Establishment of Zebrafish Models for Studying Mesenchymal Stromal Cell Therapy for Cardiac Disease

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ABSTRACT

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Bone marrow (BM)-derived mesenchymal stromal cells (MSCs) can be induced to express cardiac-specific markers by embryonic cardiomyocytes in vitro. To determine whether this phenomenon occurs in vivo, we have developed a cell transplantation system using zebrafish embryonic recipients. We were unable to isolate expandable zebrafish kidney stromal (ZKS) cells from the kidney, the human BM equivalent; hence, we analyzed the established ZKS1 cell line. We found that ZKS1 expresses stromal genes, but also expresses hematopoietic genes not normally expressed by MSCs. Furthermore, we were unable to differentiate ZKS1 cells into adipocytes, osteoblasts or cardiomyocytes in vitro. We created a transgenic ZKS1(CMV:eGFP) cell line which, after transplantation into zebrafish blastulae, was observed within the host heart, among other tissues. Finally, pT2/s2tnnt2-GM2 and pT2/s2tnnt2-DsRed transposons were generated to mark ZKS1 cardiac differentiation. The zebrafish model established here will be useful for studying the molecular mechanisms of exogenous MSC cardiac differentiation in vivo.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>α-MHC</td>
<td>mammalian alpha-myosin heavy chain</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMC</td>
<td>bone marrow cell</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>bone marrow-derived mesenchymal stromal cells</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>CFU-F</td>
<td>colony-forming unit fibroblast</td>
</tr>
<tr>
<td>cmlc2</td>
<td>zebrafish cardiac myosin light chain-2</td>
</tr>
<tr>
<td>CM</td>
<td>cardiomyocytes</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>cry</td>
<td>β-crystalline</td>
</tr>
<tr>
<td>cTnI</td>
<td>mammalian cardiac troponin I</td>
</tr>
<tr>
<td>cTnT</td>
<td>mammalian cardiac troponin T</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dpf</td>
<td>days post-fertilization</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-injection</td>
</tr>
<tr>
<td>DTA</td>
<td>diphtheria toxin A</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescently-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post-fertilization</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post-injection</td>
</tr>
<tr>
<td>HS</td>
<td>horse serum</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>ISCT</td>
<td>International Society for Cellular Therapy</td>
</tr>
<tr>
<td>krt8</td>
<td>keratin 8</td>
</tr>
<tr>
<td>LTMC</td>
<td>long-term marrow culture</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stromal cell</td>
</tr>
<tr>
<td>nt5e</td>
<td>ecto-5’-nucleotidase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>pT2/s2</td>
<td>Sleeping Beauty transposon</td>
</tr>
<tr>
<td>rCM</td>
<td>rat embryonic cardiomyocytes</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RmT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal cell-derived factor 1</td>
</tr>
</tbody>
</table>
TGF-β - transforming growth factor-β

thy-1 – thymocyte differentiation antigen 1

tnni-HC – zebrafish cardiac troponin I-HC

tnnt2 – zebrafish cardiac troponin T2

TTC – troponin-tropomyosin complex

vcam1 – vascular cell adhesion molecule 1

VEGF – vascular endothelial growth factor

WKM – whole kidney marrow

ZKS – zebrafish kidney stromal

ZKS1 – zebrafish kidney stromal cell line
CHAPTER 1.0 – GENERAL INTRODUCTION

1.1 The Burden of Heart Disease

Cardiovascular disease is the leading cause of death worldwide and hospital admissions for heart failure are on the rise with a predicted 50% increase over the next 25 years [1]. In 2005, 54% of cardiovascular deaths in Canada were due to ischemic heart disease [2]. In the United States, approximately 550,000 patients are diagnosed with heart failure each year [3]. As adult cardiomyocytes are generally considered terminally differentiated and unable to proliferate, areas of cardiac tissue necrosis will form scar tissue which reduces myocardial elasticity and stimulates ventricular remodelling, ultimately leading to progressive loss of cardiac function [4,5]. The standard of care for heart failure usually involves administration of drug therapy including diuretics, beta-blockers, and ACE-inhibitors [6]. However, this strategy does not repair the damaged myocardium nor does it restore myocardial function. The only clinically effective corrective therapy for end-stage heart failure today is cardiac transplantation. Yet, this strategy is limited by the availability of donor hearts. By the end of 2009, 168 cardiac transplants were performed in Canada, while 136 patients remained on the transplant waiting list [7]. Therefore, the development of more effective corrective therapies is a significant priority.

1.2 Bone Marrow Cell Therapy for Cardiac Disease

Research towards the discovery of novel corrective therapies for cardiac disease has concentrated on regenerative medicine. Specifically, cell-based therapy using adult stem or progenitor cell transplantation to replace damaged cardiac tissue with new, functional
cardiomyocytes has surfaced as a promising approach. Cell-based therapy has proven to be effective in the past for restoring tissue compartments. Popular examples currently in use clinically include bone marrow cell (BMC), umbilical cord blood stem cell or peripheral blood stem cell transplantation to reconstitute bone marrow tissue in patients with blood cancers or other hematological disorders [8, 9, 10]. A significant amount of research is being devoted to unveiling the potential application of various adult cell types in the reconstitution of cardiac tissue and functional cardiac recovery of patients presenting with heart disease. Although a number of cell types have been proposed, cells of the BM have the advantage of being easily accessible and multipotent. In 2001, one of the first studies investigating BM cell therapy for acute myocardial infarction (MI) was conducted by Orlic and colleagues [11]. They transplanted autologous, fluorescently-labelled bone marrow cells into infarct regions of mouse models of MI. Results of this study suggested that transplanted BMCs can differentiate into cardiomyocytes (CMs) during infarction in vivo. These optimistic results led to the initiation of clinical trials of intracoronary BM transplantation therapy for patients with acute MI in 2002 [12]. Although BMC therapy did not elicit any significant side effects in MI patients, many clinical trials concluded that this therapy only results in modest improvements in heart function based left ventricular functional parameters [13]. These modest results suggest that preclinical studies are required to determine which specific cell populations within the BM are most suitable for a more effective cell-based therapy for cardiac disease. The BM-derived mesenchymal stromal cell (BM-MSC) has emerged as an excellent cell type for cardiac therapy due to its many unique advantages.
1.3 The Bone Marrow-derived Mesenchymal Stromal Cell

Mesenchymal stromal cells represent a miniscule fraction, 1/10 000 to 1/100 000, of all mononuclear cells within the BM [14]. They were first identified in the bone marrow by Friedenstein et al. in 1976, who described this cell population as adherent and fibroblast-like with the capacity for osteogenic differentiation [15]. Since there has been no discovery of a genetic marker specific to the BM-MSC to-date, the International Society for Cellular Therapy (ISCT) developed minimal criteria for defining BM-MSCs as to ensure studies involving BM-MSCs are comparable. The criteria are as follows: 1) Plastic adherence in standard tissue culture; 2) Expression of surface antigens CD105 (endoglin), CD73 (ecto 5’ nucleotidase) and CD90 (Thy-1), and lack of expression of hematopoietic or endothelial markers CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR; and 3) In vitro differentiation to osteoblasts, adipocytes, and chondroblasts [16].

1.4 The Advantages of BM-MSCs for Cardiac Regenerative Therapy

The BM-MSC represents a strong candidate cell-type for regenerative medicine, particularly due to the 6 main advantages that these cells offer:

1.4.1 Multipotency

Both in vitro and in vivo studies have demonstrated the ability of BM-MSCs to differentiate towards a number of different tissue types, including skeletal muscle, smooth muscle, cardiac muscle, neurons, dermal tissues, retinal photoreceptors, bone, adipose tissue, cartilage, tendons, and possibly others [reviewed in 14]. That said, evidence of true functional differentiation remains controversial for certain tissue types, such as myocardial tissue.
1.4.2 Accessibility
BM-MSCs are easily accessible from bone marrow aspirations extracted from healthy donors. The extraction is not an invasive procedure and complications are very rare and often mild, which encourages bone marrow donation.

1.4.3 Expandability
Primary MSCs purified from BM tissue are easily expanded ex vivo to generate cell numbers required for transplantation [14]. Ex vivo expandability also allows for genetic or chemical manipulations of BM-MSCs prior to transplantation to enhance the outcome of cell therapy.

1.4.4 Low Immunogenicity
Allogeneic BM-MSCs can be transplanted into patients with acute MI without eliciting a host immune response or causing any significant side effects [17]. There are a few proposed reasons as to why BM-MSCs are non-immunogenic in allogeneic settings. First, BM-MSCs do not stimulate the proliferation of allogeneic lymphocytes in vitro, as demonstrated by co-culture studies between BM-MSCs and lymphocytes of a mixed lymphocyte reaction [reviewed in 14, 18-20]. Furthermore, these cells suppress the activation of T lymphocyte subsets CD2, CD4 and CD8, and therefore suppress the T cell-mediated immune response [reviewed in 14,21,22]. BM-MSCs also lack expression of co-stimulatory molecules, such as CD80, CD86, CD40, CD40 ligand and CD154 [reviewed in 14,23]. Finally, BM-MSCs can escape lysis by cytotoxic lymphocytes and natural killer cells [24].

1.4.5 Immunomodulatory Properties
BM-MSCs are thought to promote an anti-inflammatory response in hostile environments, a property that may help reduce inflammation within diseased or injured tissue. More specifically, both the formation of mature T lymphocytes and the cytotoxic T lymphocyte response are
inhibited by BM-MSCs [24]. It has been proposed that these suppressive effects are mediated by soluble factors secreted by BM-MSCs, such as hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), indoleamine oxidase, human leukocyte antigen-G and interleukin-10 [reviewed in 14,21,25-27].

1.4.6 Homing to Injured Tissue

Damage to body tissue activates molecular pathways causing the expression of surface chemokines by tissue surrounding the injured area. BM-MSCs can express receptors for these chemokines which attracts the cells to the site of injury. For example, both postoperative chemokine stromal cell-derived factor 1 (SDF-1) expression within the heart and the numbers of intravenously (IV) transplanted BM-MSCs homing to the host heart were significantly higher in rat models of MI than sham operated animals [28]. Currently, the only identified receptors of SDF-1 are CXCR4 and CXCR7 [29,30]. CXCR4 was found to be expressed on the surface of a small proportion of BM-MSCs [31]. As the expression of CXCR4 by MSCs has shown to correspond with an increased migration of MSCs to SDF-1 [32], the chemoattractive nature of CXCR4 and SDF-1 plays a role in MSC homing to infarcted myocardium. This attribute of homing to tissue injury enables BM-MSCs to be delivered to patients with minimal invasiveness through IV injection.

Altogether, BM-MSCs are an excellent source for cellular therapy as the procedures for both donor collection and delivery to recipients are not invasive and the cells can be made widely available for patients due to their accessibility and ability for allogeneic transplantation. BM-MSCs provide an avenue for a more efficient corrective therapy for heart disease than cardiac transplantation.
1.5 Pre-clinical Studies and Clinical Trials of BM-MSC Therapy for Myocardial Diseases

Several preclinical trials have demonstrated a therapeutic benefit of BM-MSC therapy using animal models of myocardial disease. In a rat model of ischemic heart disease, BM-MSCs engineered to express Akt, a pro-survival gene, were transplanted intramyocardially and found to be associated with the observed attenuation of cardiac remodeling, increased area of myocardial regeneration, and near normalization of systolic and diastolic cardiac function in the host heart [33]. Furthermore, using a rat model of acute MI, IV-delivered BM-MSCs were shown to preferentially attract to necrotic areas within the myocardium and mediate cardiac functional repair through increased capillary formation within the heart, decreased infarct size, and decreased left ventricular end-diastolic pressure [34]. BM-MSCs directly injected into the infarcted myocardium of porcine models of MI were likely attributable to the marked reduction in cardiac contractile dysfunction and prevention of pathological ventricular wall thinning that was observed in these animals [35].

Although there are a limited number of reports on clinical trials using BM-MSCs for cardiac therapy, several Phase I and Phase II trials testing the safety and efficacy of this therapy are currently ongoing [36]. One reported randomized clinical trial involving intracoronary injections of autologous BM-MSCs into patients with acute MI found that the treatment group showed statistically significant improvements in several left ventricular parameters by 3 months post-transplantation, ultimately ameliorating overall cardiac function in this group of patients [37]. An increase in wall movement velocity over the infarct area was observed in the treatment group (2.17±1.3cm/s to 4.2±2.5cm/s) as compared to the control group receiving saline injections (2.19±1.5cm/s to 2.7±1.7cm/s). Similarly, left ventricular ejection fraction (67±11%
after treatment vs. 49±9% before treatment) and perfusion defects (20±5% after treatment vs. 36±6% before treatment) were both significantly improved in the treatment group. Moreover, end-diastolic and end-systolic volumes were reduced after receiving BM-MSC therapy. Thus, both extensive preclinical and preliminary clinical evidence conclude that BM-MSC transplantation improves cardiac function and proposes a novel therapeutic approach for corrective cardiac therapy in patients with ischemic cardiac disease.

Despite this evidence of MSC-mediated cardiac improvements, much work is still required to uncover the underlying mechanisms of the MSC-mediated repair to enhance the beneficial outcomes of this therapy.

1.6 Mechanisms of Cardiac Regeneration and Repair by Exogenous BM-MSCs

There are a few proposed mechanisms by which transplanted BM-MSCs induce cardiac repair which are categorized as either direct or indirect mechanisms. Direct mechanisms include BM-MSC differentiation into cardiomyocytes, differentiation into vascular endothelial cells leading to neovascularisation, and fusion with endogenous functional cardiomyocytes. Indirect mechanisms include both BM-MSC release of paracrine factors that support cardiac repair and MSC-mediated immunomodulatory effects that promote cell survival within the hostile disease environment.

1.6.1 Paracrine Mechanisms

The BM-MSC paracrine effect observed during cell therapy for cardiac disease is predominantly achieved through both cardiovascular regenerative and cardioprotective mechanisms. Factors secreted by transplanted BM-MSCs act to attenuate progressive cardiac necrosis and promote the
regeneration of functional myocardial tissue by inhibiting myocyte apoptosis and eliciting angiogenic and myogenic responses, respectively. An *in vitro* study demonstrated that BM-MSCs express paracrine factor genes known to be involved in cellular proliferation and survival when exposed to hypoxic conditions [38,39] resembling an ischemic cardiac environment. Such factors include vascular endothelial growth factor (VEGF), HGF, adrenomedullin, and insulin-like growth factor 1 [38,40,41].

**1.6.2 The Mechanism of Cell Fusion**

Cell fusion events between exogenous BM-MSCs and endogenous cardiomyocytes have been noted; however these events were found to occur at such low frequencies [42] that this mechanism will not likely have a significant contribution to the functional cardiac improvements resulting from BM-MSC therapy.

**1.6.3 The Mechanism of Exogenous BM-MSC Cardiac Differentiation**

Direct differentiation of MSCs into cardiomyocytes after transplantation remains a more controversial mechanism for cardiac repair. It is well-known that BM-MSCs can express cardiac-specific genes when co-cultured with embryonic or neonatal CMs *in vitro* [42-44,45,46], but not when co-cultured with adult CMs [46]. The most recent findings in regards to functional cardiomyocyte differentiation *in vitro* report that BM-derived MSCs can express cardiac-specific genes when co-cultured with embryonic or neonatal cardiomyocytes, yet do not form sarcomeric units nor generate action potentials and ionic currents which are necessary for cardiomyocyte contractility [42,43]. Our research group recently established a novel *in vitro* system to test cardiac differentiation [42]. Female murine transgenic BM-MSCs expressing GFP under control of the alpha-myosin heavy chain promoter were co-cultured with male rat embryonic cardiomyocytes for 5-15 days. The cells were sorted for GFP, indicative of cardiac protein
expression. GFP-expressing cells were observed to retain their stromal morphology. After staining with a number of cardiac and stromal antibodies, it was found that these cells express both cardiac and stromal markers after co-culture. Finally, the electrophysiology of the GFP+ cells, as assessed by sodium (Na+) and calcium (Ca2+) ionic currents, was not characteristic of cardiomyocyte electrophysiology. Thus, it was concluded that BM-MSCs retained a stromal phenotype, expressed both stromal and cardiac proteins, but did not functionally resemble cardiomyocytes in vitro. On the other hand, another relatively recent study by Li et al. found that when rat BM-MSCs were co-cultured with rat neonatal ventricular myocytes, some BM-MSCs expressed cardiac specific markers, initiated contraction and demonstrated an ultrastructure characteristic of cardiomyocytes [44]. Since this group used a transwell co-culture system in which BM-MSCs were separated from the neonatal CMs by a semipermeable membrane, cell fusion could be ruled out as the mechanism of cardiac antigen expression by BM-MSCs. Consequently, it can be concluded that BM-MSCs can be induced to express cardiomyocyte-specific markers when co-cultured with embryonic or neonatal cardiomyocytes in vitro, but whether they differentiate into fully functional cardiomyocytes remains inconclusive.

In vivo studies have concentrated on assessing MSC differentiation after transplantation into adult animal models. The general consensus is that transplanted BM-MSCs that engraft and persist in the host heart are capable of expressing cardiac-specific markers [35,47-49]. For example, when human BM-MSCs were transplanted into the left ventricle of immunodeficient mice, a limited number of MSCs survived and a fraction of these surviving cells expressed cardiac markers at levels comparable to host cardiomyocytes while displaying a sarcomeric organization of contractile proteins [47]. Moreover, in a swine model of MI, autologous BM-MSCs that were transplanted directly into the infarct region showed robust engraftment in the
host heart and expressed muscle-specific proteins as early as 2 weeks post-transplantation [35]. Many *in vivo* studies assess cardiac protein expression by BM-MSCs according to standard immunohistochemical techniques. New studies using more direct forms of immunofluorescent marking are required, such as transgenic BM-MSCs expressing a fluorescent reporter protein under the direction of a cardiomyocyte-specific promoter which is a strategy that I have incorporated into my research project.

1.7 Justification and Advantages for Using Zebrafish Embryonic Recipients to Study Cardiac Differentiation by Exogenous BM-MSC *in vivo*

To-date, there have been no studies assessing the cardiac differentiation of transplanted BM-MSCs in an embryonic or post-natal-staged animal model to determine whether a more primitive cardiac microenvironment will stimulate functional cardiac differentiation at higher frequencies than in an adult cardiac microenvironment. Therefore, in our study, we propose a unique animal model system to efficiently assess the extent of cardiac differentiation by BM-MSCs at various stages of development using the zebrafish.

Cellular and molecular events occurring during embryonic and fetal stages of development cannot be observed directly *in vivo* when using mammalian models as the embryo is dependent on maternal circulation and nutrition for survival. Therefore, in my study I employed the zebrafish model which, as a result of several specific advantages over mammalian models, enables large-scale *in vivo* experimentation at early stages of development followed by direct visualization of cellular and molecular outcomes. Several experiments can be performed and results observed within days, making the zebrafish an efficient and cost-effective model. The
specific advantages for using the zebrafish to study the cardiac differentiation capacity of transplanted MSCs at embryonic and post-natal stages of development in vivo are as follows:

1.7.1 External embryonic development

The zebrafish embryo is externally fertilized and develops external to the mother’s body within an aqueous environment. The embryo does not require exogenous nutrition for the initial stages of development and inherently relies solely on innate immunity for defence mechanisms [50]. This enables embryo survival independent of maternal support and limits host embryo immune reaction to allogeneic cell transplantation. External embryonic development allows for both the manipulation and direct observation of the zebrafish embryo during development.

1.7.2 Rapid embryonic development

Zebrafish embryonic development occurs rapidly and organogenesis is completed around 2 days post-fertilization (dpf) [51]. As early as 24 hours post-fertilization (hpf), the developing heart begins to beat. The heartbeats are irregular as there are no coordinated contractions. At this stage of development, the circulatory system has begun to develop. By about 30hpf, the heartbeat becomes more rhythmic and a weak circulation has initiated. At 36hpf, the circulation is much stronger and becomes progressively more widespread throughout the embryo. At around 42hpf, there is a prominent division of the atrial and ventricular chambers of the heart which yields a coordinated, two-part heartbeat of 180 beats per minute. The atrium contracts first and is followed by a ventricular contraction. After 60hpf, the heart has a distinct structure and is beating strongly.
1.7.3 Transparent embryonic development

The zebrafish embryo and larva are transparent for about one week after fertilization. Development of pigmentation in the zebrafish begins at 24hpf in the retina and skin, but is very light and appears transparent [51]. At 36hpf, the tail begins to develop pigmentation and pigmentation in the eye is dark and prominent. Embryonic transparency enables the tracking of cellular and molecular events over time by direct observation using a bright field microscope.

1.7.4 High fecundity

One zebrafish breeding pair can yield hundreds of fertilized embryos. Zebrafish reach sexual maturity at 10-12 weeks, but generate the highest clutch size when they are between 7-18 months old [52]. High fecundity enables large-scale experimentation in a short period of time as well as more statistically significant results due to the large sample size.

1.7.5 Ease of embryo manipulation

Since the zebrafish embryo is easily accessible, it can be chemically, mechanically, or genetically manipulated. My study specifically takes advantage of the latter source of manipulation. Many techniques for generating transgenic zebrafish have been reported, all involving the delivery of small concentrations of DNA into embryo recipients [53]. In my study, I have used transposon systems for delivery of specific DNA fragments into the genome of zebrafish embryo recipients in attempts to generate zebrafish lines with tissue-specific expression of fluorescent reporter proteins and a zebrafish model of cardiac necrosis. Transposon systems have the advantage of offering one of the highest frequencies (30-50%) for generating transgenic founder fish [53]. Additionally, they can be used to create stable transgenic lines with tissue-specific reporter expression.
1.7.6 Conserved Cardiac Developmental Processes

Most organ systems within the zebrafish develop according to similar genetic patterning events as those observed in mammals, which leads to a similar organ structure and function. Of particular interest for my study was the homology observed between zebrafish and human cardiology and hematopoiesis. As in mammals, the heart is the first organ to form in the zebrafish and it forms through a similar series of cellular specializations and migrations that are regulated by gene expression patterns [54]. The heart is positioned at the ventral midline of the zebrafish embryo facilitating clear observation of cardiac development. The stages of zebrafish heart development are described in Trinh et al [54]. Initial cardiac progenitor cells align along the yolk-blastoderm interface during the blastula stage of embryonic development and occupy the first few tiers of cells. During gastrulation, the progenitor cells arrange themselves into 2 bilateral strips, known as heart fields, within the anterior lateral plate mesoderm (LPM) region. These strips consist of 3 rows of progressively specialized progenitor cells. The most medial layer consists of endocardial progenitors and the most lateral layer consists of atrial progenitors, leaving ventricular progenitors in the middle. The cardiac progenitor cells migrate towards the midline of the embryo where they fuse to form a cardiac cone with endocardial precursor cells located on the luminal surface and myocardial precursors on the exterior. The ventricular precursors are located at the apex of the cardiac cone. The cone tilts and then elongates to yield a single heart tube which has been positioned to the left-side of the embryo. The heart tube begins to bend to form an S-shaped tube with 2 distinct cardiac chambers, the atrium and the ventricle, separated by an atrioventricular boundary. The ventricle is positioned to the right of the atrium upon completion of zebrafish embryonic heart development.
Similarly, the genetic pathways involved in cardiac development are conserved in vertebrates [55]. Several transcription factors involved in mammalian cardiomyocyte differentiation are conserved in the zebrafish, including nkx2.5, hand2, gata-4, -5 and -6, and tbx-5 and -20 [54,55]. In the zebrafish, Nkx2.5 is first expressed in the anterior LPM and continues to be expressed throughout development in cardiac progenitor cells [56]. Gata4/5/6 are members of the zinc finger transcription factor GATA family and are important genes for normal embryonic heart formation in multiple animal model systems [57]. Furthermore, several sarcomeric proteins are conserved between humans and zebrafish, such as actin thin and thick filament and myosin heavy and light chain proteins [55]. Zebrafish cardiomyocyte-specific markers implicated in my study include cardiac myosin light chain-2 (cmlc2), troponin I-HC (tnni-HC) and cardiac troponin T2 (tnnt2). Cmlc2 gene expression initially overlaps with nkx2.5 expression at the anterior boundary of the notochord [54,58]. It marks myocardial progenitors within the cardiac cone and continues to be expressed by cardiomyocytes throughout development and into adulthood. Tnni-HC and tnnt2 are both thin filament proteins within the sarcomere units of adult cardiomyocytes and play important roles in regulating cardiac contraction in response to intracellular calcium levels [59,60]. The troponin genes begin to be expressed within myocardial progenitors during the somitogenesis phase of development and continue to be expressed in cardiomyocytes within the adult heart [61].

Moreover, several genetic zebrafish models which phenotypically mimic human cardiac diseases carry a genetic mutation within a gene that is also implicated in the human disease. For example, the homozygous silent heart (sih) zebrafish experiences myocyte disarray and sarcomere loss within cardiomyocytes [60], consistent with that observed in human cases of familial hypertrophic cardiomyopathy in which TNNT2 mutations were implicated [62].
Likewise, the *sih* mutation affects the *tnnt2* gene in zebrafish [60]. *Tnnt2* encodes the sarcomeric thin filament protein, TNNT2, in cardiomyocytes. TNNT2 is part of the troponin-tropomyosin complex (TTC) which regulates cardiac muscle contractions in response to calcium. Other examples include the zebrafish *pickwick* (*pik*) and *heartstrings* (*hst*) mutations [63,64]. The *pik* mutation manifests as a thin-walled, dilated and poorly contractile heart, which resembles that of patients with dilated cardiomyopathy [63]. This mutation affects the zebrafish *titin* (*ttn*) gene. TTN is a sarcomeric protein which acts as a scaffold for thick and thin filament assembly. Some cases of human familial dilated cardiomyopathy have been attributed to mutations in *TTN* [65]. Thus, the zebrafish and human *titin* gene play an equivalent role in upholding sarcomere integrity and myofibrillar elasticity. Finally, the zebrafish *heartstrings* (*hst*) mutation, which affects the zebrafish *tbx5* gene, manifests as heart and fin defects [64]. These defects are characteristic of the human genetic disorder, Holt-Oram syndrome, which is also caused by a mutation in the human *TBX5* gene [66]. Thus, several zebrafish genes play an equivalent role as their human homologues in maintaining normal cardiac cell structure and function.

The structure of the adult zebrafish heart is closely matched to that of the mammalian heart. One major difference is that the zebrafish heart consists of only two chambers, one atrium and one ventricle. As in mammals, the ventricle has a thick myocardium and trabeculae and is responsible for pumping blood into the circulatory system to deliver oxygen to the body tissues [67]. Blood is pumped from the ventricle into the dorsal aorta and then returns to the atrium via the sinus venosus. There are a number of genetically mutated zebrafish lines available that model human cardiac disease phenotypes and will be useful for studying novel therapies for cardiac disease, such as stromal cell therapy [68,69]. Uniquely, the zebrafish embryo is able to survive
up to 7 days without a heart beat via diffused oxygen which is beneficial for studies investigating repair of non-functional myocardium in vivo [70].

1.7.7 Conserved Hematopoietic Developmental Processes

Similarly, zebrafish hematopoiesis closely resembles that of mammals. However, one important distinction is that the adult hematopoietic organ in the zebrafish is the kidney. The zebrafish kidney functions equivalent to the mammalian bone marrow in generating stem and progenitor cells capable of producing all mammalian blood cell lineages [71]. Zebrafish blood cell lineages are morphologically and functionally similar to the mammalian hematopoietic cells with the exceptions that mature erythrocytes are nucleated and thrombocytes are structurally and functionally analogous to the human platelet cell [72-74].

Although both the zebrafish kidney marrow-derived hematopoietic stem cell and the mammalian BM-MSC have been widely studied for many years, a zebrafish kidney marrow-derived MSC has not yet been described. However, a zebrafish kidney marrow-derived MSC-like cell, the zebrafish kidney stromal (ZKS) cell, was identified in 2009 by Stachura et al. as reported in Blood [75]. This group developed ZKS cell lines, including ZKS1 which was applied in my research, through continuous passage of primary ZKS cells. The focus of this research was to develop an in vitro system that could support zebrafish hematopoiesis and hematopoietic differentiation. Prior to this study, hematopoietic cells would not persist in culture alone which limited in vitro characterization and in vivo transplantation of this cell population. This group showed that co-culture of whole kidney marrow (WKM) cells atop a ZKS cell line at 80-90% confluence enables hematopoietic proliferation and differentiation in vitro. Culture conditions for generating ZKS cell lines from long-term cultures of WKM tissue was established by this group. They also described the ultrastructure of ZKS cell lines as fibroblastic and revealed that the cells
lacked expression of 2 zebrafish hematopoietic genes, *cd45* and *gata1*. As the ZKS cell shares some common cellular properties to BM-MSCs, the next step is to determine whether the ZKS cell can be classically defined as an MSC according to the criteria established by ISCT [16].

To complement both the study on ZKS cell lines and our recent findings demonstrating the ability of BM-MSCs to express cardiac-specific markers in an embryonic cardiac microenvironment *in vitro*, I have performed a more extensive characterization of the ZKS cell line and have initiated the development of an *in vivo* embryonic system for testing the capacity of marrow stromal cells to differentiate into cardiomyocytes in an embryonic *in vivo* setting using the zebrafish.
CHAPTER 2.0 – HYPOTHESIS AND SPECIFIC AIMS

HYPOTHESIS:
Zebrafish possess a kidney marrow-derived mesenchymal stromal cell with the capacity for cardiac differentiation in an embryonic environment *in vivo*.

SPECIFIC AIMS:

SA1 – Characterize a zebrafish cell-type equivalent to the mammalian BM-MSC

SA2 – Test cardiac differentiation of zebrafish stromal cells using embryonic CMs *in vitro*

SA3 – Establish an embryonic zebrafish cell transplantation system to test cardiac differentiation of zebrafish stromal cells *in vivo*

SA4 – Mark zebrafish stromal cell cardiac differentiation by fluorescent reporter gene expression using transgenic vectors
CHAPTER 3.0 – SA1: Characterizing a Zebrafish Cell-type Equivalent to the Mammalian BM-MSC

3.1 Introduction

The BM-MSC has been identified in several mammalian species; however, the existence of a zebrafish MSC has not yet been proven. An MSC-like cell has recently been identified and labelled as the zebrafish kidney stroma (ZKS) cell [75]. Optimal culture conditions for generating ZKS cells were determined to consist of incubation at 32°C and 5% CO₂ within ZKS culture media consisting of 10% ES cell qualified fetal bovine serum (FBS), 55% L-15, 32.5% DMEM and 12.5% Ham’s F-12 and supplemented with 150mg/L sodium bicarbonate, 2% penicillin/streptomycin (10U/mL), 1.5% HEPES, 1% L-glutamine and 0.1mg/mL gentamycin. Upon 60-80% confluence, ZKS cells were detached from culture using 0.25% trypsin and re-plated under the same conditions to expand cell numbers. ZKS cells appeared to be density dependent and grew optimally at 25-50% confluency.

This same group generated a ZKS cell line (ZKS1) which demonstrates morphological and functional consistency with MSCs. These cells were described as fibroblastic in morphology as they were irregularly branched with large filamentous processes, and contained a heterochromatic nucleus and a prominent endomembrane system. ZKS1 does not express the hematopoietic genes cd45 and gata1, but expresses a number of transcripts, cytokines and Notch ligands that support hematopoietic progenitor cell maintenance and differentiation in vitro. Moreover, ZKS1 is able to support hematopoietic cell proliferation and differentiation in vitro when co-cultured with WKM cells.
Although some properties of the ZKS cell have been described, it has not yet been defined as a zebrafish MSC. Since an MSC-specific marker has not been discovered thus far, the International Society for Cellular Therapy (ISCT) has developed a set of defining properties for MSCs to ensure consistency among studies [16]. First, the cell must adhere to plastic tissue culture dishes in vitro. To test for plastic adherence, mammalian BM-MSCs are normally cultured in media consisting of Dulbecco’s modified Eagle’s Medium (DMEM) or α-MEM supplemented with 10% fetal calf serum in an incubator set at 37°C and 5% carbon dioxide (CO₂) [14]. MSCs must also express the cell surface antigens CD90, CD73 and CD105 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. Other common antigens found on MSCs include CD106 and CD54 [14]. Detection of surface antigen expression is achieved by immunocytochemical staining using antibodies against the desired antigen [16]. Finally, MSCs must possess a minimal in vitro differentiation capacity consistent of adipogenic, osteogenic and chondrogenic differentiation. Osteogenic differentiation is usually detected in vitro by cellular staining with Alizarin Red or von Kossa, adipogenic with Oil Red O, and chondrogenic with Alcian blue. Chondrogenic differentiation can also be detected by immunocytochemical staining for collagen type II.

Therefore, a goal in this study was to produce primary ZKS cells in culture and determine whether they can be defined as zebrafish MSCs through evaluation of their cellular properties. Due to the difficulties in generating primary ZKS cells in culture, my next goal was to determine whether the ZKS1 cell line could be defined as a zebrafish kidney marrow-derived MSC line.
3.2 Methods

3.2.1 Generation of primary zebrafish kidney stromal (ZKS) cell cultures

a. Isolation and Dissociation of the Whole Kidney Marrow from Adult Zebrafish

Adult zebrafish were anaesthetized in 100ppm of clove oil. Surgical scissors were used to make an incision along the ventral side of the fish from the abdomen up to the jaw. The digestive organs and swim bladder were carefully removed from the fish using #3 forceps (Fine Science Tools, Item No.11231-30) to expose the spine. The kidney is located just atop the spine and is distinguishable by its dark red colouration. The hematopoietic cells are most abundant within the head of the zebrafish kidney (the anterior end), while renal cells are more abundant in the tail kidney [76]. Therefore, starting from the head, the kidney was carefully removed from the spine using the forceps and placed into 1mL of phosphate-buffered saline (PBS). The whole kidney marrow (WKM) was dissociated by drawing the cell suspension up and down with either a P-200 pipette or a 1-mL disposable syringe (BD, Product No.309602) with a 26G tip and then filtered through a cell strainer cap atop a 5mL Falcon tube (BD, Cat No.352235). WKM cells were plated in a 24-well tissue culture dish at a density of 5x10⁵ cells/cm². Cells were also plated at densities of 1x10⁶ and 2x10⁶ cells/cm² in three experiments.

b. Tissue Culture of Primary Zebrafish Kidney Stromal (ZKS) Cells

Dissociated WKM cells were cultured in zebrafish kidney stroma (ZKS) medium, developed in collaborator Dr. Traver’s Lab, at 32°C, 5% CO₂ according to methods described previously for generating ZKS cell lines [75]. Appendix-1 lists the composition of ZKS medium. As another attempt, I coated the tissue culture plates with fibronectin before plating the WKM cells. Next, as the ZKS1 cell line obtained from the Traver Lab grew efficiently in ZKS medium, I generated primary ZKS cultures using a combination of fresh ZKS medium and either 25% or
50% conditioned medium from ZKS1 cultures. Medium was removed from ZKS1 culture after 3, 4 and 9 days of culture. Moreover, I cultured WKM cells according to the recently established protocol [75] with the exception that the 10% FBS within the ZKS medium was replaced with horse serum (HS). ZKS medium was also supplemented with either 10% embryo extract or 10% hydrocortisone. Finally, I tried plating higher densities of WKM cells (1x10⁶ and 2x10⁶cells/cm²) using original culture conditions [75]. In all trials, the media was replaced every 3-4 days and plated cells were left to grow for 2-3 weeks, at which time cultures were assessed for confluency and potential for first passage (T₁).

c. Passaging Primary ZKS Cells

Adherent ZKS cells were detached by incubation in 0.25% trypsin-EDTA at 32°C for 5-10 minutes or until cells began to detach from the surface of the plate. Trypsin was then deactivated by adding either 10X ZKS media [75] or 1X FBS to each well. The cell suspension was centrifuged at 1200rpm for 10min at room temperature and supernatant was carefully removed by aspiration. The cell pellet was resuspended in ZKS media and re-plated at a ratio of either 1:3 or 1:1 in either a 24-well or 96-well plate. After 1-2 weeks of growth, T₁ cultures were assessed for confluency and potential for second passage (T₂).
3.2.2 Maintenance of the ZKS1 and ZKS1(CMV:eGFP) cell lines

The ZKS1 cell line provided by the Traver Lab in San Diego, CA and the transgenic ZKS1(CMV:eGFP) cell line created in this study were maintained according to similar conditions described previously [75]. Briefly, ZKS1 was grown in ZKS medium within vented tissue culture flasks (BD Falcon, Ref No.353112) and incubated at 32°C in 5% CO₂. The medium was changed every 3-4 days. The cells were passaged using 0.25% trypsin-EDTA and neutralized with 1X FBS. Aliquots of the established ZKS1 cell line and the transgenic ZKS1 cell line created in this study were frozen down for long-term storage in liquid nitrogen. To freeze the cells, they were first detached from culture using 0.25% trypsin-EDTA, neutralized with 1X FBS and centrifuged to generate a cell pellet. The supernatant was removed and cells were resuspended in a freezing medium consisting of 50% ZKS medium and 50% FBS-diluted dimethyl sulfoxide (DMSO) (4:1). The cell suspension was distributed into freezing tubes at concentrations of 1 million cells per mL. The tubes were placed into an isopropanol jacketed freezing chamber at -80°C overnight and transferred to liquid nitrogen the next day. To thaw the frozen cells for growth in tissue culture, the freezing tube was removed from liquid nitrogen and placed in a 37°C waterbath for 5 min. Immediately after the cell suspension had thawed, the solution was pipette out and slowly released drop-by-drop into 5mL of fresh ZKS medium. The cell suspension was centrifuged at 1,200 rpm for 5 min to remove any traces of DMSO. The cells were resuspended in fresh ZKS medium and transferred to vented tissue culture flasks.
3.2.3 Characterization of the ZKS1 cell line

a. RT-PCR

RNA was extracted from ZKS1 according to the TRIzol protocol (Cat. No.15596-018). Briefly, ZKS1 cells were grown to confluency in 6-well plates, washed with 1X PBS twice, and then treated with 1mL of TRIzol. TRIzol was pipetted up and down to cover all cells and then the solution was transferred to an eppendorf tube. After leaving the tube at room temperature (RmT) for 3min, 200μL of chloroform was added and the solution was mixed well. Again, the suspension was left at RmT for 3min and then centrifuged at 12,000g for 15min at 4°C. The clear aqueous phase was carefully transferred to a new tube. 500 μL of isopropyl alcohol was added to the new tube, which was then mixed by inversion 5-10 times and left at RmT for 10min. The solution was centrifuged at 12,000g for 10min at 4°C and the supernatant was removed. 750 μL of 75% ethanol was added and the tube was mixed by inversion 3-5 times. The solution was centrifuged at 7,500g for 5min at 4°C and the supernatant was removed. The RNA pellet was left at RmT for 10min with the eppendorf lid open in order to allow residual ethanol to evaporate. The RNA was resuspended in double-deionized (ddH2O) and stored at -20°C. RNA concentration was determined by spectrophotometry.

To generate cDNA from 1μg of extracted RNA, the RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Part No.4368814, Lot No.0806056). The RNA was added to a master mixture of 2.0μL of 10X RT buffer, 0.8μL of 10mM deoxynucleotide triphosphates (dNTP), 2.0μL of 10x RT Random Primers, 1.0μL Reverse transcriptase and ddH2O to produce a total volume of 20μL. The RNA solution was run in a thermal cycler under the following conditions: 25°C for 10min, 37°C for 60min, 37°C for 1min, 85°C for 5min and 4°C for short-term storage.
Finally, to detect expression of stromal genes by ZKS1, 3 different RT-PCR oligonucleotide primer sets were designed using the electronic Primer3 program [77] to bind specifically to zebrafish stromal cell markers cd106 (vcam1), cd73 (nt5’e), and cd90 (thy1). These primer sets were designed to span 2 adjacent exons which flank an intron of considerable size within the gene of interest. This design strategy is used to prevent any DNA contamination from confounding RT-PCR results. Primer sets designed for zebrafish stromal markers as well as primer sets previously created to detect zebrafish hematopoietic markers are listed in Table-1.

2μL of the cDNA product was added to a mixture of PCR reagents consisting of 2.5μL 10X PCR reaction buffer (Invitrogen, Lot 620874), 0.75μL 50mM MgCl2 (Invitrogen, Lot 401309), 0.5μL 10mM dNTP, 0.5μL 10μM forward primer, 0.5μL 10mM reverse primer, 0.125μL Taq DNA polymerase (Invitrogen, Cat 10342-020, Lot 644287) and ddH20 to yield a total volume of 25μL. PCR conditions for primers against zebrafish stromal genes were as follows: 94° C for 45s, 35 cycles of denaturation at 94° C for 20s, annealing at 57° C for 30s and elongation at 72° C for 45s, 72° C for 7min, and 4° C for storage. Conditions for primers against zebrafish hematopoietic genes were similar with the exception that the annealing temperature was raised to 60° C. The PCR results were analyzed using gel electrophoresis and the CloneSize 100bp DNA ladder (NORGEN, Biotec Corp., Cat No.11600, Lot No.2D99B8).

b. Immunocytochemical Staining and Flow Cytometry

The following antibodies were used in this experiment: Anti-human CD73-PE (BD Pharmingen, Cat-550257, Lot-24750), anti-human CD90-FITC (Serotec, Batch-0305), and the isotype control FITC-mouse-IgG1 (Pharmingen, Cat-20604A, Lot-M045141). Immunocytochemical analysis was performed using two different methods. Slides generated by
cytospin of ZKS1 were analyzed using the Zeiss LSM510 Confocal microscope and suspended ZKS1 cells were assessed using flow cytometry (FC500 Flow Cytometer, Beckman Coulter).

For cytospin analysis, ZKS1 cells were detached from tissue culture flasks and centrifuged for 10 min at 1,000 rpm onto glass slides. Adherent cells were fixed to the slides by treatment with 2% paraformaldehyde for 10 min. The slides were then washed three times for 5 min each wash using Earls Buffered Salt Solution. Meanwhile, the monoclonal antibodies were diluted 1:20 in ddH$_2$O. Three slides of fixed cells were co-stained with both CD90-FITC and CD73-PE antibodies, while another slide was stained with an IgG1-FITC antibody to serve as a negative control. Cells were stained with 50 μL of diluted antibody for 1 hr at room temperature in a moist environment. After staining, slides were washed again three times for 5 min. For slides that were co-stained, the last 2 steps were repeated using the second antibody. To seal the immunostained cells and mark the cell nuclei for easy detection of cells during analysis, one drop of ProLong antifade with DAPI (Invitrogen, Cat No.P-36931) was spread evenly above the cells which were then sealed with a glass coverslip. The cells were then ready to be observed for FITC, PE and DAPI fluorescence using confocal microscopy. Human T4 BM-MSCs were stained with all 3 antibodies to serve as a positive control.

Immunofluorescent staining was also performed on live suspended ZKS1 cells and analyzed using fluorescently-activated cell sorting (FACS). ZKS1 cells were detached from tissue culture and washed twice with 1X PBS and once with FACS buffer (1X PBS + 1% FBS + 5 mM EDTA). The cells were resuspended in FACS buffer at a concentration of 5x10$^5$-1x10$^6$ cells per sample. Anti-human antibodies were diluted 1:10 in FACS buffer. 50 μL of the dilute antibody was added to a ZKS1 cell pellet of approximately 1x10$^6$ cells and left at 20°C for 30 min in a dark compartment. After 30 min, the cells were centrifuged at 2,000 rpm for 4 min and washed twice.
with FACS buffer. The cells were then resuspended in 500μL of FACS buffer (or 2% paraformaldehyde for short-term storage) and transferred to FACS tubes, ready to be analyzed using the FC500 Flow Cytometer. Cells staining positive for CD90-FITC were detected by the FL1 channel and those positive for CD73-PE by the FL2 channel. Flow cytometry recordings were analyzed using the FloJo software.

**c. In vitro Adipogenic and Osteogenic Differentiation Assays**

Differentiation assays were carried out in 12-well plates and induction media was added when plated ZKS1 reached approximately 80% confluency. Murine balb-c T4 BM-MSCs were used as a positive control and ZKS1 cultured in ZKS medium alone was used as a negative control. The induced murine BM-MSCs were incubated at 37°C, 5% CO₂, while induced ZKS1 was incubated at 32°C, 5% CO₂ for all *in vitro* differentiation experiments and induction medium was changed every 2-3 days for 2 weeks.

**Adipogenic Assay:** Mammalian adipogenic assays were performed according to procedures described previously [78]. The murine BM-MSC control and ZKS1 were induced by a base medium (DMEM-high glucose [hg] + 10% FBS) supplemented with 10μg/mL bovine insulin, 60μM indomethasin, 1μM dexamethasone, and 0.5mM 3-isobutyl-1methylxantina (3-IBMX). After 3 days, the induction media was removed and the base medium supplemented with 10μg/mL insulin was added to the cells. After 2 weeks of adipogenic induction, analysis of adipogenesis by ZKS1 and the murine BM-MSC control was carried out by Oil-Red-O staining according to methods described by Zhao et al [79]. Briefly, cells were washed twice with PBS, fixed with 10% neutral buffered formalin for 30min, and stained with 0.5% Oil-Red-O in isopropanol:distilled water (60:40) for 30min. Stained cells were photographed under a bright field microscope at 20X magnification. A modified adipogenic differentiation assay was also
tested for ZKS1 using ZKS medium as the base medium in place of MSC medium. All other steps of the procedure remained constant for this assay. Finally, I began conducting a preliminary experiment of inducing ZKS1 adipogenesis in vitro according to a protocol used to differentiate stromal-vascular cells of red sea bream fish, Pagrus major, into adipocytes [80]. At about 80% ZKS1 confluency, medium consisting of DME/F12 (1:1) supplemented with 65mM NaCl, 10% FBS, 100μg/mL streptomycin and 100U/mL penicillin, 0.042mg/mL linoleic acid, 50μg/mL transferrin, 5ng/mL sodium selenite, 50ng/mL hydrocortisone, and either 25μg/mL, 50μg/mL or 100μg/mL of insulin was added. In the previous study, 50μg/mL was sufficient for clear visualization of adipogenesis after analysis. After 2 weeks of induction, adipogenesis was evaluated by Sudan Black B staining according to methods described previously [80]. Briefly, cells were washed with 1X PBS, fixed in 10% neutral-buffered formalin, and stained with Sudan Black B.

**Osteogenic Assay:** Mammalian osteogenic assays were performed according to procedures described previously [81]. The murine BM-MSC control and ZKS1 were induced by a base medium (DMEM-low glucose [lg] + 10% FBS) supplemented with 50μM ascorbic acid 2-phosphate, 10mM β-glycerophosphate, and 0.1μM dexamethasone. After 2 weeks of induction, osteogenesis was evaluated using Alizarin Red S staining according to similar methods described by Zhang et al [78]. In short, cells were washed in phosphate-free saline solution, fixed with 10% neutral buffered formalin or 4% paraformaldehyde for 30min, and stained with 1% Alizarin Red S dye for 10min. Stained cells were photographed under a bright field microscope at 10X magnification. A modified osteogenic differentiation assay was also trialed for ZKS1 using ZKS medium as the base medium. All other methods remained the same for this assay.
In total, 7 different trials of both ZKS1 adipogenic and osteogenic differentiation assays, using on average 8 wells per trial, were performed using standard mammalian MSC in vitro adipogenic and osteogenic differentiation conditions, respectively. Only one trial was performed using adipogenic differentiation conditions designed for red sea bream stromal-vascular cells.

d. Colony-forming Unit Fibroblast (CFU-F) Assay

ZKS1 was plated in 5 wells of a 12-well tissue culture plate using a limited dilution plating technique starting with 160 cells in well #1 and ending with 10 cells in well #5. Cells were left to grow in ZKS medium for 8-10 days, after which they were fixed with methanol for 5 min and then stained with Giemsa (Fisher Scientific, UN1993) for 5 min. After gently washing away excess stain, the number of blue-coloured, Giemsa-stained adherent colonies consisting of at least 50 cells were counted under the microscope using 10X magnification. The Microsoft Excel software was used to plot collected data and calculate the rate of CFU-F formation by ZKS1. An average number of colonies formed for each limited dilution level (10, 20, 40, 80 and 160 ZKS1 cells plated) was calculated from the 5 trials and plotted in a graph. The standard deviation of this average was also calculated and incorporated into the graph as error bars. A trendline was drawn based on the plotted points and the average rate of CFU-F formation per number of cells plated was determined by calculating the slope of the trendline.
3.3 Results

3.3.1 Primary ZKS cell cultures do not survive past first passage (T1)

To study the BM-MSC in zebrafish, an MSC population within the adult zebrafish kidney, which is equivalent to the bone marrow in mammals needed to be identified. As the recently identified ZKS cell resides in the adult zebrafish kidney, I wanted to isolate expandable primary ZKS cells in tissue cultures and characterized them according to the ISCT definition of MSCs. Table-2 lists the various combinations of serum, cell concentrations and other media reagents used in attempts to generate primary ZKS cell cultures. In total, I generated 96 T₀ tissue culture wells from plating dissociated WKM cells (Figure 1a). I was able to passage 14 wells of the 96 T₀ cell cultures (14.6%) (Figure 1b) and carry out a second passage for 3 wells of the 14 T₁ cell cultures (3.1% of all T₀ cultures) (Figure 1c). Very few T₂ ZKS cells were adherent to the bottom of the tissue culture dish and those that did adhere took on an irregular morphology and were not clonogenic. After adult zebrafish WKM isolation, separation and plating, spindle-shaped adherent cells began to emerge from colony forming unit fibroblasts (CFU-F) between 3-5 days after the initial plating and would expand in a radial fashion. ZKS cell cultures were grown for around 2 weeks at which point they were assessed for confluency and selected for passage if confluency exceeded approximately 25%. Adherent cells within T₁ cultures could be visualized as early as 24 hours after re-plating. Using the recently published protocol for generating ZKS cell lines from dissected adult zebrafish kidneys [75], only 17% of all T₀ ZKS cultures could be passaged to T₁ and 3% passaged to T₂. Modifications to the established ZKS medium also did not appear to generate T₁ ZKS cultures at a rate consistent with that of mammalian MSC cultures generated from bone marrow cells. Modifications to the ZKS medium included a substitution of 10% FBS with 10% HS, an addition of either 10% zebrafish embryo
extract or 10% hydrocortisone, or a substitution of a fraction of ZKS medium with conditioned ZKS media extracted from ZKS1 cultures after 3, 4, and 9 days. Finally, coating the tissue culture dishes with fibronectin did not appear to greatly improve the frequency of successful T1 ZKS cultures (20% of T₀ vs. 17% of T₀ with original conditions). Table-2 lists the results of the primary ZKS cultures generated under 7 different culture conditions.

3.3.2 ZKS1 is MSC-like in that it is plastic adherent in culture and expresses classical stromal cell genes.

Due to the difficulties in generating primary ZKS cells using the culture conditions tested here, I attempted to characterize the recently identified zebrafish kidney stromal cell line (ZKS1) using the standard criteria for defining mammalian BM-MSCs: plastic adherence in culture, specific stromal and hematopoietic antigen expression patterns, and in vitro differentiation into adipocytes, osteoblasts, and chondroblasts [16]. When ZKS1 cells are in suspension, they have a circular morphology. However, when grown in ZKS medium within vented tissue-culture flasks and incubated at 32°C, 5%CO₂, the cells adhere to the bottom of the flask and become spindle-shaped as shown in Figure 2a.

ZKS1 stromal and hematopoietic gene expression profiles were determined by RT-PCR. ZKS1 expresses zebrafish genes homologous to the classical mammalian stromal cell markers CD106, CD73, and CD90 (Figure 2.b.i). Furthermore, ZKS1 does not express hematopoietic genes cd45, gata1, lmo2, mpo, igM, and pax5 (Figure 2.b.ii).
3.3.3 Unlike MSCs, ZKS1 expresses a subset of hematopoietic genes not normally expressed by MSCs and is unable to differentiate into adipocytes or osteoblasts within mammalian *in vitro* differentiation assays.

According to RT-PCR results, ZKS1 appears to express a minority of hematopoietic genes at low levels, including *cmyb*, *pu.1*, *gata3*, *rag2*, and *runx1* as compared to the whole kidney marrow (WKM) control (Figure 2.b.ii).

Furthermore, since optimal culture conditions for induction of adipogenic or osteogenic differentiation by zebrafish progenitor cells *in vitro* have not been established, I tested whether the standard *in vitro* adipogenic or osteogenic differentiation assays currently established for mammalian BM-MSCs would be compatible with ZKS1. According to Oil Red O staining, I found that ZKS1 does not differentiate into adipocytes after 2 weeks of culture in mammalian adipogenic induction medium when compared to a murine BM-MSC control (Figures 2.c.i & 2.c.iii). Moreover, it appeared that ZKS1 cells were unhealthy and dying after 2 weeks in the assay. Thus, I also tested a modified protocol in which the base medium was replaced by ZKS medium; the aim was to improve ZKS cell survival by inducing ZKS cells in medium that is optimal for their growth *in vitro*. However, results of these trials were comparable to those using standard mammalian MSC adipogenic induction medium. I also conducted a preliminary adipogenic assay on ZKS1 using a protocol designed to induce adipogenic differentiation by stromal-vascular cells of the red sea bream, *Pagrus major* [80]. My preliminary findings were comparable to the results obtained when using mammalian MSC adipogenic assays in that ZKS1 did not differentiate into adipocytes *in vitro* as determined by absence of Sudan Black B cell staining. Similarly, we found that ZKS1 does not differentiate into osteoblasts after 2 weeks of culture in mammalian osteogenic induction medium (Figure 2.c.ii) compared to the murine BM-
MSC control (Figure 2.c.iv) as determined by Alizarin Red S staining. Again, ZKS1 within the differentiation assay appeared to be dying and using a base ZKS medium did not improve the survival or osteogenesis of ZKS1.

3.3.4 Antibodies against human stromal cell antigens CD90 and CD73 do not cross react with zebrafish stromal cell antigens.

Immunofluorescent staining is a standard method for defining BM-MSCs according to stromal and hematopoietic antigen expression profiles and for generating purified MSC populations [14]. In an attempt to characterize and purify the ZKS1 cell line, I tested whether monoclonal antibodies reactive against human MSC antigens cross-react with zebrafish stromal cell antigens. As I have demonstrated by RT-PCR that ZKS1 expresses the cd73 and cd90 genes, I selected monoclonal anti-human antibodies specific for these two stromal markers. ZKS1 was double-stained with both anti-human CD90-FITC (green fluorescence) and anti-human CD73-PE (red fluorescence) monoclonal antibodies. The cell nucleus was also stained using DAPI (blue fluorescence). Human T4 BM-MSCs were used as a positive control. I found that neither antibody was reactive with stromal cell antigens on ZKS1 (Figure 3.i) as compared to the positive control (Figure 3.ii). An antibody against IgG1 was used as a negative control and confirmed that the positive staining for human T4 BM-MSCs was indeed a result of the interaction between the antibodies and the surface stromal cell antigens (Figures 3.iii & 3.iv).
3.3.5 **ZKS1 forms colonies at a rate of 8:1.**

To determine the clonogenic capacity of the ZKS1 cell line, I conducted CFU-F assays. ZKS1 was plated using a limiting dilution technique starting with an initial cell count of 160 cells per well and ending with 10 cells/well. After 8 days in tissue culture, Giemsa-stained ZKS1 colonies consisting of at least 50 cells were counted. Data accumulated from 5 ZKS1 CFU-F assays suggests that one ZKS1 CFU-F will form for every 8 ZKS1 cells plated (Figure 4).

3.4 **Discussion/Conclusions**

The human bone marrow includes hematopoietic stem/progenitor cells and MSCs. Although it is well-known that hematopoietic stem/progenitor cells reside within the zebrafish kidney marrow, the human bone marrow equivalent, no studies have sought to discover a zebrafish kidney marrow-derived cell with the defining properties of MSCs. Therefore, my aim was to identify, isolate and characterize a zebrafish cell-type equivalent to the human BM-MSC. Initially, I attempted to generate primary zebrafish MSC cultures by plating adult WKM cells in long-term culture medium established for human MSCs (Appendix-2) and incubating the cells at 37°C, 5% CO₂ [82] (data not shown). After 85 attempts, I concluded that these conditions were insufficient for generation primary ZKS cell cultures as T0 ZKS cells could neither expand nor survive past 5 days in culture. However, I had conducted these experiments prior to learning that zebrafish cells generally survive best at lower temperatures *in vitro* [75,83,84]. In general, zebrafish cell cultures are often propagated at 26°C [83], however blastula-staged zebrafish embryonic stem cell cultures have also been generated at 22°C [84]. Fortunately, around this same time, a zebrafish kidney stromal (ZKS) cell had been identified in collaborator Dr. Traver’s Lab which appears to share morphological and functional properties with human MSCs [75].
Their research concluded that prolonged culture of ZKS cell lines at 37°C was toxic to the cells and that ZKS appeared healthy in cultures at physiological temperature (28°C), but expanded optimally at a temperature of 32°C [75]. Thus, I attempted to isolate primary ZKS cells in culture by plating WKM cells in culture conditions described previously for generating ZKS cell lines, which generally involves culturing the cells in ZKS medium (Appendix-1) at 32°C, 5% CO₂ [75]. Under these conditions, adherent, spindle-shaped cells morphologically similar to mammalian BM-MSCs were observed beneath a cobblestone network of cells by 5-7 days in culture. This observation is consistent with what is observed when mammalian bone marrow cells are grown under standard long-term culture conditions [82]. As studies assessing cardiac differentiation by mammalian BM-MSCs generally use fourth passaged (T4) BM-MSCs [42,43,45], generation of primary T4 ZKS cells is necessary for consistency. However, in 83% of primary T0 ZKS cultures produced, the adherent cells were not able to expand to high enough cell numbers for passage. Furthermore, only 3% of primary ZKS cells were able to reach second passage (T2) and none of these T2 cultures formed colonies of ZKS cells. Conversely, when mammalian BMCs are plated in culture, virtually all primary T0 cultures can be expanded to at least fourth passage (T4), as demonstrated by colleagues in the Keating Lab. Therefore, the culture conditions used previously for generating ZKS cell lines appear to be inadequate for generating expandable primary ZKS cells.

Consequently, I experimented with new culture conditions involving different modifications to the original ZKS medium. I attempted to grow ZKS cell cultures within ZKS medium which was supplemented with either 10% HS instead of FBS, 10% hydrocortisone, 10% zebrafish embryo extract or 25-50% ZKS1 conditioned media. Fish embryo extract has been used previously to successfully generate zebrafish embryonic cell cultures [83-85]. Although
only a limit number of primary ZKS cultures were generated using modified ZKS media, it appears as though none of the modifications make a distinct improvement in expansion of ZKS cells after WKM was cultured for 2 weeks. Similarly, neither increasing the initial WKM cell number plated by 2-4X nor coating tissue culture plates with fibronectin greatly improved the frequency of successful T1 ZKS cultures.

Difficulties in generating primary ZKS cell cultures may be attributed to a number of factors. First, there may be a restricted capacity for ZKS proliferation due to the intrinsic biology of the zebrafish. Zebrafish kidney marrow-derived hematopoietic cells and WKM cells were also very difficult to culture in vitro initially. It was not until the ZKS1 cell line was discovered and used as feeder cells in co-cultures with hematopoietic or WKM cells that these cells could persist, proliferate and differentiate in tissue culture [75]. Next, both the previously established and currently tested culture conditions may not be adequate for supporting ZKS cell proliferation in vitro. The antibiotic composition within the ZKS medium could be impairing the expandability of ZKS cells in vitro as fibroblast cells have been found to show sensitivity to antibiotic exposure [86]. Moreover, the culture medium may require supplementation with a combination of soluble growth factors to support ZKS growth and proliferation. Some growth factors known to promote MSC expansion in vitro include epidermal growth factor (EGF) [87], fibroblast growth factors (FGFs) [88,89], TGF-β and platelet-derived growth factor (PDGF) [89].

Since primary ZKS cells proved difficult to generate, I took a different approach to identifying a zebrafish MSC. As the Traver Lab had developed ZKS cell lines with indefinite expansion, I chose to determine whether one of their cell lines (ZKS1) could be defined as a zebrafish MSC line according to the criteria established by the ISCT [16]. First, I confirmed that ZKS1 was plastic adherent in culture using standard ZKS culture conditions [75]. Next, I looked
at the stromal and hematopoietic gene expression profile of ZKS1 using RT-PCR. The criterion for defining MSCs involves expression of CD73, CD90 and CD105 [16]. However, as the zebrafish genome does not carry a CD105 homolog according to The Zebrafish Model Organism Database (www.zfin.org), I selected another gene commonly expressed by MSCs, CD106 [14]. Furthermore, RT-PCR primer sets for zebrafish cd73 (nt5e), cd90 (thy1) and cd106 (vcam1) genes had not yet been created. Thus, we designed RT-PCR primer sets to bind to the cDNA of each of these 3 genes. The forward and reverse primers were designed to bind to adjacent exons which span one large intron within the desired gene. The primers were also designed to be specific to the zebrafish species. Through RT-PCR, we showed that ZKS1 expresses the stromal cell genes cd73, cd90 and cd106. However, these results are limited by the absence of an adequate negative control. Most studies assessing stromal marker expression by mammalian MSCs use hematopoietic cells as a negative control. Therefore, to overcome this limitation, mRNA should be extracted from zebrafish hematopoietic cells and tested for cd73, cd90 and cd106 expression using RT-PCR. Isolation of zebrafish hematopoietic cells from the WKM can be performed in a number of ways; however, the most efficient and reliable method is to extract the WKM tissue from adult transgenic zebrafish in which a hematopoietic-specific promoter, such as lmo2 or gata1, directs the expression of a fluorescent reporter protein. The extracted tissue would then be sorted for fluorescent protein expression using FACS and the fluorescently-labelled cells would be placed in TRIzol to extract RNA for gene expression analysis.

The next criterion for defining MSCs requires that the prospective cell lacks expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR [16]. These antigens are expressed on either hematopoietic or endothelial cells in mammals. For this study, I selected a panel of zebrafish genes that have been extensively used to mark various zebrafish hematopoietic
cell lineages [reviewed in 90] for characterization of hematopoietic gene expression by ZKS1. While ZKS1 did not express cd45, gata1, lmo2, lck, mpo, igM, and pax5, it did express a minority of hematopoietic genes not normally expressed by MSCs including cmyb, pu.1, gata3, rag2 and runx1. In almost all cases, expression levels of hematopoietic genes in ZKS1 were much lower than those in the positive control WKM cells. ZKS1 was generated from multiple passage of a primary ZKS cell culture and these cells have undergone more than 200 passages [75]. MSCs that have undergone more than 7 passages are at risk of experiencing a change in their global gene expression pattern [91]. Therefore, the finding of hematopoietic gene expression by ZKS1 could be attributable to potential replicative senescence experienced by ZKS1.

The final criterion for defining a cell population as MSCs required that the cells possessed the capacity for adipogenic, osteogenic and chondrogenic differentiation in vitro [16]. Zebrafish possess both adipocytes and osteoblasts, however no studies have assessed the differentiation of a zebrafish progenitor cell into either of these cell types in vitro. In fact, prior to 2009, zebrafish adipocytes remained uncharacterized [92]. Flynn III et al. demonstrated the homologies between zebrafish and mammalian adipocytes. As with the development of mammalian white adipocytes [93], zebrafish adipocytes contain several small lipid droplets during early-stages of terminal adipocyte differentiation. Furthermore, mature zebrafish adipocytes carry a single large lipid droplet and an eccentric nucleus, consistent with mature mammalian adipocytes [92,93]. Due to the conserved biology between zebrafish and mammalian adipogenesis, the capacity of ZKS1 to differentiate into adipocytes in vitro was assessed using mammalian BM-MSC adipogenesis assays, with the exception that the incubation temperature remained at 32°C instead of 37°C to support ZKS cell survival overtime. The adipogenesis induction medium primarily consisted of a
base DMEM media supplemented with bovine insulin. Under these conditions, ZKS1 was unable to differentiate into adipocytes in vitro as determined by Oil Red O staining. Additionally, I conducted a preliminary ZKS1 adipogenic differentiation experiment using an adipogenesis induction medium that was established for adipogenic differentiation of red sea bream stromal-vascular cells [80]. Although only single trial was conducted using this medium, my preliminary findings suggest that this medium does not induce the adipogenic differentiation of ZKS1 in vitro as determined by lack of Sudan Black B staining. Sudan Black B staining was used instead of Oil Red O in this experiment in order to keep consistency with the protocol used for differentiating red sea bream stromal-vascular cells.

Similarly, clear homologies exist between bones of mammals and zebrafish, both in terms of bone structure and patterns of bone development [94,95]. Therefore, induction medium used to differentiate mammalian BM-MSCs into osteoblasts was used to assess ZKS1 osteogenesis in vitro. As determined by Alizarin Red S staining, which stains the calcified matrix associated with bone tissue, ZKS1 was not able to differentiate into osteoblasts under these conditions.

The reasoning behind the lack of adipogenic and osteogenic differentiation by ZKS1 observed in this study remains elusive. One explanation is that there is a restrictive capacity for differentiation by the ZKS cell due to discrepancies between zebrafish and mammalian biology. Furthermore, ZKS1 is likely a permanent cell line and is thus at risk of acquiring a genetic mutations affecting its differentiation capacity [91]. Therefore, the observed lack of adipogenic and osteogenic differentiation by ZKS1 may not reflect the normal physiological situation. To rule out this option, the DNA of ZKS1 should be screened for genetic mutations. As MSCs are at risk of changes in cell differentiation potential after undergoing more than 7 passages [91] and ZKS1 has undergone more than 200 passages [75], ZKS1 cell differentiation potential may not
be reflective of primary ZKS cells. Next, the induction conditions may not be appropriate for adipogenic and osteogenic differentiation by zebrafish progenitor cells. Finally, the agents within the induction medium could, in fact, be appropriate for ZKS1 adipogenesis or osteogenesis, but may not be fish-specific. However, my preliminary findings suggest that this explanation is less likely as ZKS1 appeared to be unable to differentiate into adipocytes when using \textit{in vitro} induction conditions specific for induction of adipogenesis by a stromal-vascular cell derived from a fish species [80]. This experiment should be repeated to confirm this suggestion.

Through additional characterization of ZKS1, I found that the clonogenic capacity of ZKS1 is $8:1$, with one CFU-F forming per 8 ZKS1 cells plated. Therefore, the ZKS1 cell line must be heterogenous as a completely homogenous population of MSCs would have a CFU-F frequency of $1:1$. This finding is consistent with findings of mammalian MSC clonal assays which continually demonstrate that MSC populations are heterogeneous with individual cells holding varying clonogenic and expansion capacities [96]. The heterogeneity of the ZKS1 cell line could either be inherent, or could have arisen due to acquired mutations over passages, or a combination of both.

Finally, common strategies for purifying and characterizing MSCs are through immunohistochemistry (IHC) and FACS [16]. IHC is performed using both antibodies against stromal cell antigens and antibodies against hematopoietic cell antigens. Cells within a BMC culture that stain positive for stromal cell markers and negative for hematopoietic cell markers can be considered a purified population of MSCs. Currently, there are no available antibodies specific for zebrafish stromal and hematopoietic antigens. Additionally, there has been no investigation into whether antibodies against mammalian stromal markers cross-react with zebrafish stromal antigens. Hence, I tested the cross-reactivity of human monoclonal anti-CD90
and -CD73 antibodies on ZKS1 as ZKS1 was found to express both CD90 and CD73 genes. I found that both antibodies were not reactive with zebrafish stromal antigens. However, while the anti-CD90 antibody used in this study shows species cross reactivity with the cynomolgus monkey (Serotec, [www.abdserotec.com](http://www.abdserotec.com)), the anti-CD73 antibody has not yet been shown to react with multiple species (BD Pharmingen, [wwwbdbiosciences.ca](http://wwwbdbiosciences.ca)). Thus, the anti-CD73 antibody may be specific only to human antigens. A better approach would be to test polyclonal anti-CD90 and anti-CD73 antibodies that have an application across a wide range of species. This will increase the probability of discovering an antibody which cross-reacts with zebrafish stromal antigens. In this study, I tested monoclonal antibodies as they were both easily accessible from the Keating Lab and consistently shown to be successful for characterizing and purifying human MSCs by my colleagues.

In sum, ZKS1 cells cannot yet be defined as zebrafish MSCs due to their positive expression of certain hematopoietic genes and the lack of evidence for adipogenic and osteogenic differentiation by these cells. Moreover, ZKS1 may carry a genetic mutation which could explain the persistence of these cells *in vitro* while primary ZKS cells rarely expand past the T0 culture. Therefore, it would be best to develop an efficient strategy for reproducible generation of primary T4 ZKS cells *in vitro* in order to test ZKS cells for MSC-defining properties in a setting that is more reflective of normal zebrafish physiology.
CHAPTER 4.0 – SA2: Test Cardiac Differentiation of ZKS1 Cells using Embryonic Cardiomyocytes in vitro

4.1 Introduction

Several studies have shown that mammalian BM-MSCs can be induced to express cardiac-specific markers when co-cultured with embryonic or neonatal cardiomyocytes in vitro [42-44]. Our lab recently developed a novel co-culture system to study cardiac differentiation by BM-MSCs in an in vitro cardiac microenvironment [42]. Female murine transgenic BM-MSCs expressing GFP under the control of the alpha-myosin heavy chain (α-MHC) promoter were co-cultured with male rat embryonic cardiomyocytes (rCM) for 5-15 days. Cardiac-specific antigen expression by the co-cultured MSCs was determined by GFP expression as well as immunohistochemical staining against cardiac-specific markers on GFP-expressing cells. Co-cultured MSCs expressed a number of cardiomyocyte-specific markers, such as α-MHC, cardiac troponin T and troponin I. ZKS-CM co-culture systems have not been described in the literature thus far. Therefore, due to the availability of primary rCM cultures in our lab, I chose to conduct a preliminary evaluation of cardiac gene expression by ZKS1 after co-culture with rCM. Unlike findings from the murine MSC-rCM co-culture experiments, my preliminary findings suggest that ZKS1 cannot be induced by rCMs to express zebrafish cardiac-specific genes in vitro.
4.2 Methods

4.2.1 Generation of a ZKS1-rCM in vitro co-culture system

Approximately 2x10^6 rCMs were plated in a 6-well plate and grown at 37°C for 24hrs in rCM medium composed of DMEM/F12 (1:1) supplemented with 5% FBS, 10% HS, 0.5% PenStrep. After 24hrs in culture, the medium was removed and ZKS1 cells were added to the rCM culture. A range of ZKS1 cell numbers was tested (7.5x10^4, 1x10^5, 2.5x10^5, 5x10^5, 7.5x10^5, and 1x10^6). 50% ZKS medium and 50% rCM medium were added to the co-culture system which was maintained at 32°C, 5% CO₂ for 5 days. After 5 days, mRNA was extracted from the cells within the co-culture system using TRIzol as described in methods from Chapter-3.2.3(a).

4.2.2 RT-PCR analysis of co-cultured ZKS1

Primer sets specific for zebrafish tnt2, tnni-HC and cmlc2 genes are listed in Table-1. mRNA from 5 day co-cultured cells was extracted using the TRIzol method described in Chapter-3.2.3(a). The extracted mRNA consisted of both ZKS1 mRNA and rCM mRNA. Therefore, to ensure that zebrafish cardiac primers would not specifically bind to the mRNA of rCMs, I obtained rCM mRNA from a colleague and performed RT-PCR using zebrafish cardiac primers. As a positive control, I dissected 3 adult zebrafish to collect samples of heart tissue and again used TRIzol to extract the mRNA. As a negative control, I used mRNA extracted from ZKS1. RT-PCR conditions were the same as those described in Chapter-3.2.3(a), with the exception that the annealing temperature was raised to 59°C. All RT-PCR products were analyzed using gel electrophoresis and a 1-kb DNA ladder.
4.3 Results

4.3.1 ZKS1 does not express cardiac-specific genes when co-cultured with rat embryonic cardiomyocytes (rCMs) for 5 days.

To determine whether ZKS1 can be induced to express cardiac-specific markers in an \textit{in vitro} embryonic cardiac microenvironment, consistent with properties of mammalian BM-MSCs [42-44], preliminary ZKS1-rCM co-culture experiments were conducted. Cardiomyocytes extracted from hearts of embryonic rats were plated in 6-well plates for 24 hours prior to addition of ZKS1 (procedure performed by a colleague). Between $1 \times 10^5 - 1 \times 10^6$ ZKS1 cells were added to the rCM culture dish. In most experiments, rCMs began to contract after 24 hours in culture, even when the incubation temperature was reduced to 32°C. RT-PCR was performed to determine whether ZKS1 had been induced to express zebrafish cardiac-specific genes \textit{cmlc2}, \textit{tnnt2}, and \textit{tnni-HC}. I, first, confirmed that primers designed against zebrafish \textit{cmlc2}, \textit{tnnt2}, and \textit{tnni-HC} genes did not bind specifically to rat myosin light chain, troponin T, and troponin I genes, respectively, using RT-PCR (Figure 5.a). I also confirmed that ZKS1 alone does not express zebrafish cardiac genes (Figure 5.b). Finally, my preliminary findings from the ZKS1-rCM co-culture experiments suggest that ZKS1 cannot be induced to express \textit{cmlc2}, \textit{tnnt2} or \textit{tnni-HC} after 5 days of co-culture with rCMs (Figure 5.c).
4.4 Discussion/Conclusions

Mammalian BM-MSCs have been shown to express cardiac-specific markers when co-cultured with embryonic or neonatal cardiomyocytes in vitro [42-46]. However, cardiac differentiation by zebrafish stromal cells has never been assessed in vitro. Therefore, I first sought to perform an initial assessment of in vitro cardiac differentiation by ZKS1 using a modified version of a co-culture system previously established in our lab. The original co-culture system consisted of male rCMs and female murine T4 BM-MSCs isolated from transgenic B-a-Fvb mice expressing GFP under the cardiac-specific α-MHC promoter [42]. The MSCs were added to rCMs that had been cultured for 24 hours at 37°C and then the entire co-culture was incubated at 37°C. Co-cultured MSCs were assessed for cardiac-specific marker expression using RT-PCR or IHC between days 5 and 15, as it takes 7-10 days for spontaneous electrical activity to be observed in developing mouse embryos [97]. Likewise, in my co-culture experiment, the ZKS1 cells were added to rCMs that had been cultured alone for 24 hours and cardiac gene expression by co-cultured ZKS1 was determined by RT-PCR using oligonucleotide primers specific for zebrafish cardiac genes. I analyzed co-cultured ZKS1 cells for cardiac-specific gene expression after 5 days of co-culture as the heart is fully formed and functional in the developing zebrafish embryo by 2-3dpf [54]. Another difference between co-culture systems was that the co-culture of ZKS1 and rCM was incubated at a lower temperature of 32°C to support zebrafish cell survival. rCMs were still able to spontaneously contract when cultured at this temperature. In contrast to the findings of the original co-culture study which demonstrated that co-cultured BM-MSCs express a panel of cardiac-specific markers, ZKS1 could not be induced by rCMs to express tnnt2, tnni-HC, or cmlc2. However, these findings have a few limitations due to time constraints. First, only a small number of experiments were repeated. Second, cardiac gene
expression by ZKS1 is only assessed at one specific time-point. It is possible that cardiac expression by ZKS1 was either induced earlier than 5 days after initiation of the co-culture and could not be maintained, or required a longer incubation with rCM. Finally, a positive control co-culture system is lacking in this experiment. Therefore, an appropriate design would involve assessment of cardiac gene expression by co-cultured ZKS1 at multiple time points throughout the culture paralleled by a murine MSC-rCM co-culture control. Additionally, a co-culture system consisting of ZKS1 and zebrafish embryonic cardiomyocytes could eliminate the cross-species confounder involved with the ZKS1-rCM co-culture system. However, dissection for the primitive heart of embryonic zebrafish proved to be exceedingly difficult due to the microscopic size and fragility of the heart at this stage of development. A better approach would be to digest transgenic zebrafish embryos which have cardiomyocytes genetically labelled by fluorescent reporter gene expression, such as the Tg(\textit{cmlc2}:GFP) zebrafish [98,99].
CHAPTER 5.0 – SA3:

Establish an Embryonic Zebrafish Cell Transplantation System to Test Cardiac Differentiation of Zebrafish Stromal Cells in vivo

5.1 Introduction

Cell transplantation experiments have widely been used in mammals to assess the in vivo multipotency of stem and progenitor cells. While few zebrafish cell transplantation experiments have been conducted, a variety of different transplantation methods have been established for the transplantation of hematopoietic cells [10]. Donor hematopoietic cells can be isolated from either zebrafish embryos at the 8-10 somite stage of development or the kidney of an adult zebrafish. Donor cells are resuspended in 0.9X PBS containing 3U Heparin and 1U DnaseI prior to transplantation. The Heparin and DnaseI help prevent coagulation and aggregation of the cells. The cell suspension is backloaded into a glass, filament-free, fine-pulled capillary needle and the tip of the needle is broken on a bevel to create an opening of approximately 20 μm. The needle is loaded into an air-pressurized injection station, ready for cell transplantation. Donor hematopoietic cells can be transplanted into recipient zebrafish at three different developmental stages: (i) Blastula; (ii) 48hpf larval; and (iii) Adult [10].

(i) Blastulae – Embryos at the 500- to 1000-cell stage of development are dechorionated by light pronase treatment or manually using forceps within an agarose-coated Petri dish. Dechorionated blastulae are transferred to a fresh agarose-coated transplantation plate. The loaded injection needle is inserted into the center of the cell mass and the cell suspension is dispensed into the embryo using a positive pressure. The maximum number of cells that can be transplanted into
embryonic recipients was estimated to be $5 \times 10^3$. Injected recipients are transferred to an agarose-coated Petri dish that is submersed in embryo medium [52] and then incubated at 28.5°C.

(ii) 48hpf embryos – Zebrafish embryos are dechorionated at the blastulae stage and staged on an agarose mold at the 48hpf stage of development for transplantation. Recipients are anaesthetized in tricaine as they are able to move at this stage of development. Donor cells are expelled into the sinus venosus of recipients using positive pressure. The cell suspension being injected into 48hpf recipients must be highly concentrated as only a small volume can be released into the sinus venosus. For example, a concentration of $5 \times 10^5$ cells/μL can be used for transplantation if proper de-coagulation steps are taken. Up to $5 \times 10^3$ cells can be transplanted into 48hpf recipients. Again, recipients are transferred to embryo medium and incubated at 28.5°C.

(iii) Irradiated adult zebrafish – Adult zebrafish recipients are anesthetised with tricaine and then transferred to a Petri dish containing fish water for irradiation. Recipients are irradiated using a $^{137}$Cesium source irradiator at a dose of 20-Gy. Irradiated fish are, again, anesthetised and placed into a moist sponge ventral side up. The scales covering the heart region are removed with forceps. Using a pulled glass, filament-free capillary needle with an opening of approximately 50μm, the donor cell suspension is transplanted directly into the recipient heart, which is the most efficient area for delivering cells to the blood circulation. Typically, between $1 \times 10^6$ and $2 \times 10^6$ cells are transplanted into adult zebrafish. Recipients are left to recover in fresh fish water and are not fed until the following day to reduce the risk of infection.

These transplantation methods have been applied to study hematopoietic cell fate. One study isolated lmo-2-expressing hematopoietic precursors from transgenic LMO-2\textsuperscript{eGFP} zebrafish embryos and transplanted the donor cells into 1000-cell stage blastulae recipients. It was
observed that the transplanted cells were able to survive the procedure well and GFP expression was visible for over several days in the developing host [reviewed in 100]. This group also isolated lmo-2-expressing hematopoietic precursor cells from double transgenic LMO-2eGFP;GATA-1DsRed embryos and determined that these precursors were capable of generating erythrocytes post-transplantation, as marked by DsRed expression within the host circulation, after transplantation [reviewed in 100, 101]. Thus, the transplantation strategies developed by Traver et al., in combination with transgenically-labelled donor cells, is an effective tool for studying hematopoietic stem and progenitor cell differentiation in vivo.

Referring to the established transplantation methods for zebrafish hematopoietic cells, I have initiated the development of a transplantation system for delivering adult kidney marrow-derived ZKS cells into zebrafish blastulae recipients to determine whether these cells are capable of cardiac differentiation in vivo.
5.2 Methods

5.2.1 Generation of a transgenic ZKS1(CMV:eGFP) cell line

To create a transgenic ZKS1(CMV:eGFP) cell line, a lentiviral vector carrying the transgenic pHR-cPPT-CMV-IRES-eGFP-WPRE vector [102] was transduced into ZKS1. The pHR-cPPT-CMV-IRES-eGFP-WPRE vector was developed in collaborator Dr. Jeffrey Medin’s Lab and was constructed so that a cytomegalovirus (CMV) promoter directs GFP expression ubiquitously. Prior to transduction, ZKS1 cells were grown to confluency in a 12-well tissue culture plate with 1.5mL of ZKS medium/well. Viral transduction of ZKS1 was conducted by Tania Felizardo of the Medin Lab. Briefly, ZKS medium was removed from ZKS1 and replaced with ZKS medium supplemented with 10-MOI of the lentiviral vector and protamine sulfate. The cells were incubated with the virus overnight at RmT, after which the medium was replaced with fresh ZKS medium. At 48hrs post-transduction, transduced cells were analyzed for GFP expression using the Zeiss Axiovert 200M widefield fluorescence microscope with the FITC filter. If GFP+ cells were detected, the population of transduced cells was sorted for GFP+ cells by FACS using the FL1 channel for positive selection. For this experiment, FACS was performed by the UHN Flow Cytometry facility. After sorting for a cell population with >99% of the cells expressing GFP, the new ZKS1(CMV:eGFP) cell line was maintained according to ZKS1 maintenance procedures and aliquots of the cells were stored in liquid nitrogen according to ZKS1 freezing procedures, both described in Chapter-3.2.2. Furthermore, ZKS1(CMV:eGFP) was stained with DAPI as described in Chapter-3.2.3(b) and photographed using the Zeiss Axiovert 200M widefield fluorescence microscope with the FITC filter.
5.2.2 Transplantation of Transgenic ZKS1(CMV:eGFP) and CAG/GFP-LEW rat T4 BM-MSC into zebrafish embryos

Transgenic ZKS1(CMV:eGFP) and transgenic rat T4 BM-MSCs previously isolated from a CAG/GFP-LEW rat line [103] by a colleague in my lab were removed from tissue culture using 0.25% trypsin-EDTA and neutralized with 1X FBS. Cells were pelleted by centrifugation at 1,200rpm for 5-10min and resuspended in an injection solution composed of 0.9X PBS, 5% FBS, 3U heparin, and 1U DNaseI [100]. 3μL of cell suspension was loaded into a pulled, filament-free, thin-walled glass capillary needle (World Precision Instruments, Order#: TW120-4) and the tip was broken open. The needle was then loaded into a stationary air-pressurized injection station. Meanwhile, a 0.7% agarose mold was made to hold the recipient zebrafish embryos while being injected with cells. Zebrafish embryos were lined up vertically along the mold, ready to be injected. The mold was placed atop a petri dish and the embryos were pushed into the loaded capillary needle. For transplantation into blastula recipients, the tip of the injection needle was inserted within the center of the embryo near the yolk interface. For transplantation into 48hpf recipients, the injection needle was inserted into the recipient sinus venosus. The cell suspension was expelled from the needle using a positive pressure. Approximately 10-20nL of cell suspension was injected into zebrafish recipients. Between 100-200 ZKS1(CMV:eGFP) cells were transplanted into blastulae recipients at the 500- to 1000-cell stage of development. Three different transplantation experiments were conducted (each involving between 80-160 embryo recipients). Using donor rat BM-MSCs, approximately 40-70 cells were transplantation into blastulae-staged recipients and 3-5 cells into 48hpf recipients. Four transplantation experiments using blastulae recipients (each involving between 20-40 recipients) and three using 48hpf recipients (each involving approximately 10 recipients) were
conducted. Injected recipients were placed in a clean petri dish of ddH$_2$O or embryo medium [52] and incubated at 30°C overnight. The next day, infected or deceased embryos were discarded and the remaining healthy embryos were placed in a clean dish. This step was repeated each day for up to 7dpf. Recipient embryos were assessed for GFP expression using a bright field fluorescent microscope immediately after injection and every 24hrs after for up to 7dpf. Pictures of injected embryos were taken using the Leica DFC320 microscope camera.
5.3 Results

5.3.1 T4 BM-MSCs isolated from CAG/GFP-LEW rats were transplanted into zebrafish blastulae and 48hpf larvae as an initial attempt to study MSCs in an embryonic environment *in vivo*.

Prior to obtaining the ZKS1 cell line, I conducted preliminary transplantation experiments using GFP-labelled CAG/GFP-LEW rat T4 BM-MSCs. The MSCs were transplanted into both zebrafish blastula-staged embryos and 48hpf embryos to determine whether these cells could survive over time within the host and whether they could engraft into zebrafish heart tissue without eliciting an immune response. However, rat BM-MSC transplantations into zebrafish blastulae and 48hpf larvae recipients proved to be extremely technically challenging to perform when transplanting between 40-70 cells and 3-5 cells, respectively. Frequently the cells would clog the injection needle and only injection solution was expelled. If the opening of the needle was made larger to accommodate the larger size of rat BM-MSCs as compared to ZKS1 cells, the injected embryos were usually infected or deformed due to the increased puncture wound area (Figure 6.a.ii). Only a small number of cells could be injected into a single embryo as increasing the concentration of rat BM-MSCs within the needle promoted clogging.

In regards to the transplantation experiments using blastulae recipients, injecting 40-70 rat BM-MSCs resulted in only a small number of cells being expelled into the cell mass of the recipients (Figure 6.a.i). Moreover, rat BM-MSCs were only observed in a miniscule fraction of injected blastulae embryos immediately post-injection. After performing 4 separate trials, each consisting of 20-40 recipient embryos, approximately 0.8% (n = 120) of all injected embryos survived to 3dpf and carried GFP* cells (Figure 6.a.iii). After conducting 3 separate
transplantation experiments using approximately ten 48hpf recipients per experiment, I was unable to visualize any GFP-expressing cells within the host immediately post-transplantation. Furthermore, no injected embryos were able to survive for more than 24hrs post-transplantation. These techniques were also very time-consuming as only 10-40 embryo recipients could be injected over a period of 2-3 hours. Table-3 lists the cell transplantation trials performed in blastulae recipients. I stopped working with rat BM-MSC transplantations at this point as I had obtained the ZKS1 cell line [75] and would use these zebrafish cells for transplantation studies.

5.3.2 A transgenic ZKS1(CMV:eGFP) cell line was created with ubiquitous GFP expression

To be able to track ZKS1 cell migration and survival in vivo, I generated a novel ZKS1 cell line with ubiquitous GFP expression. The ZKS1 cell line was transduced with a lentiviral vector carrying the pHR-cPPT-CMV-IRES-eGFP-WPRE transgenic vector and sorted for GFP+ cells using FACS. Initial FACS analysis confirmed that only 2% of transduced ZKS1 cells expressed GFP when using 10-MOI of the lentiviral vector. After two rounds of FACS, 99.45% of the cell population showed ubiquitous and enhanced GFP expression (eGFP) (Figure 7.a.i). The cell line was coined ZKS1(CMV:eGFP). These transgenic cells are easily visualized both in vitro (Figure 7.a.ii & 7.a.iii) and in vivo (Figures 8 & 9) using a fluorescent microscope. Multiple vials of ZKS1(CMV:eGFP) cells were frozen down and are now stored in liquid nitrogen.
5.3.3 A high concentration of ZKS1(CMV:eGFP) can be transplanted into zebrafish embryo recipients with 16.7% of recipients with GFP+ cells surviving at least 3dpf.

To determine whether ZKS1 cells can be used as donor cells in transplantation experiments using zebrafish embryo recipients, I transplanted ubiquitously-labelled ZKS1(CMV:eGFP) cells into 500- to 1000-cell stage zebrafish embryos. In contrast to rat BM-MSCs, higher concentrations of ZKS1(CMV:eGFP) cells could be prepared for injection due to their smaller size. Between 100-200 ZKS1(CMV:eGFP) cells were successfully injected into the center of recipient embryos near the yolk interface (Figure 8). After injection, approximately 16.7% (n = 263) of recipient embryos were able to survive for more than 72 hours post-injection (hpi) and carried GFP+ cells (Table3; Figure9). ZKS1(CMV:eGFP) cell transplantation experiments were very time-efficient taking only 1-2 hours to inject between 80-160 zebrafish embryos. Furthermore, the procedure was also technically very efficient as the cells never clogged the injection needle in any of the trials performed.

5.3.4 Transplanted ZKS1(CMV:eGFP) can reside within the recipient embryonic heart, CNS, trunk and/or yolk tissues after organogenesis and can form a band-like structure over the pericardial sac.

To determine whether transplanted ZKS cells can survive throughout embryogenesis and whether ZKS cells can reside in the host heart tissue, ZKS1(CMV:eGFP) was transplanted into zebrafish blastula-staged embryo recipients. Approximately 100-200 ZKS1(CMV:eGFP) cells were transplanted into the center of the embryos near the yolk interface. Immediately after injection, GFP-expressing cells were observed in the cell mass of recipients. In surviving embryo recipients, GFP-expressing cells were visualized within the host tissue for at least 7 days post-
transplantation. Moreover, transplanted ZKS1(CMV:eGFP) cells were able to reside within several different host tissue types by 3dpf and remain at that specific site for at least 7dpf or until the embryo was deceased (Figure 9). Table-4 records the results of three separate ZKS1(CMV:eGFP) transplantation trials. Of importance to the aims of this study, the ZKS1(CMV:eGFP) cells were found to reside within the beating host heart in 11% of the injected zebrafish embryos (n=179) (Figure 9.i, video-1). Interestingly, transplanted ZKS1(CMV:eGFP) cells resided within the host CNS region in 36% of the injected embryos (n=179) (Figure 9.ii). ZKS1(CMV:eGFP) cells were also observed within the trunk tissue and yolk of some recipient fish post-transplantation (Figures 9.iii & 9.v). Finally, a unique observation made during these transplantation experiments was the tendency of the transplanted ZKS1(CMV:eGFP) cells to form a band of tissue spanning across the pericardial sac making attachments around the anterior of the yolk sac and the mandible of the recipient (Figure 9.iv). In one trial, it was found that this band-like tissue of ZKS1(CMV:eGFP) cells was observed in 16.7% of the recipient fish.
5.4 Discussion/Conclusions

In order to study cardiac differentiation by ZKS cells in an embryonic environment in vivo, it was important to determine a transplantation strategy amenable for the delivery of ZKS1 into zebrafish embryo recipients. The protocol designed for zebrafish hematopoietic cell transplantation into zebrafish blastula recipients was trialed for ZKS1 transplantation into zebrafish blastulae [100], with the main exception that the recipient embryos were not dechorionated. In order to track transplanted hematopoietic cells in vivo, Traver et al. used donor cells derived from transgenic zebrafish in which hematopoietic progenitor cell-specific promoters directed fluorescent protein expression, such as Tg(LMO-2<sup>eGFP</sup>) zebrafish [100]. Unfortunately, a ZKS cell-specific gene has not yet been discovered, nor has an MSC-specific gene [16]. Moreover, as primary ZKS cells cannot yet be sustained in tissue culture, it is not possible to derive ZKS cells from transgenic zebrafish with ubiquitous fluorescent reporter protein expression for in vivo cell tracking. Yet it is necessary to follow transplanted ZKS cells in recipient embryos during development to ensure ZKS cells can survive in the host long enough for assessment of cardiac differentiation. Thus, an alternative strategy for labelling the ZKS1 cell line in vivo was essential. For simplicity, I chose to generate a transgenic ZKS1 cell line which ubiquitously expresses GFP, enabling a continuous supply of GFP-labelled ZKS cells. Conveniently, a lentiviral vector carrying a transgene in which the CMV promoter directs ubiquitous expression of GFP (the pHR-cPPT-CMV-IRES-eGFP-WPRE lentiviral vector) had already been established within our collaborator, Dr. Jeffrey Medin’s Lab [102]. Through collaboration with Tania Felizardo in the Medin Lab, we generated a stable transgenic ZKS1(CMV:eGFP) cell line with ubiquitous eGFP expression after transduction of ZKS1 with the pHR-cPPT-CMV-IRES-eGFP-WPRE lentiviral vector and subsequently sorting for GFP+.
ZKS1 cells. This transduction strategy had very low efficiency as only 2% of the transduced ZKS1 cells expressed eGFP. Conducting a dose-response experiment by transducing ZKS1 with different MOIs of the lentiviral vector would determine whether the transduction efficiency can be improved by increasing the concentration of lentiviral vector in the transduction medium. However, for the purpose of this study, the transfection strategy used was appropriate for the task of labelling ZKS1 in vivo.

Subsequently, transgenic ZKS1(CMV:eGFP) was transplanted into zebrafish embryos at the blastula-stage of development. Transplantation of ZKS1(CMV:eGFP) was technically much easier than transplantation of rat BM-MSCs as ZKS1 cells are significantly smaller in size than rat BM-MSCs and can easily pass through the opening of the injection needle into the cell mass of recipient embryos. In fact, compared to only 0.8% of zebrafish receiving rat BM-MSCs, 16.7% of recipients receiving ZKS1(CMV:eGFP) were able to survive for at least 72hpi. Whether the survival rate of rat BM-MSCs transplant recipients can be improved by reducing the concentration of the cell suspension prior to injection remains unknown. A dose-response test using different concentrations of rat BM-MSCs for transplantation while holding the injection volume constant could be performed to answer this question. However, as high cell concentrations are required for transplantation into 48hpf embryos [100] and future direction of this research involves transplantation into zebrafish embryos at various developmental stages, the rat BM-MSC does not appear to be the most optimal cell type for this research. The smaller size of ZKS1(CMV:eGFP) cells, in comparison to rat BM-MSCs, allows for a high concentration of cells to used for transplantation. Hence, the focus of my study was directed towards the evaluation of the ZKS1 cell as a donor MSC for the transplantation system proposed in this study. Another advantage of ZKS1(CMV:eGFP) transplantation for in vivo assessment of cardiac
plasticity in an embryonic setting is that the transplanted GFP+ cells can be visualized over a period of at least 7dpf. This suggests that the ubiquitous GFP expression by ZKS1(CMV:eGFP) does not fade overtime in vivo and that ZKS1(CMV:eGFP) can survive within the host embryo for at least 7 days, results consistent with previous zebrafish kidney marrow cell transplantation experiments [100]. As the embryonic zebrafish heart is fully formed and functional around 2-3dpf [54], any cardiac differentiation events occurring by transplanted ZKS1(CMV:eGFP) should be identifiable by at least 7dpf.

ZKS1(CMV:eGFP) transplantation experiments were also analyzed for trends in ZKS cell engraftment patterns. ZKS1(CMV:eGFP) cells were transplanted at the midline of recipient embryos near the yolk-embryo surface, which is where the host cardiac progenitors reside [54]. In general, most injected cells did not disperse throughout the developing embryo, but remained in close contact with one another within certain host tissues. Observation of recipient embryos at 3dpf showed that GFP+ cells resided within the host heart in 11% of recipients. Thus, there is a possibility that ZKS1 cells have been induced to undergo cardiac differentiation within an embryonic environment in vivo. There are two methods for determining whether this hypothesis is correct. First, transplant recipient embryos with GFP-expressing cells residing within the heart at 3dpf can be disrupted as described previously [99] and the heart tissue can be digested to generate a cell suspension according to methods described in Warren et al. [104]. This suspension would then be sorted for GFP-expressing cells using FACS. mRNA would be extracted from the population of GFP+ cells and analyzed for cardiac gene expression by RT-PCR. The other strategy would be to generate tissue sections of the heart of 3-5dpf zebrafish transplant recipients with GFP-expressing cells residing in the host heart. The sections would then be stained with monoclonal antibodies specific for zebrafish cardiac antigens, such as the
Ab-s46 antibody which binds the zebrafish myosin heavy chain-6 (myh6) antigen found on atrial cardiomyocytes [61].

Transplanted ZKS1(CMV:eGFP) cells were also found to reside within the host CNS in 36% of all transplant recipients. Mammalian BM-MSCs have been found to differentiate into neural cell types after transplantation into adult recipients [105]. Therefore, transplanted ZKS1(CMV:eGFP) cells which reside in the recipient CNS should be assessed for expression of neural markers using RT-PCR or immunohistochemical techniques. It is also interesting to note that there was an observed trend for the GFP+ cells to coalesce into a band-like structure which spanned across the ventral surface of host pericardial sac, attaching from the yolk sac to the mandible region. It could be suggested that the band-like structure formed by GFP+ cells is a form of dense connective tissue as the structure appears as a network of dense fibers under the Leica DFC320 microscope using approximately 10X magnification. To determine whether this hypothesis is correct, tissue sections of the recipient embryo could be stained with Van Gieson which stains collagen tissue red [106]. Mammalian BM-MSCs are defined according to their capacity for differentiation into bone, adipose and cartilage tissues, which can all be found in certain types of connective tissues. Therefore, if it is confirmed that the band-like structure is, indeed, a form of connective tissue, the next step would be to determine whether the connective tissue is composed of ZKS1(CMV:eGFP)-derived bone, adipose or cartilage tissue by staining with Alizarin Red S, Oil Red O and von Kossa stains, respectively. This will support my original hypothesis which states that zebrafish possess a cell population within the kidney marrow that can be defined as an MSC.
CHAPTER 6.0 – SA4:

Mark Zebrafish Stromal Cell Cardiac Differentiation by Fluorescent Reporter Gene Expression using Transgenic Vectors

6.1 Introduction

6.1.1 Transposon Systems

Transposon systems are useful tools for creating transgenic zebrafish lines and cell lines. They can generate transgenic founder fish at frequencies of 30-50% and stable transgenic lines with tissue-specific reporter expression [53]. I have employed both the Sleeping Beauty (pT2/S2) and mifepristone-inducible LexPR transposon systems in my study [107-110]. In general, the technique of transposon-mediated DNA delivery in vivo involves co-injection of mRNA encoding transposase and the transposon vector into fertilized zebrafish oocytes at the 1-cell stage of development [107-110]. The transposase enzyme is essential for successful delivery of the transposon transgene into the host genome after DNA-mRNA co-microinjection [110].

a. The Sleeping Beauty Transposon System

The pT2/S2 Sleeping Beauty transposon system has a transgenesis rate of approximately 30% [110]. The pT2/S2 transposon consists of short terminal inverted repeat sequences (T2/S2) in cis upon which the co-injected transposase mRNA can act to deliver the promoter-reporter construct within the transposon vector into the host genome following a cut-and-paste mechanism [108]. Additionally, a poly(A) sequence within the transgene also helps with transgene integration into the host genome after microinjection [108,110]. The pT2/S2 transposon is applied in this study to generate a vector with a cardiac-specific promoter directing fluorescent
reporter gene expression. In turn, this transgenic transposon vector can be used to mark cardiac gene expression by ZKS cells.

b. The mifepristone-inducible LexPR Transposon System

The mifepristone-inducible LexPR transposon system is a relatively new system being explored in the zebrafish. It has the additional advantage of tissue-specific, chemical-inducible gene expression [109]. This system also provides flexibility in transgene construction as it consists of 2 separate vectors, a driver line and an effector line. The driver line can either be used independently to generate transgenic fish with chemical-inducible expression of a fluorescent reporter in a specific tissue type or used in combination with the effector line to generate double-transgenic zebrafish with chemical-inducible expression of both a fluorescent reporter and a target gene within a desired tissue type. The key elements of this system are a chimeric transcription factor (LexPR), which is expressed under the control of a tissue-specific promoter within the driver line, and a cis-acting operator (LexOP)-promoter sequence located in both the driver and effector lines upon which the tissue-specific transactivator can bind to promote fluorescent reporter and target gene expression [109]. However, the transcription factor can only bind to the LexOP in the presence of mifepristone (RU-486), a progesterone antagonist. Mifepristone binds to the progesterone receptor (LBD) located on the transactivator which, through unknown mechanisms, activates the transactivator enabling it to bind LexOP. Therefore, in general, the LexPR system is an inducible system in which a tissue-specific promoter within the reporter transposon can be induced to direct the expression of either a fluorescent reporter gene or both the reporter gene and a target gene within an effector transposon. This aspect is useful for developing a model of cardiac necrosis in zebrafish embryos. Although both surgical removal of cardiac tissue and laser-mediated cellular ablation have been well-established in the
zebrafish, these methods are labour-intensive, time-consuming and not genetically reproducible [111]. Thus, a genetic model of inducible cardiomyocyte ablation, such as that proposed in this study, would be a more efficient tool. A cytotoxic gene can be inserted into the effector transposon and an exogenous cardiomyocyte-specific promoter can be inserted into the reporter transposon to enable temporal and spatial control of cytotoxic gene activation. Cell ablation will be specific to cardiomyocytes and can be timed to initiate after the heart has fully formed.

6.1.2 The Zebrafish Cardiac Troponin T2 Promoter, Gene and Gene Product

The cardiomyocyte-specific promoter designed in this study is the cardiac troponin T2 (tnnt2) promoter. Naturally, the endogenous zebrafish tnnt2 promoter directs the expression of the tnnt2 gene which encodes the zebrafish cardiac tnnt2 protein. Zebrafish tnnt2 is a thin filament protein within the troponin-tropomyosin (TTC) complex of sarcomeres and is homologous to the mammalian TNNT2 protein [60]. The role of the TTC is to coordinate cardiac muscle contractions in response to intracellular calcium levels. More specifically, troponin T is essential for the inhibition of contraction by the TTC when calcium levels are low. Without troponin T, muscle contractions occur regardless of intracellular calcium concentrations [112]. Interestingly, 15% of all cases of familial hypertrophic cardiomyopathy can be attributed to mutations of the TNNT2 gene [113]. Likewise, the silent heart (sih) zebrafish mutant, which phenocopies hypertrophic cardiomyopathy, carries a mutation within the zebrafish tnnt2 gene [60]. We also chose to construct an exogenous tnnt2 promoter for this study because tnnt2 is expressed in zebrafish myocardial precursors during embryonic development starting at the 10-13 somite stage (14-16hpf) and continues to be expressed in adult cardiomyocytes [60,61]. Thus changes in cardiac gene expression can be monitored over a broad timeframe.
6.1.3 The Cytotoxic Diphtheria Toxin A (DTA)

The cytotoxic gene chosen to ablate cardiomyocytes in a temporally and spatially regulated manor is the diphtheria toxin A-chain (DTA) gene. DTA is an exotoxin that is secreted by the bacteria which causes Diphtheria: *Corynebacterium diphtheria* [114,115]. When DTA is produced within cells, it will cause cell death by inhibiting endogenous RNA translation [115]. DTA has been used previously to generate transient transgenic zebrafish with DTA-mediated ablation of either the lens [116] or the exocrine pancreas [117] tissue. These groups used tissue-specific promoters to confine DTA expression to the tissue type being studied.

In this study, I generate 2 transgenic pT2/s2 vectors with either a green or red fluorescent reporter gene expressed under the control of an exogenously built zebrafish *tnnt2* promoter: pT2/s2*tnnt2-GM2* and pT2/s2*tnnt2-DsRed*. These vectors can be used in the future to mark cardiac differentiation by ZKS cells. Moreover, I have constructed 2 transgenic transposons of the *mifepristone-inducible LexPR* system, a pDs(*tnnt2*LPR-LOP:G4) reporter and a pDs(*cry*:ECFP-LOP:DTA) effector vector. The reporter vector is capable of directing cardiomyocyte-specific GFP expression in zebrafish when mifepristone is present. The effector is capable of directing CFP expression in the lens while carrying a silent DTA gene. Combining these two pDs transposon vectors will create a model of mifepristone-inducible cardiac ablation which can be used to study ZKS cell contributions in a disease setting.
6.2 Methods

6.2.1 Generation of transgenic F0 zebrafish

a. Transposase mRNA

As I employed both the Sleeping Beauty and mifepristone-inducible LexPR transposons in my study, I generated both pSB10 and pAc2 transposase mRNA, respectively, using the mMESSAGE mMACHINE T7 Kit (Applied Biosystems, AM1344). First, the template pSB10 and pAc2 DNA provided by my lab was linearized by restriction enzyme digestion. Complete linearization of the DNA was confirmed by gel electrophoresis using a 0.7% agarose gel. A 1kb DNA ladder was run alongside the DNA in the gel as a size reference. The resulting band was excised and dissolved at 50°C for 10min to release the DNA. The extracted DNA was then purified using the QIAquick Gel Extraction Kit (QIAGEN, Cat #28704). According to the mMESSAGE mMACHINE T7 Kit protocol, the following reagents were added to approximately 1ug of the purified DNA at RmT: 10μL of 2X NTP/CAP, 2μL of 10X reaction buffer, and nuclease-free water to a total volume of 20μL. The solution was mixed thoroughly, centrifuged briefly to collect reagents at the bottom of the tube, and then incubated at 37°C for 2hrs. After this time, 1μL of TURBO DNase was added and the mixture was incubated again at 37°C for 15mins. Next, the RNA was recovered from the transcription reaction by lithium chloride (LiCl) precipitation. Briefly, 30μL of both nuclease-free water and LiCl Precipitation Solution (7.5M LiCl, 50mM EDTA) were added to the transcription reaction mixture and mixed thoroughly. The solution was chilled at -20°C for 30-60min and then centrifuged at 4°C for 15min at 12,000g to pellet the RNA. Supernatant was carefully removed and the pellet was washed with 1mL of 70% ethanol. The RNA was again collected at the bottom of the tube by centrifugation and the ethanol
was carefully removed. The RNA was resuspended in 30-50μL of nuclease-free water and stored at -80°C.

**b. Co-microinjection of Transposon DNA and Transposase mRNA**

An air-pressurized injection apparatus was used to perform microinjection experiments. Between 100-300ng/μL of transposon DNA and 100-200ng/μL of transposase mRNA were combined in an ependorff tube and diluted 10X by addition of 1μL of KCl, 0.5μL of Fast Green dye, and double-deionized water (ddH₂O) to a total volume of 10μL. Approximately 2-3μL of the solution was back-loaded into a thin-walled glass capillary needle (World Precision Instruments, Order#: TW120-4F), which was then loaded into the air-pressurized injection device. Meanwhile, adult wildtype zebrafish were mated and healthy embryos were collected. When the embryos reached the 1-cell stage of development, the embryos were aligned along a trough of a 0.7% agarose mold which was stabilized by a petri dish. The embryos were pushed into the stationary needle so that the tip of the needle went through the yolk to the yolk-blastoderm interface. Air pressure was used to expel approximately 3-5nL of DNA solution into the embryos. Injected F0 embryos were placed in a clean petri dish with ddH₂O and incubated at 30°C overnight. Over the next 3 days, infected or deceased F0 embryos were removed from the dish and fresh ddH₂O was added. When the F0 larvae were able to swim, they were transferred to a small tank and fed brine shrimp by the Wen Lab technician, Youdong Wang. Once F0 transgenic zebrafish reached maturity, they were mated to wildtype zebrafish and the resulting F1 offspring were screened for germline transmission of the transgene. For each transgenic strain, I screened approximately 300-400 zebrafish F1 offspring.
6.2.2 Generation of an exogenous zebrafish cardiac troponin T2 (tnnt2) promoter

To generate an exogenous tnnt2 promoter, I used a zebrafish genomic DNA template and oligonucleotide primers designed specific for the endogenous zebrafish tnnt2 promoter for PCR. I also subcloned the tnnt2 promoter into the pGEM-T Easy vector to increase the copy number.

a. Extraction of Zebrafish Genomic DNA

Adult zebrafish were anesthetised with 100 parts-per-million (ppm) clove oil and then a section of the tail was removed using surgical scissors and incubated in Proteinase K at 50°C for tissue digestion, releasing the genomic DNA. Subsequently, chloroform was added to the solution at a 1:1 volume ratio. This mixture was centrifuged at 12,000g for 10min. The top clear aqueous layer was carefully transferred to a new tube and the DNA was precipitated by adding 500μL/mL of isopropanol. The solution was centrifuged and the supernatant was removed. The DNA pellet was washed in 70% ethanol and again centrifuged. Supernatant was removed and the tube was left to air dry. Finally, the DNA pellet was dissolved in ddH₂O to a concentration of 0.5μg/μL.

b. PCR

The tnnt2 primer set used in this experiment is listed in Table-5. PCR was performed as follows. 0.5μg of zebrafish genomic DNA was diluted 10X with ddH₂O and cycled once at 92°C for 2min in a thermal cycler. Next, 3μL of 10X PCR reaction buffer, 3μL of 10mM dNTP, 0.5μL of 10mM tnnt2 forward primer (FP), 0.5μL of 10mM tnnt2 reverse primer (RP) and 0.8μL of Taq DNA polymerase were added to 10μL of the DNA product to a total volume of 20μL. PCR was performed in a thermal cycler under the following settings. The sample was first heated to 92°C for 2min to activate Taq DNA polymerase. Next, the sample underwent 35 cycles consisting of 3 separate temperature-specific phases: 1) Denaturation of template DNA strands at
92°C for 30sec; 2) Annealing of the primers to the template DNA at 63°C for 30sec; and 3) Elongation of the new complementary DNA strand at 72°C for 2min. After the 35 cycles, there is a final elongation step at 72°C for 10min to ensure all new single stranded DNA fragments are fully extended. The final resting temperature is set at 4°C to store the DNA until ready to use. The PCR product was separated according to base-pair length using gel electrophoresis. The band corresponding with a size of 1.87-kb was excised from the gel and then purified using the QIAquick Gel Extraction Kit (QIAGEN, Cat #28704).

6.2.3 Cloning of the *tnnt2* promoter into the PGEM-T Easy vector

**a. DNA Ligation**

The *tnnt2* promoter was ligated within the PGEM-T Easy vector (Promega, Cat-A1360) at the insertion site which is bound by two T-overhangs. For DNA ligation, the *tnnt2* promoter and T-vector DNA were combined and treated with 2X rapid ligation buffer and 1U of T4 DNA ligase enzyme (Invitrogen, Cat.15224-017) to reach a total volume of 10μL. The ligation mixture was cooled in a 16°C waterbath for 5hrs.

**b. Bacterial Transformation, Bacterial Culture and Plasmid Selection and Purification**

5μL of the ligated product was transformed into 50μL of DH5-α bacteria by cooling the mixture on ice for 30min, followed by incubation in a 42°C waterbath for 45s, and then immediate transfer back to ice for another 2min. As a negative control, 1μL of the original *tnnt2* promoter DNA was transformed into 50μL of the bacteria. After bacterial transformation, 1mL of LB medium was added to the transformed bacteria which were then agitated within a 37°C rotational incubator at a speed of 150rpm for 5hrs to promote bacterial growth. Subsequently, 200μL of the bacterial solution was mixed with 100μL of X-gal (Promega, Cat-V3941) and then
spread evenly atop an ampicillin\(^+\) (amp\(^+\)) LB-agar-based growth media within a petri dish. This plating strategy allows for an enhanced selection of plasmids in which the \textit{tnnt2} promoter fragment has been successfully integrated. First, an ampicillin-resistant gene resides within the T-vector, therefore only bacterial colonies carrying the T-vector will grow on the agar plate. Additionally, X-gal is used as a selection tool to delineate whether surviving colonies carry plasmids containing the \textit{tnnt2} promoter fragment. When X-gal binds to \(\beta\)-galactosidase, a series of reactions lead to the production of a blue insoluble compound, 5,5\(^{\prime}\)-dibromo-4,4\(^{\prime}\)-dichloro-indigo. \(\beta\)-galactosidase is encoded by the \textit{lacZ} gene which is actively expressed in the T-vector alone. However, when the \textit{tnnt2} promoter enters the T-vector at the T-overhang insertion site, it disrupts the \textit{lacZ} gene and inhibits the formation of the blue compound. Therefore, bacteria colonies carrying the \textit{tnnt2} promoter will appear white in colour whereas those lacking the promoter will appear blue. Plated bacteria were incubated overnight at 37\(^\circ\)C. The next day, white bacteria colonies were selected using a 200-\(\mu\)L pipette tip which was subsequently place in amp\(^+\) LB aqueous media and rotated overnight at 37\(^\circ\)C and 250rpm. A clean pipette tip dropped into the LB media served as a negative control. Plasmid DNA was then extracted from bacteria colonies and purified using QIAprep Spin Miniprep Kit (QIAGEN, Cat. 27104).

c. Enzyme Digestion and DNA Sequencing

Enzyme digestion was performed according to the protocol provided by the manufacturer of the enzyme (Fermentas). Briefly, 2\(\mu\)L of 10X digestion buffer, 10U/\(\mu\)g of EcoR1 enzyme and ddH\(_2\)O were added to 5\(\mu\)L of the purified plasmid DNA to reach a total reaction volume of 20\(\mu\)L. The reaction mixture was incubated in 37\(^\circ\)C waterbath for 1hr to activate the EcoR1 enzyme. The resulting fragmented DNA was separated according to base-pair length using gel electrophoresis to confirm correct insertion of the \textit{tnnt2} promoter. Samples showing bands of the
correct size were selected for sequencing by the UHN Gene Profiling Facility. Sequencing results were compared to the DNA sequence of the endogenous zebrafish \textit{tnnt2} promoter obtained from the Zebrafish Model Organism Database (ZFIN) website [61] using the BLASTN application from the NCBI website [118].

\subsection*{6.2.4 Generation of transgenic pT2/s2 transposons of the \textit{Sleeping Beauty} system and Ds transposons of the mifepristone-inducible LexPR system}

To create the transgenic transposons, either the \textit{tnnt2} promoter or an exogenous diphtheria toxin A (DTA) gene was subcloned within one of 3 transposon vectors according to methods described in Chapter-6.2.3(a). The copy number of each constructed vector was exponentially expanded and then purified according to methods described in Chapter-6.2.3(b), with the exception that plasmid selection does not involve use of X-gal for these vectors. Aliquots of transformed bacteria carrying the correct transposon sequence were stored for long-term in glycerol at a volume ratio of 1:1 and at a temperature of -80°C. Restriction enzyme digestions were performed according to methods described in Chapter-6.2.3(c). For double digestions using 2 restriction enzymes with different activation temperatures, the 2 enzymes can be added to the digestion mixture at the same time, but the solution should be incubated at the lower activation temperature first to avoid degradation of the enzyme at higher temperatures.

\textbf{a. Generation of pT2/s2\textit{tnnt2}}-GM2 or pT2/s2\textit{tnnt2}-DsRed Transposons

The \textit{ef1\alpha} promoters within the pT2/s2\textit{EF1\alpha}-GM2 (pDB371) and pT2/s2\textit{EF1\alpha}-DsRed transposon vectors [110] were excised using Sma1 and Sph1 restriction enzyme digestion. The \textit{tnnt2} promoter was similarly excised from the pGEM-T(\textit{tnnt2}) vector previously generated using Sma1 and Sph1 enzyme digestion. Sma1 requires incubation at a temperature of 30°C for 1hr for
activation, whereas Sph1 requires an incubation temperature of 37°C for 1hr. The digested DNA was separated by size using gel electrophoresis. The 4.5kb product of the digested pSB transposon and the 1.87kb product (tnnt2 promoter) of the digested pGEM-T(tnnt2) were excised and purified using QIAquick Gel Extraction Kit. The tnnt2 promoter and pT2/s2 transposon backbone DNA fragments were then ligated at the Sma1 and Sph1 restriction sites to yield either pT2/s2tnnt2-GM2 or pT2/s2tnnt2-DsRed transposon vectors. Each vector was multiplied within DH5α bacteria and then extracted and purified using the QIAprep Spin Miniprep Kit. A final Sma1 and Sph1 restriction enzyme digestion of the pT2/s2tnnt2-GM2 or pT2/s2tnnt2-DsRed transposons was performed to confirm that they carried the 1.87kb zebrafish tnnt2 promoter at the desired location.

b. Generation of the pDs(tnnt2:LPR-LOP:EGFP) Transposon

pDs(tnnt2:LPR-LOP:EGFP) was generated by replacing the original keratin8 (krt8) promoter within the previously developed pDs(krt8:LPR-LOP:EGFP) reporter transposon [109] with the zebrafish tnnt2 promoter that I developed. 250ng of pDs(krt8:LPR-LOP:EGFP) DNA was digested with Xho1 and Asc1 restriction enzymes to remove the krt8 promoter. The digested DNA product was separated according to size using gel electrophoresis and the 7.5kb band was extracted from the gel and purified using the QIAquick Gel Extraction Kit. Next, to modify the tnnt2 promoter so that it would be compatible with the digested ends of the pDs(LPR-LOP:EGFP) DNA fragment, I performed PCR using the pT2/s2(tnnt2:DsRed) transposon DNA as a template and a new tnnt2 primer set designed with 5’-Xho1 and 3’-Asc1 restriction sites. PCR conditions were similar to those used for generating the original tnnt2 promoter described in Chapter-6.2.2(b), with the exception that the annealing temperature was decreased to 60°C. The PCR product (the 5’-Xho1-tnnt2-Asc1-3’ promoter) was purified and digested with Xho1
and Asc1 restriction enzymes. The digested \textit{tnnt2} promoter was then ligated within the digested pDs(LPR-LOP:EGFP) transposon. The new pDs(\textit{tnnt2}:LPR-LOP:EGFP) transposon was replicated in bacteria and then purified. Correct insertion of the \textit{tnnt2} promoter was confirmed by Xho1 and Asc1 enzyme digestion of pDs(\textit{tnnt2}:LPR-LOP:EGFP) with expected DNA fragments of 1.87-kb and 7.5-kb in length.

c. Generation of the pDs(\textit{cry}:ECFP-LOP:DTA) Transposon

First, 325ng of the previously developed pDs effector transposon vector, pDs(cry:ECFP-LOP:Ch) [109], was digested with NotI and SmaI restriction enzymes to remove the \textit{mCherry} gene that is downstream of the Lex operator (LexOP). The sizes of the digested DNA fragments were analyzed by gel electrophoresis and the 6.5kb band was excised and purified. Next, an exogenous DTA gene was excised from the previously developed BigT(GFP-DTA) vector [119] using restriction enzyme digestion. BigT(GFP-DTA) was first digested with NheI which produces sticky ends when it cuts DNA; however, a blunt end is required for ligation with pDs(cry:ECFP-LOP). Thus, to fill in the sticky end with nucleotides, 2.5U of DNA polymerase I/Large (Klenow) Fragment and 0.5μL of 10mM dNTPs were added to the NheI digested solution and incubated at RmT for 20min. The resulting DNA products were separated by gel electrophoresis and the 7.7kb band was excised and purified. The linear 7.7kb DNA fragment was next digested with NotI and the digested product was again run on a gel. The 0.6kb band (DTA gene) was excised and purified. Finally, the 0.6kb DTA gene was ligated within the pDs(cry:ECFP-LOP) DNA fragment to generate a pDs(cry:ECFP-LOP:DTA) transposon. After copy numbers were expanded using bacterial transformation and the DNA was extracted and purified, both NotI and EcoR1 restriction enzyme digestion and DNA sequencing of the
pDs(cry:ECFP-LOP:DTA) transposon DNA were performed to confirm that the DTA gene had been successfully transferred from the BigT vector to the pDs effector vector.

6.3 Results

6.3.1 A functional, exogenous 1.87-kb zebrafish tntt2 promoter has been created.

In order to label ZKS cells undergoing cardiac differentiation by fluorescent reporter expression in vivo, an exogenous tntt2 promoter was created. A tntt2 primer set was previously designed in my lab and is listed in Table-5. The primer set spans a 1.87-kilobase pair (kb) DNA sequence within the endogenous zebrafish tntt2 promoter region. The primer set was designed to include a SmaI enzyme restriction site so that it could be fused to a fluorescent reporter gene within a transposon vector in subsequent experiments. Using PCR and the tntt2 primer set, I developed an exogenous zebrafish tntt2 promoter of 1.87-kilobase pair (kb) in length (Figure 10.a). To further amplify the exogenous tntt2 promoter and incorporate a Sph1 restriction site within the promoter, it was ligated within the pGEM-T Easy vector, which was then transformed into bacteria. The pGEM-T(tntt2) plasmid construct is illustrated in Figure 10.b.i. The pGEM-T(tntt2) plasmid DNA was digested with the EcoR1 restriction enzyme to confirm that the tntt2 promoter had been correctly inserted. As there are 2 EcoR1 restriction sites flanking the tntt2 promoter and one site within the promoter region, it was expected that EcoR1 enzyme digestion would result in 3 fragments of DNA of 3kb, 1.22kb and 650bp in length as detected by gel electrophoresis. Figure 10.b.ii displays the results of the EcoR1 digestion which are consistent with the expected lengths of the digested DNA fragments. Next, the tntt2 promoter region within the pGEM-T(tntt2) vector was sequenced and found to have 94% identity with the DNA sequence of the endogenous zebrafish tntt2 promoter using BLASTN analysis.
6.3.2 Transgenic pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 transposons have been created to mark cardiac differentiation by fluorescence.

To label cardiac-specific gene expression by ZKS1, I have generated pT2/S2 transposon vectors with a fluorescent reporter gene fused to a cardiac-specific promoter, which can be transfected into ZKS1 in future studies. I have subcloned the 1.87kb exogenous zebrafish tnnt2 promoter into the two pT2/S2 Sleeping Beauty transposons by removing the original ef1α promoter using Sma1 and Sph1 restriction enzyme digestion and ligating the tnnt2 promoter in its place. Thus, I created both pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 transposon DNA vectors and the structure of each are displayed in Figure 11.a. Furthermore, I have generated pSB10 transposase mRNA using mMACHINE mMESSAGE to help integrate the tnnt2:GM2 and tnnt2:DsRed DNA fragments into the genome of recipient zebrafish embryos after microinjection of the pT2/S2 transposons.

6.3.3 Transgenic F0 pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 zebrafish were created.

To assess whether the transgenic pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 transposon vectors are capable of labelling cardiomyocytes by fluorescence in vivo and to generate novel transgenic zebrafish strains, the pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 transposon vectors were independently co-microinjected with pSB10 transposase mRNA into zebrafish embryos at the 1-cell stage of development. Recipients were subsequently monitored for DsRed (red fluorescence) or GM2 (green fluorescence) expression, respectively. Co-microinjection of the transposon DNA and transposase mRNA led to fluorescent reporter gene expression within the heart of a small fraction of recipient F0 zebrafish embryos at 2-3dpf (Figure 11.b). In total, I generated 15 pT2/S2tnnt2-DsRed and 10 pT2/S2tnnt2-GM2 transgenic F0 zebrafish which showed
fluorescent reporter gene expression within the heart at 3dpf and survived to the reproductive stage. To generate stable transgenic pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 zebrafish models, adult transgenic F0 zebrafish were mated to wildtype adult zebrafish and resulting F1 progeny were examined for fluorescent reporter gene expression in the heart. After crossing each transgenic F0 zebrafish to a wildtype zebrafish mate and screening between 300-400 F1 offspring under the fluorescent microscope at 2-4dpf, no F1 progeny expressed the fluorescent reporter protein within the heart, indicating that neither the tnnt2:DsRed nor the tnnt2:GM2 transgene was passed through the germline to the next generation in these F0 fish.

6.3.4 Transgenic pDs(tnnt2:LPR-LOP:G4) and pDs(cry:ECFP-LOP:DTA) transposons and F0 zebrafish have been created for development of a zebrafish model of cardiac necrosis.

The krt8 promoter was removed from the pDs reporter transposon by XhoI and AscI restriction enzyme digestion and replaced with the exogenous zebrafish tnnt2 promoter with the help of DNA ligase. The resulting transgenic pDs(tnnt2:LPR-LOP:G4) transposon is illustrated in Figure 12.a.i. Furthermore, I constructed pAc2 transposase mRNA using the mMESSAGE mMACHINE T7 Kit to enable successful integration of the transgene within transposons into the host genome after DNA-mRNA co-microinjection. Several zebrafish embryos were co-microinjected with 60-150pg of pDs(tnnt2:LPR-LOP:G4) and 30-100pg of pAc2 transposase mRNA at the 1-cell stage of development. By 2-3dpf, green fluorescence was observed within the beating heart of a small fraction of the recipients after mifepristone was added to the fish water (Figure 12.b.i). In total, 15 F0 zebrafish embryos expressing GFP specifically in the heart were able to survive to adulthood. These transgenic F0 pDs(tnnt2:LPR-LOP:G4) zebrafish were
crossed to wildtype zebrafish mates and approximately 300-400 resulting F1 progeny were screened under a fluorescent microscope at 2-3dpf. However, again, there were no F1 offspring expressing GFP within the heart, thus the *tnnt2*::LPR-LOP:GM2 transgene was not transmitted through the germline to their offspring.

Next, a pDs(*cry*:ECFP-LOP:DTA) effector transposon was created by replacing the *mCherry* gene within the original pDs(*cry*:ECFP-LOP:Ch) transposon with the cytotoxic DTA gene. The 0.6kb DTA gene was successfully isolated from the BigT(GFP-DTA) vector by SmaINot1 restriction enzyme double digestion. The *mCherry* gene within pDs(*cry*:ECFP-LOP:Ch) was similarly excised by SmaINot1 double digestion. The DTA gene was successfully ligated to the digested pDs(*cry*:ECFP-LOP) as confirmed by both SmaINot1 double digestion and DNA sequencing. SmaINot1 restriction enzyme digestion resulted in 2 separate DNA fragments of 0.6kb (DTA) and 6.5kb (pDs transposon). DNA sequencing confirmed that the DTA gene had been successfully incorporated into the pDs reporter vector. The vector diagram of pDs(*cry*:ECFP-LOP:DTA) is depicted in Figure 12.a.ii. Next, zebrafish embryos were co-microinjected with pDs(*cry*:ECFP-LOP:DTA) and pAc2 transposase mRNA at the 1-cell stage of development. Recipient F0 embryos carrying the transgene were detected by cyan fluorescence within the eye by 2-3dpf using a fluorescent microscope (Figure 12.b.ii). Many injected embryos carrying the transgene developed abnormally and did not survive to adulthood after injection of 30-100pg of DNA and 30-100pg of mRNA. In total, 5 F0 pDs(*cry*:ECFP-LOP:DTA) zebrafish embryos expressing CFP in the eye were able to survive to adulthood, at which point they were mated to wildtype zebrafish. The resulting F1 progeny were screened under a fluorescent microscope at 2-3dpf for cyan fluorescence within the eye. Again, there were
no F1 offspring carrying the cry:ECFP-LOP:DTA transgene after screening over 300 F1 embryos.

6.4 Discussion/Conclusions

Deriving stem or progenitor cells from transgenic animal models with tissue-specific expression of a fluorescent reporter protein is a reliable strategy for direct observation of cell differentiation events over a period of time. As described previously, this strategy was used to assess the capacity of HSC to differentiation into erythrocytes after transplantation into zebrafish embryo recipients by extracting donor HSCs derived from transgenic LMO-2\textsuperscript{EGFP};GATA-1\textsuperscript{DsRed} zebrafish [100]. Another example involves the use of BM-MSCs derived from transgenic B-a-fvb mice to assess cardiac gene expression by MSCs after co-culture with rCMs in vitro [42]. Therefore, to mark cardiac gene expression by ZKS cells after transplantation into zebrafish embryo recipients, I developed transgenic transposon vectors in which an exogenous zebrafish cardiac-specific \textit{tnnt2} promoter directs fluorescent protein expression. An exogenous zebrafish \textit{tnnt2} promoter had not yet been created, hence the construction of a functional 1.87-kb zebrafish \textit{tnnt2} promoter in this study. To generate the transgenic transposon vectors, the exogenous \textit{tnnt2} promoter was fused to a fluorescent reporter gene within a transposon vector. We selected to use the \textit{pT2/s2 Sleeping Beauty} transposon system as it has been shown to have a high rate of efficiency for integration into the host genome [110]. The two transposon vectors used in this study were created by Balciunas et al: 1) \textit{pT2/s2EF1α-GM2} (pDB371) and 2) \textit{pT2/s2EF1α-DsRed} transposon vectors [110]. GM2 is a green fluorescent reporter gene, while DsRed is a red fluorescent reporter gene. After removal of the \textit{ef1α} promoter from the \textit{pT2/s2} vectors and fusion of the \textit{tnnt2} promoter to the GM2 or DsRed gene within the vectors, the \textit{pT2/s2tnnt2-GM2} and
pT2/s2tnnt2-DsRed transposon vectors were successfully created. The two new transgenic transposons were also shown to be capable of labelling cardiomyocytes in vivo as determined by the cardiac-specific fluorescence observed within transgenic F0 pT2/s2tnnt2-GM2 and pT2/s2tnnt2-DsRed zebrafish embryos. Originally, the created vectors were to be used to generate transgenic zebrafish lines marking cardiomyocytes. These transgenic zebrafish were to be used to derive primary ZKS cells in which cardiac differentiation would be marked by fluorescent protein expression. However, primary ZKS cells could not be established in this study. Instead, the vectors can be used in the future to generate transgenic pT2/s2tnnt2-GM2 and pT2/s2tnnt2-DsRed cell lines by transfection of the transposon vector into ZKS1 cells. These transgenic cell lines can be used as donor cells in transplantation experiments assessing the capacity of ZKS cells to differentiate into cardiomyocytes in vivo.

The mechanisms of cardiac repair by MSC therapy, including MSC cardiac differentiation, are typically evaluated using adult animal models of MI. Myocardial infarction is often created in mammalian models by manually ligating the left anterior descending coronary artery [120]. However, it is not possible to ligate arteries of the zebrafish model manually due to the small size of the vessels. A better approach for creating an MI in zebrafish is the development of a genetic zebrafish model of inducible cardiac ablation. Due to the availability of the mifepristone-inducible LexPR transposon system and a vector carrying the cytotoxic DTA gene in the Wen Lab, as well as the exogenous zebrafish tntt2 promoter developed here, we chose to generate transposon vectors that can be used to develop a transgenic zebrafish model with chemical-inducible cardiac ablation. I have generated two pDs transposon vectors that can be applied in trans to accomplish this task. A reporter pDs(tntt2:LPR-LOP:G4) vector and an effector pDs(cry:ECFP-LOP:DTA) vector were developed. Extension to this area of my research should
include the initial generation of both a transgenic \textit{pDs}(\textit{tnnt2}:LPR-LOP:G4) and a transgenic \textit{pDs}(\textit{cry}:ECFP-LOP:DTA) zebrafish line and the subsequent generation of a double transgenic \textit{pDs}(\textit{tnnt2}:LPR-LOP:G4;\textit{cry}:ECFP-LOP:DTA) zebrafish line through mating of transgenic \textit{pDs}(\textit{tnnt2}:LPR-LOP:G4) and \textit{pDs}(\textit{cry}:ECFP-LOP:DTA) zebrafish. By addition of mifepristone to the surrounding aqueous environment, \textit{pDs}(\textit{tnnt2}:LPR-LOP:G4;\textit{cry}:ECFP-LOP:DTA) zebrafish embryos will express both GFP (G4) and DTA (endotoxin) within cardiomyocytes as directed by the exogenous \textit{tnnt2} promoter. DTA is cytotoxic and its expression will lead to gradual cardiomyocyte necrosis, similar to that observed by metrodinazole-nitroreductase (Mtz-NTR)-mediated cardiac ablation \cite{111}. Cardiac-specific DTA and GFP expression can be induced at any developmental stage in the zebrafish through controlled timing of mifepristone exposure to the fish. The length of mifepristone incubation should be varied to determine the most appropriate incubation length for causing sufficient cardiac necrosis without killing the animal. Ultimately, donor \textit{ZKS1}(\textit{tnnt2}:DsRed) cells can be transplanted into both 48hpf \textit{pDs}(\textit{tnnt2}:LPR-LOP:G4;\textit{cry}:ECFP-LOP:DTA) zebrafish larvae and adult \textit{pDs}(\textit{tnnt2}:LPR-LOP:G4;\textit{cry}:ECFP-LOP:DTA) zebrafish recipients with mifepristone-induced cardiac necrosis to study cellular changes by zebrafish MSCs in a cardiac disease setting. A significant limitation of this model, which is part of the intrinsic nature of the zebrafish, is that the zebrafish has the capacity for cardiac regeneration after 20\% ventricular resection \cite{121} or 24hrs of NTR-Mtz induced cardiac ablation \cite{111}. Therefore, evaluations of cardiac repair by cell therapy for zebrafish models of cardiac necrosis should focus on the rate of regeneration as compared to untreated zebrafish models of cardiac ablation.
CHAPTER 7.0 – GENERAL DISCUSSION

Although cardiac differentiation by BM-MSCs has been extensively investigated in vivo using adult recipient models, few studies have looked at the impact of an embryonic cardiac milieu on BM-MSC cardiomyogenesis, which has been found to be a more effective environment for cardiac differentiation by these cells in vitro [42-46]. It is difficult to study cardiac differentiation by BM-MSCs within an embryonic microenvironment in vivo using mammalian model systems as the mammalian embryo is not easily accessible. It is challenging and invasive to transplant stromal cells into the embryo and observe the cellular and molecular changes experienced by these transplanted cells over time. In contrast, the zebrafish provides an excellent model for studying cardiac differentiation by MSCs in embryonic recipients in vivo. Zebrafish embryonic development is rapid, external and transparent which enables direct observation of the cellular and molecular changes experienced by transplanted cells at various stages of developmental within days [51]. The zebrafish also has a high fecundity which allows for a large sample size. Finally, the zebrafish embryo can survive several days without a beating heart, which is beneficial for studying cardiac differentiation by transplanted stromal cells in a cardiac disease model. For these reasons, I selected the zebrafish model to develop a cell transplantation system whereby cardiac differentiation by donor MSCs could be assessed in embryonic zebrafish recipients before (blastulae recipients) and after the embryonic heart is formed (48hpf recipients). As this area of research is extremely novel, much of the work performed in this study had either not been attempted previously or had been repeated according to protocols recently developed.
While the transplantation system proposed in this study has developmental potential, a few significant limitations to the system must first be resolved. These include: (i) Discovery of a suitable donor marrow-derived MSC for transplantation into blastulae and 48hpf zebrafish recipients; (ii) Development of co-culture systems to examine cardiac differentiation by ZKS cells \textit{in vitro}; and (iii) Establishment of a strategy for distinguishing cardiac differentiation from cell fusion by the donor cells \textit{in vivo}.

(i) **Finding a suitable marrow-derived MSC for transplantation into embryonic zebrafish recipients** – Initially, I attempted to transplant rat BM-MSCs into zebrafish blastulae and 48hpf recipients using similar transplantation methods as those described previously [100]. It would have been convenient to use rat BM-MSCs as donor cells for the proposed transplantation system because rat BM-MSCs have been well-characterized and shown to express cardiac markers in adult transplant recipients [35,47-49]. However, transplanting 40-70 rat BM-MSCs into zebrafish blastulae recipients was technically challenging and inefficient as less than 1% of the recipient embryos survived this procedure. As only a small volume of cell suspension can be injected into the sinus venosus of 48hpf zebrafish recipients, a high concentration of cells is required for transplantation at this stage of development [100]. Thus, lowering the concentration of BM-MSCs may improve recipient survival rate, but will not be beneficial for cell transplantation experiments using 48hpf zebrafish recipients. Hence, we sought to identify and characterize a zebrafish kidney marrow-derived MSC to use as the donor cell for transplantation experiments.

In contrast to the mammalian MSC which has been studied for several decades, there has been no research aimed at discovering a zebrafish MSC. Therefore, I have conducted the first study focused on identifying a zebrafish cell type that is equivalent to the human BM-MSC. I
hypothesized that the recently established ZKS1 cell line, which was used previously as a feeder cell line for zebrafish hematopoietic stem cell proliferation and differentiation in vitro [75], was a zebrafish MSC line since ZKS1 cells shared similar cellular properties with mammalian BM-MSCs and were derived from the zebrafish kidney marrow. Since no studies have looked at the expression of zebrafish stromal cell genes \textit{cd}73, \textit{cd}90 and \textit{cd}106 by zebrafish cells \textit{ex vivo}, I developed primer sets specific for the cDNA of each of these genes and found that ZKS1 expresses \textit{cd}73, \textit{cd}90 and \textit{cd}106 genes \textit{in vitro}. This experiment should be repeated in the future using hematopoietic cells as a negative control to strengthen this conclusion. ZKS1 was also found to express a subset of hematopoietic genes that are not normally expressed by MSCs. Thus far in the literature, antibodies against zebrafish hematopoietic and stromal cell antigens are lacking. The two monoclonal anti-human antibodies against stromal cell markers CD90 and CD73 trialed in this study were unable to cross-react with ZKS1 stromal antigens. Nonetheless, screening currently available antibodies for cross-reactivity with zebrafish stromal cell antigens should be continued in the future with a focus on testing polyclonal antibodies that are known to work across different species. This will provide tools for characterizing the surface antigen expression profile of ZKS cells in future research. Finally, the minimal \textit{in vitro} differentiation capacity of MSCs could not be demonstrated by ZKS1 using standard mammalian BM-MSC adipogenic and osteogenic differentiation assays [78,81]. The differentiation capacity of ZKS1 may be altered due to senescence, thus it is important to trial these assays using primary ZKS cells. If primary cells are still unable to differentiate under induction conditions designed for mammalian BM-MSCs, the protocol established for induction of adipogenic differentiation \textit{in vitro} by stromal-vascular cells of red sea bream fish, \textit{Pagrus major} [80] should be trialed for primary ZKS cells. This will determine whether fish-specific conditions are required for ZKS
minimal differentiation \textit{in vitro}. If it cannot be shown that ZKS1 cells lack expression of the hematopoietic genes tested in this study and can differentiate into adipocytes, osteoblasts and chondroblasts, then these cells would not be an appropriate donor MSC for the proposed transplantation system.

While the ZKS1 cell line is convenient due to its indefinite expansion capacity, the possibility remains that the cells have been mutated. The cell line was derived from continuous passage of a primary ZKS cell culture generated from plating adult zebrafish WKM cells. However, expandable primary ZKS cell cultures could not be produced using the previously established ZKS culture conditions in this study. Even modifications to the original ZKS culture conditions that were experimented did not appear to improve the expandability of primary ZKS cells. Therefore, since expansion of primary ZKS cells is a rare occurrence and ZKS1 was derived from the expansion of these primary cells, it is possible that the proliferative capacity of ZKS1 is due to a mutation which activates a cell proliferation gene such as an oncogene, or inactivates a gene that normally suppresses cell proliferation, such as a tumor suppressor gene. This is comparable to mammalian MSC lines which can be generated by transfection of primary MSCs with a vector overexpressing an oncogene [122]. It is not uncommon for permanent cell lines to experience changes in their global gene expression pattern and capacity for differentiation into different tissues. Therefore, ZKS1 may not be representative of primary ZKS cells. Moreover, many studies assessing cardiac differentiation by mammalian BM-MSCs have used primary T4 BM-MSCs [42,43,45]. Thus, the best approach for identifying a zebrafish MSC for application in the proposed transplantation system is to develop appropriate culture conditions for generating primary T4 ZKS cells. This will achieve consistency between studies. However, these cells are not able to proliferate in a basic DMEM + 10%FBS medium incubated
at 32°C, 5% CO₂. It is possible that growth factors which stimulate cell proliferation are essential for the expansion of ZKS cells in vitro. Thus, future studies should focus on testing ZKS cell expansion in media consisting of different combinations of growth factors which are known to stimulate MSC proliferation, such as epidermal growth factor (EGF) [87], fibroblast growth factors (FGFs) [88,89], TGF-β and platelet-derived growth factor (PDGF) [89]. Once culture conditions for consistent generation of primary T4 ZKS cultures have been developed, primary ZKS cells can be characterized according to the defining criteria of MSCs [16] to determine whether this cell is a suitable donor MSC for transplantation experiments using the zebrafish model.

(ii) Development of co-culture systems to examine cardiac differentiation by ZKS cells in vitro – Mammalian BM-MSCs have been shown to express cardiac-specific markers when co-cultured with embryonic or neonatal cardiomyocytes in vitro [42-46], but not when co-cultured with adult cardiomyocytes [46]. Though the ultimate goal of my study was to develop an in vivo embryonic transplantation system to determine whether a primitive cardiac microenvironment provides an improved avenue for cardiac differentiation by MSCs than an adult cardiac milieu, it is necessary to first confirm that ZKS cells can reproduce the findings observed by mammalian BM-MSCs in the MSC-CM co-culture studies described above [42-46]. As cardiac differentiation by exogenous zebrafish progenitor cells has never been assessed in vitro or in vivo, I performed an initial assessment of in vitro cardiac differentiation by ZKS1 using a ZKS1-rCM co-culture system. My lab previously demonstrated that murine BM-MSCs could be induced by rCMs to express cardiac-specific markers in vitro [42], thus it was possible that ZKS1 could also be induced by rCMs to express cardiac-specific markers in vitro. However, this
was not the case as ZKS1 did not express \textit{cmlc2}, \textit{tnnt2} or \textit{tnni-HC} after 5 days of co-culture with rCMs. Since ZKS1 is a permanent cell line and may have a reduced capacity for differentiation, these experiments should be repeated using primary T4 ZKS cells once culture conditions for generating expandable ZKS cells have been discovered. Alternatively, co-culture systems combining primary ZKS cells and zebrafish cardiomyocytes would be more effective as these systems would eliminate the limitation of using a xenoculture approach. The following procedure could be used to design a co-culture system between ZKS cells and zebrafish embryonic CMs (zCMs) in future studies. First, WKM cells should be derived from adult transgenic \textit{tnnt}:DsRed zebrafish (which can be developed using the pT2\_s2\textit{tnnt2}:DsRed transposon created in this study) and cultured for primary T4 ZKS cells. Meanwhile, the hearts of 48hpf transgenic \textit{cmlc2}:GFP zebrafish embryos should be disrupted according to methods described in Burns et al. [99]. The resulting GFP-expressing fragments should be collected and digested using a solution containing collagenase I and protease XIV to yield a single cell suspension [104]. The zCMs should then be cultured for 24hrs according to either culture conditions described for zebrafish embryonic cell cultures [85], or conditions described for adult zebrafish cardiomyocyte cultures [104]. The transgenic primary ZKS cells should then be added to the zCM culture. The co-culture cells should be assessed at multiple time points for expression of DsRed, indicative of cardiac expression by the ZKS cells.
(iii) Strategies for distinguishing cardiac differentiation from cell fusion by the donor cells post-transplantation – When using mammalian models for assessing in vivo differentiation by donor progenitor cells after transplantation, cell fusion can be assessed by transplanting cells derived from female donors into male recipients and then analyzing the donor cells for the presence of a Y chromosome post-transplantation [123]. However, this method for distinguishing cell fusion from differentiation cannot be applied for the transplantation system proposed in this study as the zebrafish does not possess sex chromosomes [124]. Nevertheless, a few alternative options exist for determining the frequency of cell fusion events that occur when zebrafish MSCs are transplanted into zebrafish embryo recipients. Embryos of a transgenic zebrafish line in which a LacZ transgene is expressed under the control of a cardiomyocyte-specific promoter could be used as recipients when using donor cells derived from Tg(cmlc2:GFP) zebrafish [98]. GFP+ cells found within the host post-transplantation could then be stained with X-gal and formation a blue precipitate within the cell cytoplasm would indicate that the donor cells had fused to the recipient cardiomyocytes. A similar strategy was used previously to demonstrate cell fusion of donor hematopoietic cells with host cardiac cells in a mouse infarct model [125]. Cell fusion can also be confirmed by staining GFP+ donor cells with a nuclear stain to determine whether the cell is binucleated. Nygren et al used the Hoechst 33342 nuclear stain for additional confirmation of cell fusion events by the donor hematopoietic cells discussed above [125]. Moreover, donor MSCs from Tg(cmlc2:GFP) zebrafish can be transplanted into Tg(T2/S2tnnt2:DsRed) zebrafish embryos after establishment of a Tg(T2/S2tnnt2:DsRed) zebrafish line using the pT2/S2tnnt2:DsRed transposon developed in this study. With this approach, cells expressing both GFP and DsRed, which is observed as yellow fluorescence, are the product of a fusion event between donor MSC and a host CM.
The zebrafish model system developed in this study has prospective advantages for investigating the cellular contributions of MSCs in cardiac repair. However, the application of this system is currently limited due to cell culturing difficulties and the lack of appropriate technology for purifying MSCs in the zebrafish. Nevertheless, once ZKS cells can be defined as expandable zebrafish MSCs and more suitable reagents, such as zebrafish-specific antibodies, have been developed, the model that we have established may help to better understand the molecular processes involved during MSC-mediated cardiac regeneration and to discover novel strategies for improving the cardiac potential of MSCs in vivo.
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APPENDICES

APPENDIX-1: Zebrafish Kidney Stroma (ZKS) Medium [75]

Base medium:
55% L-15 (GIBCO, 11415)
32.5% DMEM (GIBCO, 11885)
12.5% Ham’s F-12 (GIBCO, 31765)
10% ES cell qualified FBS (American Type Culture Collection, Manassas, VA)
- or 10% FBS (Sigma, F1051)

Supplemented with:
150mg/L 7.5% (w/v) Sodium bicarbonate (GIBCO, 25080-094)
2% PenStrep (5000U/mL Penicillin, 5000ug/mL streptomycin) (GIBCO, 15070)
1.5% Hepes (1M) (BioShop, HEP003.500)
1% L-glutamine (200mM) (GIBCO, 25030)
0.1mg/mL gentamycin sulfate

APPENDIX-2: Human LTMC Medium [82]

Base medium:
DMEM (GIBCO, 11885)
10% FBS (Sigma, F1051)

Supplemented with:
160mL Vitamins
160mL Sodium bicarbonate
160mL 100mM sodium pyruvate
120mL essential amino acids
65mL nonessential amino acids
160mL L-glutamine
160mL of: 10,000U/ml Penicillin, 10,000U/ml streptomycin, 25ug/ml amphotericin
0.5mL hydrocortisone 1mg/ml in dimethyl sulfoxide
## TABLES & FIGURES

### Table 1
Oligonucleotide primers for detection of mRNA of zebrafish stromal, hematopoietic and cardiac genes in ZKS1

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sets</th>
<th>Anneal T. (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stromal</td>
<td></td>
<td>57</td>
<td>n/a</td>
</tr>
<tr>
<td><em>vcam-1 (cd106)</em></td>
<td>FP – GAAATCCAAGCAGCAGGAAGT</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>RP – TTCTCCTACTCCAGCAAGGAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nt5’e (cd73)</em></td>
<td>FP – CCTTCGAGGAGTGTCTGCTTTC</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>RP – CCCTTTCTGTTTCGCTCAATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>thy-1 (cd90)</em></td>
<td>FP – AAGCAACACATCTCATGACATTTTG</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>RP – TCTGGAGCTGGAGTGAAAGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematopoietic</td>
<td></td>
<td>60</td>
<td>[126]</td>
</tr>
<tr>
<td><em>cd45</em></td>
<td>FP – AGTTCTCTGAAAATGGAAAGC</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>RP – GCAACAGAAAATGTCAGTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>gata-1</em></td>
<td>FP – ATTATCCACACAGCCAG</td>
<td></td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td>RP – CCACTCCACTCTAGGGACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>c-myb</em></td>
<td>FP – AGTTACTCCGGAGAAGACAGG</td>
<td></td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td>RP – AGAGCAAGTGAATGGCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>runx1</em></td>
<td>FP – CTGATAGGTTGGTCTCTTC</td>
<td></td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>RP – CCGTTCTACCTTTGATTAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>gata-3</em></td>
<td>FP – CAGAGCTACAAAGCGTGCAG</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>RP – GCAGAAGTGCAAGCAGGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mpx</em></td>
<td>FP – GCTGTCTGGTGGCTCTTCA</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>RP – TTGTAGTGAGCTGGTTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lck</em></td>
<td>FP – AGATGAATGGTGTGACGAGTGA</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>RP – GATCCTGTAGTGCTTGACTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lmo2</em></td>
<td>FP – AATGAGGAGCGGGTGAGGAT</td>
<td></td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>RP – GCTCGATGGCTCCCGAAGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>pax5</em></td>
<td>FP – CTGATTACAAGCGCAGGAAC</td>
<td></td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>RP – CTCAATTATGCGCAGAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>igM</em></td>
<td>FP – AGCTCTCTCAAGCGCTTGATGG</td>
<td></td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>RP – GCTAACACATGGAAGATGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>rag-2</em></td>
<td>FP – AGCTCATTAGCTGCAACTGGAT</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>RP – TTGGAGGCGGACAGTCACCTACACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac</td>
<td></td>
<td>59</td>
<td>[129]</td>
</tr>
<tr>
<td><em>tnnt2</em></td>
<td>FP – CTTAGAGCGCAGAGGGAAGCCAAGCTGAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP – TTGCATCATCTTGCTCTTGCTTGCTTGCTGCTTGCTTGCTTGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tnni-HC</em></td>
<td>FP – ATGCCGAGCAGGAAAAAGGAGG</td>
<td></td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>RP – TTATTTTGTCTGCACTAAAAACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cmlc2</em></td>
<td>FP – GTGATGAAAGAGCTTGAGGT</td>
<td></td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>RP – GGTCATTAGGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constitutive</td>
<td></td>
<td>59</td>
<td>[126]</td>
</tr>
<tr>
<td><em>ef1-a</em></td>
<td>FP – CTTCGTCCCAATTTTCAGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP – CACACGACCACAGGAGTAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

100
Primary ZKS cells do not survive after first passage. Primary ZKS cells cultured in ZKS medium [75] at 32°C, 5% CO₂ were photographed under 32X phase contrast microscope. T0 and T1 ZKS cells had potential to form colonies, whereas T2 ZKS cells were unable to form colonies (a) T₀ ZKS cells. (b) T₁ ZKS cells. (c) T₂ ZKS cells. Cells were photographed after 5-7 days in culture.

Table 2:
The frequency of generating expandable primary T₀ and T₁ ZKS cell cultures from plating WK2 cells under various cell culture conditions

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Medium &amp; Incubation Temp</th>
<th># of wells plated</th>
<th># of T₀ cultures passaged</th>
<th># of T₁ cultures passaged</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>ZKS medium 32°C</td>
<td>64</td>
<td>11 (17%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>2</td>
<td>ZKS medium 32°C fibronectin-coated</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>ZKS medium + ZKS1 conditioned-media 32°C</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>ZKS medium (-10%FBS) + 10% FHS 32°C</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>ZKS medium + 10% Embryo extract 32°C</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>ZKS medium + 10% hydrocortisone 32°C</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>ZKS medium 32°C (ZK-4X cell #s plated)</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Σ</td>
<td></td>
<td>96</td>
<td>14 (14.6%)</td>
<td>3 (3.1%)</td>
</tr>
</tbody>
</table>

* Previously established protocol for zebrafish kidney stroma (ZKS) culture [75]
Figure 2a
ZKS1 is plastic adherent in culture with a spindle-shaped morphology. Adherent ZKS1 cells were photographed under the 32X phase contrast microscope lens.

Figure 2b
ZKS1 expresses stromal genes and a subset of hematopoietic genes. Gene expression analysis of ZKS1 (Z) was assessed by RT-PCR. (i) Results for stromal gene expression by ZKS1. (ii) Results for hematopoietic gene expression by ZKS1. Zebrafish WKM (W) cells were used as a positive control and ddH$_2$O as a negative control.
Figure 2c
ZKS1 was unable to differentiation into adipocytes or osteoblasts using standard mammalian MSC differentiation assays in vitro. ZKS1 and murine T4 BM-MCSs were induced to differentiate into adipocytes or osteoblasts using mammalian MSC adipogenic and osteogenic assays, respectively. Adipogenesis is detected by Oil Red O staining (blue arrow) and osteogenesis by Alizarin Red staining (red arrow) after 2 weeks of induction. Cells within adipogenic assays were photographed at 20X magnification, while cells of osteogenic assays were photographed at 10X magnification. Murine T4 BM-MSCs were used as a positive control and ZKS1 cultured in ZKS medium alone was used as a negative control. (i) ZKS1 adipogenic differentiation assay. (ii) ZKS1 osteogenic differentiation assay. (iii) Murine BM-MSC adipogenic differentiation assay. (iv) Murine BM-MSC osteogenic differentiation assay.
Figure 3
Monoclonal antibodies against human stromal cell antigens CD90 and CD73 do not cross react with ZKS1 cell antigens. ZKS1 was fixed to glass slides and stained with DAPI (blue) and monoclonal antibodies against CD90-FITC (green) and CD73-PE (red). Human T4 BM-MSCs were used as a positive control and anti-IgG1 antibodies were used as a negative control. Confocal microscopy was used to observe immunocytochemical staining. Anti-CD90-FITC and anti-CD73-PE antibody staining of (i) ZKS1 and (ii) human BM-MSCs. Anti-IgG1-FITC antibody staining of (iii) ZKS1 and (iv) human BM-MSCs.
Figure 4
ZKS1 forms colonies at a rate of 8:1. Data was pooled from 5 separate trials and the average number of colonies formed (y-axis) per original ZKS1 cell numbers plated (x-axis) was plotted (red points). The standard deviation was calculated for each average value (red arrow bars). A trendline was drawn based on the plotted averages (blue line). Taking the slope of the trendline, the rate of CFU-F formation per number of ZKS1 cells plated was determined to be 1:8.
Figure 5a
Oligonucleotide primers specific for zebrafish cardiac-specific transcripts did not bind to mRNA of rat embryonic cardiomyocytes. RT-PCR was performed using zebrafish cardiac primers and mRNA extracted from rat embryonic cardiomyocytes (RH). Zebrafish cardiac tissue (ZH) was used as a positive control and ddH2O as a negative control. Primers against zebrafish ef1α and rat gapdh genes were used to confirm the presence of zebrafish and rat cells, respectively.

Figure 5b
ZKS1 does not express cardiac-specific transcripts of the tnt2, tnni-HC or cmic2 genes. Cardiac gene expression by ZKS1 (ZKS) was assessed by RT-PCR using oligonucleotide primers for zebrafish cardiac-specific transcripts. RNA extracted from zebrafish cardiac tissue (ZH) was used as a positive control and ddH20 as a negative control.
Figure 5c
ZKS1 was not induced to express cardiac-specific genes *tnnt2*, *tnni-HC* and *cmlc2* by rat embryonic cardiomyocytes after 5 days of co-culture. Zebrafish cardiac gene expression by co-cultured cells (C) was assessed by RT-PCR using zebrafish cardiac-specific primers. RNA from zebrafish cardiac tissue (ZH) was used as a positive control and ddH$_2$O as a negative control. Primers for the ubiquitous zebrafish *ef1α* gene were used to confirm the presence of zebrafish cells.
Figure 6

$T_4$ BM-MSCs extracted from CAG/GFP-LEW rat can be transplanted into zebrafish blastulae with a low recipient survival rate. Zebrafish transplant recipients were photographed using a wide field fluorescent microscope. (i, ii) Recipient zebrafish blastula immediately post-transplantation of donor GFP$^+$-MSCs (red arrows). The yellow arrow indicates the location of infection post-transplantation. (iii) Zebrafish transplant recipient 72h post-injection (hpi).
Figure 7
A transgenic ZKS1(CMV:eGFP) cell line was created. ZKS1 cells transduced by the pHRC-PPT-CMV-IREs-eGFP-WPRE lentiviral vector were sorted for GFP expression using FACS. (i) Second round of FACS for transduced ZKS1 cells with enhanced GFP expression. The FL1 channel was used to detect GFP and the FL2 channel was used as a negative selector. (ii & iii) TgZKS1(CMV:eGFP) cells in vitro. TgZKS1(CMV:eGFP) cells (green) were fixed to glass slides, stained with DAPI (blue) and photographed under a fluorescent microscope at 40X (ii) and 10X (iii) magnification.
Figure 8
ZKS1(CMV:eGFP) can be easily transplanted into zebrafish blastulae recipients. The recipient zebrafish blastula was photographed using the Leica DFC320 microscope under the GFP filter immediately after injection of 100-200 cells into the midline of the embryo. The image was taken at 10X magnification.
Table 3
The number of transplant recipients able to survive for at least 3 days post-transplantation after injection with 40-70 CAG/GFP-LEW rat T4 BM-MSCs or 100-200 ZKS1(CMV:eGFP) cells

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>No. Recipients injected</th>
<th>No. Of GFP+ recipients at 74hpi</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rat GFP+ BM-MSC Transplantation into Blastulae Zebrafish</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>120</strong></td>
<td><strong>1 (0.8%)</strong></td>
</tr>
<tr>
<td><strong>ZKS1(CMV:eGFP) Transplantation into Blastulae Zebrafish</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>150</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>156</td>
<td>18</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>263</strong></td>
<td><strong>44 (16.7%)</strong></td>
</tr>
</tbody>
</table>
Figure 9
Donor ZKS1(CMV:eGFP) cells transplanted into zebrafish blastulae can reside within the host embryonic heart, brain, yolk and trunk tissue as well as form a band of tissue spanning ventral to the heart. Approximately 100-200 ZKS1(CMV:eGFP) cells were microinjected into zebrafish embryos at the 500-1000 cell stage of development. Pictures were taken of injected embryos at 3dpf using the Leica DFC320 microscope under the GFP filter. Recipient zebrafish with ZKS1(CMV:eGFP) cells within the embryonic (i) heart, (ii) brain, (iii) yolk (red arrow) and (iii, v) trunk tissues. (iv) A recipient zebrafish with a band of GFP+ cells spanning ventral to the pericardial sac.
Table 4
Trends in donor ZKS1(CMV:eGFP) cell location 3 days post-transplantation into zebrafish blastulae recipients

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Total # of Recipients</th>
<th># GFP(^*) at 72hpf</th>
<th># GFP(^*) in heart</th>
<th># GFP(^*) in CNS</th>
<th># GFP(^*) in yolk</th>
<th># GFP(^*) in trunk</th>
<th># GFP(^*) in pericardial band</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150</td>
<td>18</td>
<td>3</td>
<td>7</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>8</td>
<td>0</td>
<td>3</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>156</td>
<td>18</td>
<td>2</td>
<td>6</td>
<td>3 (16.7(^*))</td>
<td>4 (22.2(^*))</td>
<td>3 (16.7(^*))</td>
</tr>
<tr>
<td>Total</td>
<td>263</td>
<td>44</td>
<td>5 (11(^*))**</td>
<td>16 (36(^*))**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) Percentage of recipients based on value for # of GFP\(^*\) recipients at 72hpf in Trial #3

\(^*\) Percentage of recipients based on value for Total # of GFP\(^*\) recipients at 72hpf

Table 5
Oligonucleotide primer sets used in PCR to generate an exogenous 1.87kb zebrafish *tnnt2* promoter

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sets</th>
<th>Anneal T. ((^\circ)C)</th>
</tr>
</thead>
</table>
| Zebrafish       | FP - TGGCCCTAGTTTTCACACAA
                 | RP - GATCTGACCCGGGCGAGA            | 63                       |
| *Tnnt2* promoter| FP - TGGCCCTAGTTTTCACACAA
                 | RP - GATCTGACCCGGGCGAGA            | 63                       |

113
Figure 10a
An exogenous 1.87kb zebrafish *tnnt2* promoter was created by PCR. PCR was performed using a zebrafish genomic DNA template and a primer set specific for the endogenous zebrafish *tnnt2* promoter. ddH$_2$O was used as a negative control.

Figure 10b
The exogenous zebrafish *tnnt2* promoter was cloned into the pGEM-T Easy vector as confirmed by restriction enzyme digestion. (i) A diagram of the pGEM-T(*tnnt2*) vector generated. (ii) EcoR1 restriction enzyme digestion of pGEM-T(*tnnt2*).
**Figure 11a**
Transgenic pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 transposons were created. The exogenous zebrafish *tnnt2* promoter was subcloned into both pT2/S2ef1α-DsRed and pT2/S2ef1α-GM2 transposons. (i) A diagram of the pT2/S2tnnt2-DsRed transposon. (ii) A diagram of the pT2/S2tnnt2-GM2 transposon. Flanking indirect/direct repeat sequences are indicated by red arrow heads.

**Figure 11b**
Transgenic F0 pT2/S2tnnt2-DsRed and pT2/S2tnnt2-GM2 zebrafish were created. Zebrafish embryos at the 1-2 cell stage of development were co-injected with 30-150pg of pT2/S2 transposon DNA and 30-100pg of SB10 mRNA. Mosaic F0 zebrafish were photographed at 2dpf using the Leica DFC320 microscope. (i) An F0 pT2/S2tnnt2-DsRed zebrafish embryo. (ii) An F0 pT2/S2tnnt2-GM2 zebrafish embryo.
Figure 12a
Transgenic pDs(\textit{tnnt2}:LPR-LOP:G4) and pDs(\textit{cry}:ECFP-LOP:DTA) transposons were created. The exogenous zebrafish \textit{tnnt2} promoter was subcloned into pDs(\textit{krt8}:LPR-LOP:G4) to generate the pDs(\textit{tnnt2}:LPR-LOP:G4) transposon. An exogenous DTA gene was subcloned into pDs(\textit{cry}:ECFP-LOP:mCherry) to generate pDs(\textit{cry}:ECFP-LOP:DTA). (i) A diagram of the pDs(\textit{tnnt2}:LPR-LOP:G4) transposon. (ii) A diagram of the pDs(\textit{cry}:ECFP-LOP:DTA) transposon.

Figure 12b
Transgenic F0 pDs(\textit{tnnt2}:LPR-LOP:G4) and pDs(\textit{cry}:ECFP-LOP:DTA) zebrafish were created. Zebrafish embryos at the 1-2 cell stage of development were co-injected with 30-150pg of pDs transposon DNA and 30-100pg of pAc2 mRNA. Mosaic F0 zebrafish were photographed at 2dpf using the Leica DFC320 microscope. (i) An F0 pDs(\textit{tnnt2}:LPR-LOP:G4) zebrafish embryo. (ii) An F0 pDs(\textit{cry}:ECFP-LOP:DTA) zebrafish embryo.