired t-test was used to determine if significant difference in Cx43 expression between two groups of oocytes was present.
**Statistical analysis**

Statistical analysis for results of all experiment was performed in the GraphPad Prism 7.0 Software (GraphPad Software Inc, US). For experiments, where the total number of treatments was three, ANOVA was done. For the experiments with total number of treatment group was less than three, t-test was performed. A p-value <0.05 was considered statistically significant for all analyses.
3. RESULTS

The aim of this project was to elucidate a novel role of human Cx43 in the intercellular transport of miRNA. The objectives were first, using oocytes, to find whether Cx43 mediated transport occurs in the absence of vesicular transport pathways that oocytes do not possess, and then to investigate the possibility of miRNA transport through Cx43 channels in adult human cardiomyocytes.

In the following pages, first presented are data useful for constructing a suitable single cell model of *Xenopus laevis* oocytes in which miRNA transport via human Cx43 could be tested without hindrance from vesicular transport pathways that are seen in cardiomyocytes. Quantitative data for endogenous connexin expression in *Xenopus laevis* oocytes and set up in which transport via exogenously expressed human Cx43 in *Xenopus laevis* oocytes can be controlled.

In the later pages of this chapter, the results for miRNA transport in cardiomyocytes are presented. Here, the effect of La³⁺ on miRNA transport from AC16 cardiomyocytes are shown.
3.1 Xenopus laevis oocytes:

3.1.1 Endogenous Xenopus connexin expression
Quantitative RT-PCR was performed on RNA extracted from the native Xenopus oocytes to measure the expression of endogenous Cx43 (Xenopus Cx43). Three separate experiments on oocytes, each from a different frog, were performed. In each experiment, oocyte RNA used in RT-PCR was pooled from 3 oocytes of the same Xenopus laevis frog. As previously mentioned, Cx38 gene is well expressed in Xenopus laevis oocytes, and its expression was quantified for comparison with Xenopus Cx43 expression. The gene RT-PCR results show that, compared with the 0.6728 (±0.359) mean for the expression of Xenopus Cx38 (n=9, N=3) gene, the mean of the Xenopus Cx43 gene expression of 0.00029 (±0.00049; n=9, N=3) was significantly lower in the oocytes, (p < 0.05) (Figure 3.1). The expression of human Cx43 (n=3) gene was quantified as the negative control for this experiment and as expected, its expression was not detected (Figure 3.1).

![Graph](image)

**Figure 3.1 Expression of endogenous connexin genes in native Xenopus laevis oocytes.**
RT-PCR analysis data with CT values normalised to 18S control gene and plotted as quantitative measure of connexin gene expression (n= 9, N=3 for all connexin genes). Data are presented as mean ±SEM. *p < 0.05, one-way ANOVA with Tukey’s post hoc.
3.1.2 Assessment of lanthanum toxicity

To test the functional properties of Cx43 mediated transport in oocytes, it was first necessary to optimise the connexin activating and blocking solutions to ensure adequate oocyte survival. The oocytes were bathed in Ca\(^{2+}\) free solution and La\(^{3+}\) solutions and their survival was quantified by counting the number of living oocytes over time under the dissecting microscope. The images in figure 2.2 were used as the guideline for identifying dead oocytes from the cell survival count. The commonly used *Xenopus laevis* oocyte culturing solution, culOri was used as the positive control and oocytes incubated in dH\(_2\)O was used as the negative control.

Over the first 48 hours, 100% of the oocytes survived in culOri solution (Figure 3.2) but by 72 hours the oocyte survival had decreased to 64% in the culOri solution (Figure 3.2). However, at each time point, the proportion of oocyte surviving is highest for oocytes incubated in culOri. As expected, the oocytes incubated in dH\(_2\)O, that served as the negative control, were least viable, dying instantly – data not shown in graph.

To activate connexins, CaCl\(_2\) was eliminated from culOri and this modified solution is labelled as Ca\(^{2+}\) free solution. There is no significant difference in percentage of survival for oocytes incubated in culORi and in Ca\(^{2+}\) free solution, from the start (0 hour) to 72 hours (p > 0.9). At the 96 hour time point, the percentage of oocyte surviving in Ca\(^{2+}\) free solution decreased significantly to 49% compared to 62% for the oocytes bathed in culOri (p < 0.05).

To identify a tolerable La\(^{3+}\) dose for oocytes in which cell lysis is minimal, oocyte survival was tested at 50µM, 100µM, 500µM and 1000µM La\(^{3+}\). An inverse relationship was observed between oocyte survival percentage and La\(^{3+}\) concentration where oocyte survival is decreased with increases in La\(^{3+}\) concentration (Figure 3.2). When proportion of oocytes survival between oocytes incubated in varying La\(^{3+}\) concentration and culORi was compared, no significant decreases in survival was seen for oocytes bathed in 50µM La\(^{3+}\) between 0 to 72 hours (p > 0.9). The significant decrease however was noted for higher La\(^{3+}\) concentration of 100µM,
500µM and 1000µM La$^{3+}$ between 24 to 72 hour time frame (p < 0.0001). Given the lack of significant difference between percentage of oocytes surviving in 50µM La$^{3+}$ and culOri for 0 to 72 hours, the 50µM La$^{3+}$ concentration seemed to be a more tolerable La$^{3+}$ dose for oocytes. Thus, 50µM La$^{3+}$ concentration solution was chosen for blocking connexin mediated current in *Xenopus laevis* oocytes, so the cells are viable throughout the experiment.

**Figure 3.2 Survival of Xenopus laevis oocytes in connexin activating and blocking solutions.** All La$^{3+}$ concentrations were prepared in Ca$^{2+}$ free solution. n= total number of oocytes, N=total number of frogs oocytes were extracted from. Data presented as percentage mean ±SEM. Two-way ANOVA with Tuckey’s post hoc performed, *p < 0.05, ****p < 0.0001 represent significant difference in survival of oocytes in culOri versus 100µM, 500µM and 1000µM La$^{3+}$ concentrations, #p < 0.05 represent significant difference in survival of oocytes in culOri versus Ca$^{2+}$ free and 50µM La$^{3+}$ concentration.
3.1.3 TEVC current recordings

The two electrode voltage clamp (TEVC) system allows the oocyte to be clamped at a selected voltage to adjust the membrane potential and enables recording of the membrane current which reflects ion channel activity at that voltage. Here we used TEVC to determine if human Cx43 that were expressed in oocytes via exogenous mRNA expression, are functional. The whole cell current for human Cx43 cRNA injected and control (RNase free water injected) *Xenopus* laevis oocytes was measured.

The oocytes were voltage clamped at -60mV. To see if connexin expressed is functional, a connexin specific current was induced by introduction of Ca$^{2+}$ free solution for 3 minutes. In both oocyte groups this was followed by 2 minute long washing with simply oocyte Ringer’s solution containing Ca$^{2+}$ (Appendix 1, Table 6). The change in current induced by Ca$^{2+}$ free solution was recorded. The specificity of this current to functioning connexin channels was then confirmed by inhibiting the current via the application of 50µM La$^{3+}$ prepared in Ca$^{2+}$ free solution to ensure that, the inhibition of current was an effect of La$^{3+}$ and not due to Ca$^{2+}$ in the solution.

The representative trace for TEVC recording in figure 3.3A for human Cx43 cRNA injected and control oocytes, both show that Ca$^{2+}$ free solution induced a current which was fully diminished on the application of culOri. Furthermore, the application of 50µM La$^{3+}$ inhibited the Ca$^{2+}$ free induced current in both groups of oocyte, though it did not do so completely.

The change in current observed on the application of each solution illustrated in figure 3.3A is quantified in figure 3.3B. Although there was a higher mean change of Ca$^{2+}$ free induced current in Cx43 cRNA injected oocytes (1.1±0.317µA; n=6, N=3) in comparison to the control oocytes (0.764±0.011; n=11, N=3), this difference was not significant in a one-way ANOVA analysis (p > 0.9). On the application of La$^{3+}$, there is 50% decrease in current induced by Ca$^{2+}$ free in both groups of oocyte (p <0.05).
Figure 3.3 TEVC current recordings of *Xenopus laevis* oocyte. A) Representative trace of current recorded in single Cx43 cRNA injected and a control (RNase free water injected) oocyte. B) Measurement of change in current induced by Ca²⁺ free solution and subsequent inhibition by 50µM La³⁺ for Cx43 cRNA injected and control oocytes. *p < 0.05, **p<0.01, one-way ANOVA. Data are presented as mean ±SEM. (n=5 to 11).

### 3.1.4 Expression of Human Cx43 in *Xenopus laevis* oocytes

The failure to demonstrate a clear difference in current between the human Cx43– expressing oocytes and the controls raises the possibility that the oocytes were failing to express the human Cx43 channels. To test this, the expression of the human Cx43 protein was measured. Western
blot analysis was performed on protein extracted from human Cx43 cRNA injected and control oocytes (RNase free water injected) to detect human Cx43 expression. Because AC16 cells endogenously express Cx43 protein, protein extracted from these cells was used as the positive control. For negative control, 20µl of blank (RNAse free water + loading dye) was loaded.

A 43kDa band was seen for AC16 cells and for all samples of both human Cx43 cRNA injected (n=5) and control oocytes (n=5) (Figure 3.4 A). As expected, no bands were seen for the negative control.

The human Cx43 expression for each sample was quantified and normalised to GAPDH as described in Chapter 2, section 2.4, protein analysis. The normalised Cx43 value presented in figure 3.4 B, show significantly higher expression of Cx43 protein in human Cx43 cRNA injected group (0.923±0.095) in comparison to the control group (0.630±0.0387) (p <0.05).
Figure 3.4 Oocyte western blots to detect Cx43 expression. A) Each well was loaded with 40 µg protein extracted from pool of 10 oocytes that belong to the same group. Wells 1 and 2 represent positive control (Cx43 from AC16 cells). 3, 4, 5, 6 and 7 are protein samples of Cx43 cRNA injected oocytes. Wells 8, 9, 10, 11 and 12 protein samples extracted from control (RNase free water injected oocytes). Well 13 is negative control loaded with blank (20µl RNase free water and SDS combined). GAPDH was the loading control. B) Quantification of Cx43 protein expressed in Cx43 injected (n=5) and control oocytes (n=5), plotted as arbitrary unit (A.U) after normalised to GAPDH expression. Each data point represents a mean of three technical repeats. *p < 0.05, non-parametric two-tailed unpaired t-test. Mean for the overall group is represented as bar graph where the error bars represent SEM.
3.2 miRNA transport in cardiomyocytes (AC-16 cells)

3.2.1 Detection of miRNA via RT-PCR results

The hypothesis that miRNA transport occurs via Cx43 in AC16 cells was tested by looking at the transport of miRNA-1 from the lanthanum treated and non-lanthanum treated cells into the cell media (Figure 2.4). To block connexin transport, 2mM La^{3+} concentration was used as it has been shown to block 90% of Cx43 channel activity in rabbit ventricular myocytes (Kondo et al., 2000).

The RT-PCR results in figure 3.5 show minimal transport of miRNA-1 into the media from the scrambled transfected cell group. The mean for miRNA-1 expression in media was 0.4786 (±0.253) and 1.27 (±0.438) for control scramble transfected and lanthanum treated scramble group respectively. There was no significant difference between the lanthanum treated cells and control of the scramble group (Figure 3.5).

The expression of miRNA-1 in media of control miRNA-1 transfected AC16 cells (12.11±0.959; n=3) was significantly higher than the control of scramble transfected cells (1.27±0.438) (p < 0.0001). However, no significant difference was found after lanthanum exposure in miRNA-1 transport in the miRNA-1 transfected cells (figure 3.5). The mean values for miRNA-1 expression in media for lanthanum, treated of miRNA-1 transfected cells and their control were 12.01 (± 0.354) and 12.11 (±0.960) respectively.
Figure 3.5 Transport of miRNA-1 from AC16 cells into the media. The RT-PCR was performed on RNA extracted from the cell media of AC16 cells 24 hours after lanthanum or control treatment. Each data point on scatter plot represent average of triplicate C_T values, normalised to U6 and miRNA-16 expression. Errors bars on bar graph represent SEM. ****p < 0.001, one-way ANOVA with Sidak’s multiple comparisons test. n=3 for each group.

3.2.2 Quantifying Cx43 expression in AC16 cells
To confirm that lanthanum was not altering the expression of Cx43 in AC16 cells, western blots were performed on AC16 cells treated with 2mM lanthanum for 24 hours and 48 hour and the expression of Cx43 was measured. For both, 24 and 48 hour time point, AC16 cells treated with SILAC were used as the control.
A 43kDa band was detected for all samples of both control and 2mM lanthanum treated group (Figure 3.6). There was no significant difference found in the expression of Cx43 protein in lanthanum treated AC16 cells compared with the control at both time points.
Figure 3.6 Cx43 expression levels in AC16 western blots for cells treated with 2mM Lanthanum. A) Each well was loaded with 40µg of protein sample gathered from cells seeded at 800,000 density. 1 and 2 are protein from 24 hour control, 3 and 4 are protein from cells treated with 2mM La3+ for 24 hours, 5 and 6 are protein from 48 hour control, 7 and 8 are protein from cells treated with 2mM La3+ for 48 hours. B) Quantitative analysis of western blot showing Cx43 expression in AC16 control and lanthanum group for 24 hour and 48 hour time point. Expression of Cx43 plotted as arbitrary unit (A.U) after normalisation to GAPDH expression. n= 3 for each group and each data point on the scatter plot represents an average of 2 technical repeats. Shapiro-Wilk normality test was done for normalisation and then one-way ANOVA was performed. Errors bars on the bar graph represent SEM.
3.3 Results Summary

There are endogenous connexin genes present in *Xenopus laevis* where expression of endogenous Cx38 is significantly higher than endogenous Cx43. Human Cx43 cRNA was injected into *Xenopus laevis* oocytes to build a construct in which human Cx43 mediated miRNA transport can be tested. No functional difference was seen between human Cx43 cRNA injected and control oocytes when TEVC was used to assess the presence of Cx43 channels. In both groups of oocytes, a connexin specific current was induced by incubation in Ca$^{2+}$-free solution and was subsequently inhibited by lanthanum. Because of the known expression of endogenous Cx38 in *Xenopus laevis* oocytes, a Ca$^{2+}$-free induced current was expected in the control group. However, a higher Ca$^{2+}$-free induced current was expected in the human cRNA injected oocytes due to overexpression of Cx43 post cRNA injection and 24 hour incubation.

This lack of functional difference was investigated by quantifying Cx43 expression in human Cx43 cRNA and control group by western blot. It was found that Cx43 was significantly highly expressed in human cRNA injected oocytes compared with control oocytes.

For the cardiomyocyte model, no significant difference was seen in miRNA transport between lanthanum treated and non-lanthanum treated cardiomyocytes.
4. DISCUSSION

The role of miRNA in modulating cardiovascular diseases is extensively studied and their transport pathways are explored to form miRNA based therapy for CVDs. The currently known transport route of miRNAs via membrane–bound vesicles have off-target effects due to the lack of target specificity of these vesicles. Some studies have looked into a connexin protein mediated pathway as an alternative to the vesicular transport of miRNAs and as a more specific transport via gap junction proteins for miRNA transport between cells cardiac cells. The transport of miRNA through the mouse isoform of connexin Cx43 has been demonstrated in the HeLa cell line (Zong et al., 2016) and in neonatal rat cardiomyocytes (Lemcke et al., 2017). However, to the best of my knowledge this study is the first to test for the transport of miRNAs via human Cx43 that predominantly form gap junctions between ventricular cardiomyocytes.

4.1 Human Cx43 in Xenopus laevis oocytes

The expression of native connexin Cx38 and Cx43 genes was examined first. Although it is well known that the oocytes express Cx38 gene (Ebihara et al., 1989; Haefliger et al., 1992), there is minimal information on Cx43 gene expression in Xenopus laevis oocytes (Gimlich et al., 1990). The gene RT-PCR data (Figure 3.1) showed that, compared to the expression of connexin Cx38 gene, there was only minimal presence of native Cx43 gene in the non-injected oocytes (ratio of Cx43:Cx38 = 0.000431034:1). With this background information, experiments were then performed to express human Cx43 in the Xenopus oocytes. This was accomplished successfully as shown by the western blot data (Figure 3.4). The bands in each sample were detected close to 43 kDa as seen in the positive control (Cx43 from AC16 cardiomyocytes) lanes, thus confirm the presence of Cx43 in both sample groups.

However, although the gene RT-PCR data, showed virtually no human Cx43 in the native oocytes, here the western blot data showed apparent expression of human Cx43 proteins in both the human cRNA injected and, to an appreciably and significantly lesser extent (p<0.05), in the
control group. The apparent presence of Cx43 in the control group is unlikely to result from contamination, as no bands were observed in the negative control (Figure 3.4). One possible explanation for the detection of a Cx43 band in control oocytes could be a lack of specificity of the Cx43 antibody for human Cx43. The antibody in this study was a polyclonal rabbit anti-Cx43 antibody. While the use of polyclonal antibodies is advantageous in detecting protein expression as they can bind to different epitopes of the protein of interest, it can also have disadvantageous, as recognition of multiple epitopes can lead to non-specific binding in homogenous proteins (Neil et al., 2005). Therefore, the unexpected detection of Cx43 in control oocytes may reflect a lack of antibody specificity with detection of expressed Xenopus Cx43 protein. It is also possible that Xenopus Cx38 is detected as well. This is supported by findings of (Haefliger et al., 1992) which shows homogeneity between the structure of Cx38 and Cx43 proteins.

Also for the western blot experiment, as a control for cRNA injection, it would have been interesting to perform a western blot on non-injected oocytes and to investigate Cx43 expression in these oocytes. In any event, it can be confidently said that cRNA injection worked and that human Cx43 proteins were expressed in the Xenopus oocytes.

The mean current in the human cRNA injected oocytes was almost 1.5x greater than that in the RNase free water injected control oocytes. However, the difference failed to reach statistical significance. This possibly reflects the relatively small sample size (n=6 for the cRNA injected group). In addition, there may have simply been insufficient expression of the functional human connexin 43. This, together with the expression of endogenous connexins (Cx38 and Cx43) in the control and treated oocytes, would limit the magnitude of the differences in current between the two experimental groups. In support of this possibility, Ebirah (1996), has shown that the expression of Cx38 protein in Xenopus oocytes and the endogenous Cx38 in Xenopus laevis oocytes induces whole cell current upon application of Ca^{2+} free solution. Due to the lack of
significant functional difference in the present human Cx43 expressing oocytes, the hypothesis of miRNA transport via human Cx43 in *Xenopus* oocyte model could not be tested.

However, in this study it was established that connexins in oocytes can be successfully activated by Ca\(^{2+}\) free solutions and inhibited to a significant extent by concentrations of lanthanum that avoid toxicity. The highest viability of oocytes in Ca\(^{2+}\) free solution is seen in the first 48 hours (Figure 3.2). The results further support the use of 50\(\mu\)M La\(^{3+}\) which is tolerated by the cells. As the TEVC recordings show, 50\(\mu\)M La\(^{3+}\) blocked the activity of connexins in oocytes significantly. Thus, these findings demonstrate that transport via connexins can be examined by testing in the first 48 hours of incubation. This avoids hindrance in quantifying Cx43 mediated transport due to deterioration of the oocyte membrane.

### 4.2 Modifications for Xenopus oocyte model for subsequent experimental studies.

The results reported here illustrate the need to refine the *Xenopus* oocyte model to obtain more precise data. There is a need to reduce endogenous connexin expression in oocytes before using them to test miRNA transport. It is already established that ion transport via all connexins in general is Ca\(^{2+}\) and La\(^{3+}\) sensitive. Thus, to test the functionality of exogenously expressed human Cx43, the *Xenopus* oocyte must be modified to have low endogenous connexin activity. The *Xenopus* Cx38 and *Xenopus* Cx43 knockdown oocyte model would allow adequate comparison of the Ca\(^{2+}\) free current induced by human Cx43. Bao *et al.*, (2004) studies have shown knockdown of endogenous connexin has enabled concrete functional recordings of exogenous connexin.

It is also possible that the results were confounded by the quality of the oocytes as the oocytes were extracted from different frogs each week. Though visually uniform oocytes within stage V-VI were selected, the ability of the oocytes to transcribe exogenously expressed human Cx43 cRNA could vary for oocytes extracted from different frogs.
4.3 miRNA transport in cardiomyocytes

The miRNA transfected group of cells secreted miRNA into the medium. However, this secretion was not blocked by 2 mM lanthanum (Figure 3.5). Lemeke et al., (2017) found using a different experimental approach in isolated neonatal cardiomyocytes, found a 50% decrease of miRNA mimic transport following Cx43 knockdown. Other than the fact that (Lemcke et al., 2017) tested mRNA transport in neonatal rat cardiomyocytes whereas AC16 cells are representative of adult human cardiomyocytes, these contrasting results could be explained as a consequence of the different methodology used to test Cx43 mediated miRNA transport. First, in this study the transport of miRNA was tested from the cells into the media whereas Lemeke et al tested the transport of miRNA between the cells using 3D-FRAP microscopy. Their model included a Cx43 knockdown whereas, in this project connexin blocker lanthanum (2mM) was used to control transport via Cx43. The transport function of connexin is hindered by lanthanum that blocks the channel pore via a particle-receptor binding mechanism (Morley et al., 1996).

As the result illustrated in figure 3.6 show, the 2mM lanthanum concentration used here to block connexin did not affect the expression of Cx43 protein itself. Based on the demonstration that 50µM lanthanum concentration in the oocytes inhibited 50% of connexin channel activity, the use of 2mM lanthanum in cardiomyocytes should have blocked the Cx43 channel activity sufficiently.

4.4 Possible reasons for lack of miRNA transport inhibition by lanthanum in cardiomyocytes

When accessing the data in figure 3.6 it is important to recall the presence of vesicular transport pathways in cardiomyocytes. The transport of exosomes and microvesicles was not blocked in AC16 cell treatment groups. There is evidence that the half-life of the Cx43 protein in the cardiomyocytes incubated in vitro is within 1 hour to 5 hours (Laird et al., 1991). This suggests that during 24 hour incubation other pathways must account for a significant amount of the miRNA transported into the media. This offers the best explanation for the failure of lanthanum to block the movement of miRNA-1 from cardiomyocytes.
A less likely possibility that might have contributed to the lack of effect of lanthanum would be cell damage. The choice of 2 mM La$^{3+}$ was based on the work of who exposed cultured myocardial cells in vitro 2 mM La$^{3+}$ for 5 minutes (Kondo et al., 2000). This is a much shorter exposure than the 24 hours used here. If the current study were extended, the effects of different concentrations of lanthanum on AC16 cardiomyocytes would be examined.

### 4.5 Conclusion

Given the gaps in the experimental design of this study, the hypothesis of Cx43 mediated miRNA transport could not be established with the present results. Further experiments must be carried out where transport via human connexin is tested in the absence of vesicular transport pathways as well as other proteins of the connexin family. The confound of endogenous connexins present in native oocytes suggest a new proposed model for testing Cx43 mediated miRNA transport in *Xenopus* oocyte is a knockdown of *Xenopus* Cx38 and possibly *Xenopus* Cx43.

### 4.6 Future Directions

For both models, it would be wise to explore other known connexin channel blockers such as heptanol, rather than relying solely on lanthanum.

**Xenopus oocyte model**

Using an endogenous connexin knockdown model of *Xenopus* oocyte. The miRNA transport via human Cx43 can then be tested by testing the transport of miRNA-1, as this is a predominantly found miRNA in the heart and is not expressed in *Xenopus* oocytes. The mimic miRNA-1 can be injected in the oocytes and the bath solution can be collected for RT-PCR analysis to test the transport of miRNAs (see appendix 5, figure 4.1).

**AC16 Cardiomyocytes**

As the transport was tested from AC16 cells into the extracellular fluid, the methodology of this experiment tested transport through connexin 43 formed hemichannels. The junctions
between cardiomyocytes are formed by two hemichannels combined to form a gap junction. To account for variance in conductance of gap junctions and hemichannels, experiments should be performed where transport of miRNA is tested via gap junctions using fluorescence microscopy to see live movement of fluorescent tagged miRNA between the cells.
REFERENCES


Boštjančič E, Zidar N, Štajer D & Glavač D. (2010). MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. Cardiology 115, 163-169.


Appendix 1

Table 5. Solution used for oocyte survival test. The 50 µM, 100 µM, 500 µM and 1000 µM La³⁺ concentrations were prepared using the Ca²⁺ free solution.

<table>
<thead>
<tr>
<th></th>
<th>CulOri (mM)</th>
<th>Ca²⁺ free (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCl</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>LaCl₃</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Na⁺ Pyruvate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>EGTA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 6. Solutions used for TEVC recordings in Xenopus oocyte

<table>
<thead>
<tr>
<th></th>
<th>CulOri (mM)</th>
<th>Ca²⁺ free (mM)</th>
<th>0.05mM La³⁺</th>
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<tbody>
<tr>
<td>NaCl</td>
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<td>90</td>
<td>90</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaCl₃</td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>EGTA</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>
**Appendix 2**

**Table 7. Composition of lysis buffer used for oocyte homogenisation.** To extract proteins from oocytes, 10µl lysis buffer was used per oocyte.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>10 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>83 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1 %</td>
</tr>
<tr>
<td>Protease inhibitor tablets</td>
<td>1 tablet/10ml</td>
</tr>
</tbody>
</table>
### Appendix 3

Table 8: Reagents prepared for Western Blot

<table>
<thead>
<tr>
<th>Regent</th>
<th>Composition</th>
</tr>
</thead>
</table>
| **Upper Tris (1.5M Tris HCl pH 6.8)** | - 18.117g Tris (hydroxymethyl)  
- 90ml dH₂O  
- Adjust pH to 6.8 with 6M HCl |
| **Lower Tris (1.5M Tris HCl pH 8.8)** | - 22.59g Tris  
- 90ml dH₂O  
- Adjust pH yo 8.8 with 6M HCl  
- Make final volume 100ml with dH₂O |
| **10% SDS**                  | - 10g sodiumdodecyl sulphate  
- 80ml dH₂O  
- Make final volume 100ml with dH₂O |
| **50% Glycerol**             | - 50ml glycerol  
- Make final volume 100ml with dH₂O |
| **Loading Buffer (2X)**      | - 4.165ml Upper Tris  
- 10ml 20% SDS  
- 20ml 50% glycerol  
- 5ml mercaptoethanol (14.3M)  
- 500µl EDTA (200mM)  
- 10.38ml dH₂O |
| **Running Buffer (10x concentrate)** | - 60g Tris base  
- 288g glycine  
- 20g SDS (wear mask)  
- Make up to 2L with dH₂O |
| **Transfer buffer**          | - 7.57g Tris base  
- 36g Glycine  
- 500ml Methanol  
- Make to 2.4L with dH₂O and adjust pH to 8.3 with NaOH and HCl  
- Make final volume 2.5L with dH₂O |
| **TBS (10x concentrate)**    | - 80g NaCl  
- 2.0g KCl  
- 30g Tris base  
- Adjust pH to 7.4 with concentration HCl  
- Make final volume 1L with dH₂O |
| **TTBS (0.05%)**             | - 400ml TBS (1x)  
- 200µl Tween20 |
| **Stripping Buffer**         | - 15g glycine  
- 1g SDS  
- 10ml Tween20  
- Adjust pH to 2.2 with conc. HCL  
- Make final volume 1L with dH₂O |
Table 9. Component and ratios for making resolving and stacking gels used in electrophoresis stage of western blot.

<table>
<thead>
<tr>
<th>Resolving Gel</th>
<th>1 Gel (µL)</th>
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<tr>
<td>10%</td>
<td>1 Gel (µL)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>3150</td>
</tr>
<tr>
<td>Lower Tris (pH 8.8)</td>
<td>2000</td>
</tr>
<tr>
<td>10% SDS</td>
<td>80</td>
</tr>
<tr>
<td>30% AA:MBA</td>
<td>2665</td>
</tr>
<tr>
<td>10% APS (Ammonium Persulphate)</td>
<td>80</td>
</tr>
<tr>
<td>TEMED</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>8000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking Gel</th>
<th>1 Gel (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>2100</td>
</tr>
<tr>
<td>Upper-Tris</td>
<td>875</td>
</tr>
<tr>
<td>10% SDS</td>
<td>35</td>
</tr>
<tr>
<td>30% AA:MBA</td>
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</tr>
<tr>
<td>10% APS</td>
<td>35</td>
</tr>
<tr>
<td>TEMED</td>
<td>3.5</td>
</tr>
</tbody>
</table>
### Appendix 4

Primers used for gene Rt-PCR to detect endogenous connexin expression in *Xenopus laevis* oocytes.

<table>
<thead>
<tr>
<th></th>
<th>Nucleotide sequence</th>
<th>Company</th>
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<tbody>
<tr>
<td><strong>Xenopus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cx38</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Forward Primer</strong></td>
<td>AGTAATCACCAGAC</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>CTCTTC</td>
<td></td>
</tr>
<tr>
<td><strong>Reverse Primer</strong></td>
<td>TTAAGACAAGCCCA</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>TTCATC</td>
<td></td>
</tr>
<tr>
<td><strong>Xenopus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cx43</td>
<td></td>
<td></td>
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<tr>
<td><strong>Forward Primer</strong></td>
<td>TTTTGGATCTGCAA</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>GGTAAC</td>
<td></td>
</tr>
<tr>
<td><strong>Reverse Primer</strong></td>
<td>TGGGAATAGGCTTGA</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>ACTTTG</td>
<td></td>
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<tr>
<td><strong>Human Cx43</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Forward Primer</strong></td>
<td>ATCCTCCAAGGAGT</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>TCAATC</td>
<td></td>
</tr>
<tr>
<td><strong>Reverse Primer</strong></td>
<td>AATGAAAAGTACTG</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>ACAGCC</td>
<td></td>
</tr>
<tr>
<td><strong>18S</strong></td>
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<tr>
<td><strong>Forward Primer</strong></td>
<td>ATCGGGGATTGCAA</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td></td>
<td>TTATTC</td>
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<tr>
<td><strong>Reverse Primer</strong></td>
<td>CTCACCTAAACCAC</td>
<td>Sigma, USA</td>
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<td></td>
<td>CAATCG</td>
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</tbody>
</table>
Figure 4.1 A new experimental design for testing miRNA transport via human Cx43 in Xenopus oocytes based on findings of the current study. Cx38 short interfering RNA (siRNA) would ensure knockdown of endogenous Cx38 in Xenopus oocytes and will thus reduce the interference from endogenous connexin when testing the transport via human Cx43. Group A oocytes, as in Ca\(^{2+}\) free solution, will have open connexin channels whereas connexin channels in group B oocytes will be blocked due to the presence of La\(^{3+}\). Comparative RT-PCR analysis of miRNA-1 detected in the bath solution of group A (Cx43 active) and group B (Cx43 blocked) in oocytes will enable understanding of whether Cx43 has a role in miRNA transport.